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Transcriptome profiling of *Arabidopsis thaliana* roots in response to allelopathic effects of *Conyza canadensis*

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Abstract

The molecular mechanisms underlying allelopathy and their role in the interactions between invasive weeds and native species remain unclear. In this study, we aimed to explore the physiological and molecular response of plant roots of a native species to allelopathy from an invasive weed. We examined the growth and development of roots of native *Arabidopsis thaliana* for a 2-week period after being treated with aqueous extracts at different concentrations from invasive *Conyza canadensis*. Extracts with higher concentration in the Murashige and Skoog (MS) media (i.e., 4 mg of extract/mL of MS) significantly affected the root growth of *A. thaliana*. Roots of *A. thaliana* displayed weakened root tip activity and an accumulation of reactive oxygen species (ROS) in response to extracts from *C. canadensis*. The transcriptome analysis of *A. thaliana* roots exposed to phytotoxicity revealed differentially expressed genes (DEGs) involved in cell wall formation, abiotic stress, transporter genes and signal transduction. We found that genes associated with nutrient transport, such as major facilitator superfamily (MFS) and amino acid permease (AAP3) transporters as well as genes involved in stress response, including leucine-rich repeat receptor-like protein kinases (LRR-RLKs) were down-regulated. In addition, we found that many transcription factors associated with plant stress (such as APETALA2/ethylene response factors) were up-regulated while others (e.g., zinc-finger proteins) were down-regulated. Allelochemicals from *C. canadensis* also induced the up-regulation of detoxification (DTX) genes, ROS related genes, calcineurin B-like interacting protein kinases (CIPKs) and calmodulin. Overall, our findings provided insights into allelopathy in *C. canadensis* at the molecular level, and contributes to our understanding of invasion mechanisms of alien plant species.

Clinical trials registration

This study does not contain any studies with clinical trials performed by any of the authors.

Keywords Invasive plant · Allelopathy · Toxicity · Root growth · Gene expression profiling

Introduction

Invasive species are one of the major threats to the structure and function of ecosystems (Kennedy et al. 2002; Allendorf and Lundquist 2010; D'Antonio et al. 2017), and may cause huge economic losses (Pimentel et al. 2005; Caesar et al. 2012). Allelopathy is a process whereby plants release secondary metabolites into the surrounding environment, which

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may have either positive or negative effects on co-occurring plants (Putnam and Duke 1978). Allelopathy affects the ecology and evolution of plant communities, and may play an important role in plant invasion (Shackleton et al. 2015). Some introduced plant species release allelochemicals with inhibitory effects into their newly invaded ecosystem, which are relatively ineffective against co-occurring species in their original vegetative community (as these species have adapted to these chemicals over time) (Callaway and Ridenour 2004; Vivanco et al. 2010). In contrast, native species in ecosystems invaded by alien plants are not adapted to novel allelochemicals released from invasive plants (Callaway and Ridenour 2004).

Allelochemicals from alien plants can influence many basic physiological processes in other plant species in newly invaded environments, including photosynthesis, metabolism, osmotic adjustment, ion leakage, and levels of reactive oxygen species (ROS) (Reigosa et al. 2001; Gross 2003; Agati et al. 2012). The elevated levels of ROS in plants exposed to allelochemicals can disrupt antioxidant defense systems, thereby resulting in lipid peroxidation of cell membranes and the destruction of cell membrane structure (Bailey-Serres and Mittler 2006; Babula et al. 2014). Moreover, ROS play an important role in plant growth and development, physiological and biochemical reaction, hormone metabolism, as well as biological and abiotic stress responses (Saed-Moucheshi et al. 2014; Iqbal et al. 2015).

Genetic techniques can be used to improve our understanding of the role that allelopathy plays in the competitive ability of invasive plant species in their non-native ranges (i.e., regions where plants have been intentionally or accidentally introduced) (Wan et al. 2019). Previous transcriptome profiling experiments have examined the effects of allelochemicals on other plant species (Baerson et al. 2005; Chi et al. 2013). The molecular response of plants to allelopathic stress is highly complex, consisting of an interacting network of signaling pathways and the expression of numerous genes associated with metabolism, cellular defense, and transcription (Duke et al. 2008). Changes in the expression of the target genes and defense responses involved in chemical detoxification may occur later (i.e., after phytotoxin exposure) (Duke et al. 2008). As such, genetic techniques can be used to help elucidate the function of these genes at the genomic level, and may enhance our understanding of the underlying mechanisms of allelopathic stress.

The invasive plant species, *Conyza canadensis* (synonym of *Erigeron canadensis*, commonly known as horseweed), is native to North and Central America, and is widely naturalized in agricultural areas and temperate ecosystems throughout Eurasia and Australia (Gonzáleztorralva et al. 2017). Previous studies have shown that *C. canadensis* is allelopathic towards other plant species, including crops (Djurdjević et al. 2011; Queiroz et al. 2012). *Conyza canadensis* has been found to possess many allelochemicals, such as phenolics, ketones, flavonoids, alcohols, aldehydes, esters, and terpenes (Djurdjević et al. 2011; Zhou et al. 2013; Zhang et al. 2017a). Allelochemicals from *C. canadensis* in its newly invaded range have been found to inhibit germination and growth of native plant species by affecting important physiological processes, including photosynthesis, hormone metabolism and enzyme activity (Turcsányi et al. 1994), and changing soil microbial diversity and characteristics (Zhang et al. 2020). However, little is known concerning the genetic changes induced in native plants in response to the allelopathic effects of *C. canadensis*.

Arabidopsis thaliana is native to Europe, Asia, North Africa, and North America, and mainly occurs in open or disturbed rocky, sandy and loamy habitats (Mitchell-Olds 2001). The species co-occurs with invasive *C. canadensis* in European dry sandy grassland communities (Müller and Bartelheimer 2013). Previous studies have shown that *A. thaliana* may respond negatively to allelopathy from a number of plant species (Bossdorf et al. 2009; Bartelheimer et al. 2015). For instance, aqueous extracts from the invasive species, *Lonicera maackii*, inhibited growth and reproduction in *A. thaliana* (Cipollini et al. 2018). However, the allelopathic effects of extracts from *C. canadensis* on root growth in *A. thaliana* at the molecular level is currently unknown. *A. thaliana* has been studied intensively over the last 40 years and officially became a model plant for which the scientific community have developed numerous genetic and genomic resources (Koornneef and Meinke 2010). Given the availability of genomic resources for this species and that it is co-distributed with *C. canadensis*, *A. thaliana* was selected as the native species for our study. In the current study, we performed a transcriptome analysis of root tissue of the native plant, *A. thaliana*, after exposure to allelochemicals from *C. canadensis* in order to investigate the underlying molecular mechanisms of phytotoxicity and to provide information regarding its role in the invasion process.

Materials and methods

Plant materials

In August 2017, *Conyza canadensis* was collected from the grounds of Jiangsu University, China (32°12'17"N, 119°30'17"E), and air-dried at ambient temperature for storage. Seeds of *Arabidopsis thaliana* (Col-0) were purchased from Miaolingbio. Inc (China).

Preparation of aqueous extracts of *C. canadensis* and germination of *A. thaliana*

The whole plant of *C. canadensis* was cut into small pieces measuring about 0.5 cm in length. Ten grams of these small

pieces were placed inside a 100 mL beaker with 50 mL of distilled water for 2-hour ultrasonic extraction, and the obtained aqueous extracts were filtered with a 0.45 μm microporous membrane. Forty grams (containing 7 grams agar) of Murashige and Skoog (MS) medium were dissolved in 1 L water, and the solution was sterilized at 121 °C for 20 min. The filtered aqueous extracts were added to the MS medium before they were solidified, and adjusted to produce the following three concentrations: 1.0 mg/mL (T1), 2.0 mg/mL (T2), and 4.0 mg/mL (T3). In our preliminary study, the chemical compounds in extracts from *C. canadensis* were examined (Table S1), and the allelopathic effects at different concentrations of the aqueous extracts on *A. thaliana* were tested. The three concentrations (i.e., T1, T2, and T3) met our experimental requirements, and as such, these concentrations were chosen for the current study. For the control group (CK), only distilled water was added to the MS medium. The MS medium of each treatment was placed into petri dishes. Seeds of *A. thaliana* were surface sterilized with 5% sodium hypochlorite for 10 min and rinsed 10-times with sterilized distilled water. Fifty seeds of *A. thaliana* were placed into a petri dish (9 cm in diameter) containing the solidified MS medium. Each of the four treatments were replicated four times in a complete randomized design (i.e., $4 \times 4 \times 50 = 800$ seeds). The petri dishes were incubated at 25 °C, under a light intensity of 130 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (with 16 h light/8 h dark cycle). The germinated seeds (i.e., when the radicle became visible) were counted after one week, and seedling length, root viability, and reactive oxygen species (ROS) were measured after two weeks growth.

Detection of cell viability and ROS level in the root tips of *A. thaliana*

After two weeks growth, the root tips of *A. thaliana* seedlings were marked with Fluorescein diacetate (FDA, 10 μg/mL) for cell viability and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, 10 μM) to visualize ROS. The treated roots were left in darkness for 15 min using the method of Fu et al. (2017). The marked roots were washed 5 times with 0.05 mmol/L phosphate buffer (PBS, pH 7.4) and placed under a fluorescent inverted microscope (Axio Vert A1, Zeiss, Germany). The excitation and emission wavelengths were 488 and 525 nm for FDA and 502 and 530 nm for DCFH-DA.

RNA extraction and preparation

Roots of *A. thaliana* seedlings after two weeks growth from the T3 group (where growth was inhibited by extracts of *C. canadensis*), were selected for RNA extraction. Additionally, RNA was extracted from roots of the CK group and used as a comparison to the T3 group. Each group was replicated three times (i.e., included three biological replicates). A commercially available EASYspin RNA extraction kit (Aidlab Biotechnologies Co., Ltd., Beijing, China) was used to extract the RNA. RNA purity was checked using a NanoPhotometer spectrophotometer (IMPLEN, CA, USA). RNA concentrations were measured with a Qubit 2.0 Fluorometer (using the Qubit RNA Assay Kit, Life Technologies, CA, USA). RNA integrity was assessed using the Agilent Bioanalyzer 2100 system (via the Nano 6000 Assay Kit, Agilent Technologies, CA, USA).

One μg RNA per sample was used for RNA sample preparations. Samples were sent to BioMarker Technologies Co., Ltd., Beijing, China where cDNA libraries were generated. Sequencing libraries were generated using NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations. In order to select cDNA 200–250 bp length, the fragments were purified with an AMPure XP kit (Beckman Coulter, Beverly, USA) and enriched with PCR to create the final cDNA library.

Transcriptome analysis

The cDNA library was sequenced using Illumina HiSeq X-ten platform (Illumina Inc., San Diego, USA) by BioMarker Technologies Co., Ltd., Beijing, China. Filtered data were obtained by removing the adapters, the poly-structure with ambiguous 'N' nucleotides (ploy-N), and low-quality reads from the raw data using Cutadapt (parameters: cutadapt -e 0.1 -a adpter3 -A adpter5 -m 100 –cut 0 -O 13 -p) (Martin 2011). The base calling accuracy were assessed using Phred quality scores. Phred scores greater than 30 (Q30), which correspond to a probability of base-call error of 99.9%, were calculated using the method of Ewing et al. (1998).

The filtered reads were sent to Wuhan Benagen Technologies Co. Ltd., China for transcriptome mapping (using proprietary pipelines) with the latest transcriptome annotation AtRTDv2_QUASI (Zhang et al. 2017b). Transcriptional quantification was performed using Salmon (version 1.4.0) (Patro et al. 2017). Salmon is an analysis tool for RNA-seq data that can quickly complete transcript quantitative analysis without sequence alignment. The reference transcriptome AtRTDv2_QUASI was indexed using Salmon (parameter: -k 25 –keepDuplicates), and the clean reads were quantified by the Salmon quant (parameter: –validateMappings –libType A –seqBias –gcBias). The transcript expression is presented in transcripts per million (TPM) (Wagner et al. 2012). Differentially expression genes (DEGs) were assessed for each treatment using the R package DESeq2 (version 1.26.0) (Love et al. 2014), with a False Discovery Rate (FDR) < 0.05 and $|\log_2 FC| \geq 1$ to define differential genes. FDR was obtained by correcting the *p*-value using Benjamini and Hochberg's approach (Storey 2003), and the fold change was calculated as the ratio of expression between the T3 and CK groups. To

avoid the influence of analyzing artifacts or noise risk on low expression genes, the following parameter in DESeq2 was used: rnaseqMatrix = rnaseqMatrix [rowSums (rnaseqMatrix) ≥ 2].

Functional annotation of gene ontology (GO) terms (http:www.geneontology.org/) was performed using Diamond blastx (version 0.9.24) (Benjamin et al. 2015). The corresponding functional annotation information of the transcripts reconstructed by stringtie and cufflinks were not found, so Diamond blastx was used for aligning protein sequences against a protein reference database for annotation. FDR set at 0.05 was applied to calculate the statistical significance among GO terms, with a cut-off of 5 significant hits to label a GO term as significant. The Kyoto Encyclopedia of Genes and Genomes pathway analysis was performed using the KOBAS 3.0 system (http://kobas.cbi.pku.edu.cn/anno_iden.php). For KEGG enrichment, the *q*-value, obtained by multiple hypothesis correction of *p*-value (Casella and Berger 2002), was set at 0.05. GO and KEGG enrichment analysis of DEGs were carried out using the R package clusterProfiler (version 3.14.3) (Yu et al. 2012).

Quantitative real time PCR (qRT-PCR)

First strand cDNAs were prepared in a 20 µL reaction volume with extracted RNA from roots of *A. thaliana* (for the T3 and CK groups) using the M-MLV reverse transcription reagents kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. To verify our transcriptome analysis, three DEGs, namely PER1 (AT1G48130), PER71 (AT5G64120) and SCPL19 (AT5G09640) were selected for qRT-PCR analysis. These genes are involved in the phenylpropanoid biosynthesis via which allelochemicals (e.g., phenolics) are synthesized (Lattanzio 2013). The primers of the three DEGs are shown in Table S2. Actin2 (AT3G18780) was selected as the internal reference gene (Table S2). All of the qRT-PCR reactions were performed using a fluorescent quantitative detection system (BIO-RAD, USA). Reactions were carried out in a final volume of 20 µL, containing 2 µL cDNA template, 10 µL iTaq™ Universal SYBR® Green Supermix (BIO-RAD, USA), and 1 µL of each primer (10 µM). The PCR conditions were as follows: denaturation at 95 °C for 30 s, 35 cycles of denaturation at 95 °C for 10 s, annealing at 51 or 53 °C for 30 s (different annealing temperatures were used for each primer, see Table S2 for details), and extension at 72 °C for 30 s. Melt curve analysis was used to confirm the specificity for each primer and absence of primer dimers. The relative quantitative expression of the target genes was calculated using the method of Baerson et al. (2005). Three biological replicates were performed for the T3 and CK groups and were treated separately. All the primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. and the DNAMAN software (version 7.0; Lynnon Biosoft, Quebec, QC, Canada) was used to design the sequences for the primers (for PER1, PER71 and SCPL19).

Data analysis

Differences in root growth, shoot growth and relative quantitative expression of selected certified genes among treatments were assessed using one-way analysis of variance (ANOVA). Significant differences (at $p < 0.05$) between pairwise mean values were examined using Turkey's honest significant difference (HSD) test. Before each analysis, the Shapiro-Wilk test was used to assess whether the data were normally distributed. All analyses were performed using Origin 8.0.

For the germination dataset (which the Shapiro-Wilk test revealed was not normally distributed), non-parametric analyses were used to examine multiple comparisons between treatments. The Kruskal-Wallis H test was performed using SPSS 22.0 (IBM, Armonk, New York, USA) and statistical significance was set at a p-value < 0.05. The experiments were performed using 4 independent replicates for each representative assay (i.e., 50 seeds for each of the 4 conditions) and therefore in each figure, the mean and standard deviation of the replicates were shown in each assay (Fig. 1 and Fig. S1).

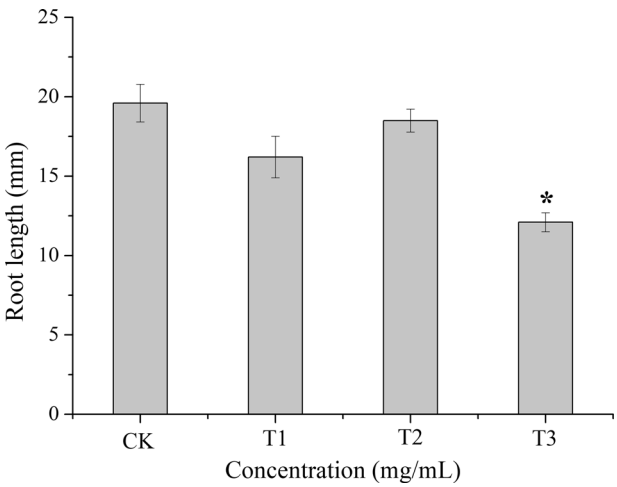

Fig. 1 The effect on the root growth of *Arabidopsis thaliana* in response to different concentrations of aqueous extracts of *Conyza canadensis* (T1, T2 and T3 represent the treatment concentrations 1.0, 2.0, and 4.0 mg/mL, respectively). The experiments were performed using 4 independent replicates for each representative assay (i.e., 50 seeds for each of the 4 conditions) and in each figure, the mean and standard deviation of the replicates was showed in each assay. Significant different values ($p < 0.05$, Tukey's HSD post hoc comparison) are indicated with an asterisk

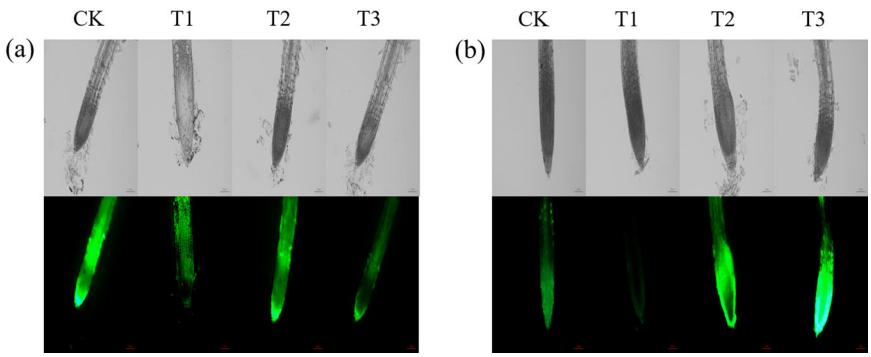

Fig. 2 The effects on (**a**) root tip viability and (**b**) ROS level of *Arabidopsis thaliana* in response to different concentrations of aqueous extracts from *Conyza canadensis* (400 × magnification). T1, T2, and T3 represent the treatment concentrations 1.0, 2.0 and 4.0 mg/mL, respectively

Results

Effects of aqueous extracts from *C. canadensis* on germination, growth, root tip viability and ROS level of *A. thaliana*

Compared with the control group (CK), root growth in *A. thaliana* was significantly reduced in response to the highest concentration (i.e., the T3 group) of aqueous extract from *C. canadensis* ($p < 0.001$, Fig. 1). However, germination and shoot elongation of *A. thaliana* were not significantly affected by aqueous extracts from *C. canadensis* ($p = 0.064$ and $p = 0.182$, respectively, Fig. S1).

The fluorescence of the FDA dye on the root tips of *A. thaliana* in the T3 group was markedly reduced (indicating lower root viability) in comparison to the other treatments (Fig. 2A). However, the application of the ROS-sensitive dye (DCFH-DA) to *A. thaliana* roots resulted in a much more visible green fluorescence in the T2 and T3 groups (indicating increased levels of ROS) compared to the T1 and CK groups (Fig. 2B).

Transcriptome of root tissues of *A. thaliana* by Illumina sequencing

The extracted RNA from roots of *A. thaliana* was determined to be of high quality as indicated by the ratio of 28 S rRNA to 18 S rRNA (which was between 1.70 and 1.90), and the values for RNA integrity number (RIN) (all exceeded 9, Table S3). Moreover, the OD (260/280) values were between 2.14 and 2.17, while OD (260/230) ranged from 1.58 to 2.05, suggesting that the extracted RNA was of high purity (Table S3). Data evaluation statistics for the clean reads identified from this study are presented in Table S4. However, there was no significant difference in the obtained number of reads of clean data between the T3 and CK groups ($p > 0.05$). A total of 607 DEGs were found from roots of *A. thaliana*, of which 339 DEGs (344 transcripts) were significantly up-regulated (FDR < 0.05, $\log_2 FC \geq 1$), while 242 DEGs (250 transcripts) were significantly down-regulated (FDR < 0.05, $\log_2 FC \leq -1$). However, 26 DEGs (56 transcripts) were also found to be either up-regulated or down-regulated (Table S5).

Functional annotation and enrichment analysis of DEGs in roots of *A. thaliana* after exposure to aqueous extract from *C. canadensis*

GO enrichment analysis was performed to assign DEGs (profiled from the CK and T3 groups) to functional groups. A total of 91 (107 transcripts) DEGs were categorized into 28 functional groups with a cut-off of 5 significant hits used to label a GO term as significant across the following main divisions: biological process, molecular function and cellular component. Of these, 51.9% of transcripts were assigned to biological processes, 3.7% to molecular function, and 44.6% to cellular components (Table S6). DEGs were significantly enriched for the following GO terms: "hydrogen peroxide catabolic process" (GO:0042744, FDR 3.68E-7), "peroxidase activity" (GO:0004601, FDR 1.06E-5), "beta-glucosidase activity" (GO:0008422, FDR 6.62E-3), "nutrient reservoir activity" (GO:0045735, 1.06E-5), "UDP-glycosyltransferase activity" (GO:0008194, FDR 1.14E-2), "cell wall biogenesis" (GO:0042546, FDR 6.42E-3) and "amino acid transmembrane transporter activity" (GO:0015171, FDR 9.27E-3) (Table S6).

The DEGs of the T3 and CK groups were compared by mapping them to 7 KEGG pathways (q-value < 0.05), and it was revealed that the phenylpropanoid biosynthesis pathway accounted for the highest proportion (where the number of DEGs was 16; Fig. 3, Table S7). We found 13 peroxidase genes, up-regulated PER1 (AT1G48130), PER2 (AT1G05250), PER7 (AT1G30870), PER8 (AT1G34510), PER35 (AT3G49960), PER44 (AT4G26010), PER56 (AT5G15180), PER60 (AT5G22410), PER61 (AT5G24070) and PER73 (AT5G67400), and down-regulated PER28 (AT3G03670), PER49 (AT4G36430) and PER71, which were involved in the phenylpropanoid biosynthesis pathways (EC number 1.11.1.7, Fig. S2). Our analyses revealed that four KEGG pathways had significantly high enrichment values (all had q-value < 0.001, Fig. S3).

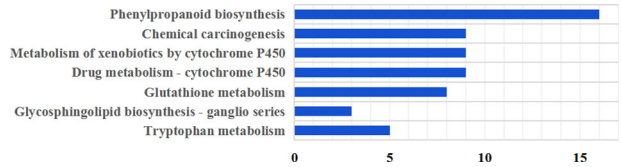

Fig. 3 KEGG classification of differentially expressed genes (DEGs) in roots of *Arabidopsis thaliana* (q-value < 0.05). The horizontal axis is the number of differentially expressed transcripts, and the vertical axis is the KEGG pathway

Functional locations of DEGs in roots of *A. thaliana* after exposure to aqueous extracts from *C. canadensis*

DEGs related to cell wall metabolism

We profiled 25 DEGs related to cell wall metabolism, among which 16 genes were up-regulated and nine were down-regulated (Table S8). Up-regulated genes were related predominantly to reassembly, including expansins (EXP), xyloglucan endotransglucosylases/hydrolases (XTH) and beta-galactosidases (BGAL) (Table S8). In addition, half of the UDP-glycosyltransferase (UGT) related genes were up-regulated in response to extracts from *C. canadensis* (Table S8).

DEGs related to ROS

We also studied the involvement of ROS-related DEGs in response to stress-induced by aqueous extracts from *C. canadensis* (from the T3 group). A total of 25 ROS response-network genes were differentially expressed, including the peroxidase (PER), glutathione-S-transferase (GST), 1-cysteine peroxiredoxin per1, thioredoxin (Trx), ferric reduction oxidase (FRO), glyoxal oxidase (GLOX), perxiredoxin, and NADPH oxidases (Table S9). Of the ROS-related genes, 19 genes were significantly up-regulated and six were down-regulated. PER genes accounted for the highest proportion (12 genes), followed by GST genes (six genes) (Table S9).

DEGs related to transporter process

Of the 31 transporter-related DEGs identified, 14 were significantly up-regulated and 17 were down-regulated after exposure to aqueous extracts from *C. canadensis* (i.e., in the T3 group) (Table S10). These transporter genes were grouped by mode of transport and energy-coupling mechanism into three types: ATP-dependent transporters, secondary transporters, and ion channels. ATP-dependent transporters included two up-regulated ATP-binding cassette (ABC) transporter genes. More than half of these secondary transporters were down-regulated, and the secondary transporters mainly included major facilitator superfamily (MFS) genes, amino acid transporter/permease genes (ATV/AAP), detoxification genes (DTX) and cation/H (+) antiporter (CHX) genes. Finally, we identified four ion channel genes, one of which was up-regulated while the others were down-regulated.

DEGs related to signalling process

We identified 33 DEGs related to signaling genes (protein kinase genes) from the root tissues of *A. thaliana* after being treated with aqueous extracts from *C. canadensis*. Of these, 18 genes were up-regulated and 15 genes were down-regulated (Table S11). The majority of responsive kinases were receptor-like kinases (RLKs), and most of the DEGs from this family were significantly down-regulated. The leucine-rich repeat receptor-like protein kinases (LRR-RLKs) made up the highest proportion of signaling genes in the RLK family and were significantly down-regulated. However, genes from the calcium receptor protein family, including calmodulin and calcineurin B-like interacting protein kinases (CIPK), were mostly up-regulated in response to extracts from *C. canadensis* (Table S11). Seven signaling genes from the protein phosphatases family were identified, three of which were down-regulated.

DEGs related to transcription factors

We found 32 transcription factors were significantly regulated by extracts from *C. canadensis*, with 17 being down-regulated and 15 up-regulated (Table S12). Transcription factors regulated by allelochemical stress predominantly belonged to the APETALA2/ET response factor (AP2/ERF), MYB, WRKY, zinc-finger proteins (ZFPs), basic helix-loop-helix (bHLH) and NAM/ATAF/CUC (NAC) families (Table S12).

Quantitative real-time PCR verification

We selected three DEGs related to stress response in the phenylpropanoid biosynthesis pathway for qPCR analysis. Compared to the CK group, we found the expression of PER1 and SCPL19 in roots of *A. thaliana* significantly increased (i.e., $p < 0.05$) after exposure to aqueous extracts from *C. canadensis*. In contrast, the expression of PER71 was lower than the CK group ($p = 0.041$, Fig. 4). The qPCR analysis validated the mRNA-seq results.

Discussion

Growth, root tip viability, and ROS in *A. thaliana* in response to extracts from *C. canadensis*

Allelochemicals in extracts from *C. canadensis* were found to be phytotoxic to native *A. thaliana* where they reduced its

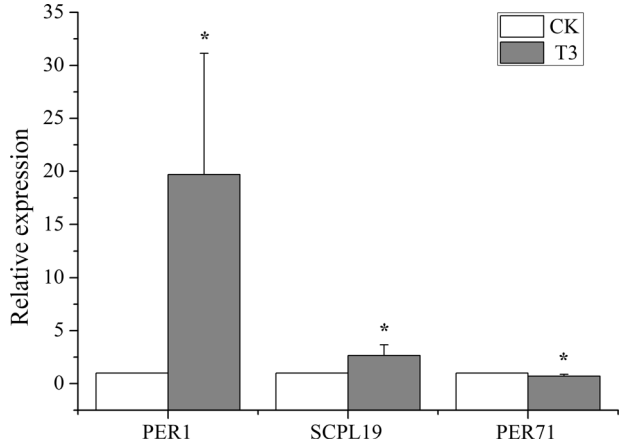

Fig. 4 Expression of three genes isolated from roots of *Arabidopsis thaliana* from the control group (CK) and after being treated with aqueous extracts from *Conyza canadensis* (T3 group, concentration 4.0 mg/mL). Bars are means ± standard deviation ($n = 3$). Significantly different values ($p < 0.05$) are indicated with an asterisk

root growth and tip viability, and increased its ROS levels (Figs. 1 and 2, Fig. S1). Previous studies have shown that the stress experienced by plants in response to allelopathy is associated with the concentration of the allelopathic substance (Iqbal et al. 2010; Silveira et al. 2012; Tigre et al. 2012). For example, as the concentration of aqueous extracts from plant tissues increases, the allelopathic effect on co-occurring plants is more severe (i.e., the 'dose inhibition' effect) (Katonoguchi et al. 1994). This was supported by the results from this study which revealed root growth, root tip viability, and ROS levels of *A. thaliana* were most significantly affected by the highest concentration of aqueous extract from *C. canadensis* (i.e., the T3 group).

Allelochemicals can affect root growth by decreasing root cell viability (Guidotti et al. 2013). Root cell viability is one of the most important physiological indicators of plant growth and directly affects plant biological processes. A plant's growth is dependent on the ability of its root to uptake water and nutrients (Dinneny 2019). As such, when roots are subjected to phytotoxins, root cell viability can be negatively affected, thereby impacting plant growth and development (Mroczek-Zdyrska and Wójcik 2012).

ROS are produced in plants as by-products of cellular metabolism and act as a key regulator of cell physiology and cellular responses to the environment (Ahmad et al. 2008). They are generated in many cellular compartments, including the chloroplast, mitochondria and peroxisomes, and can cause irreversible cell damage and trigger cell growth inhibition and death (Huang et al. 2019). Plants increase their production of ROS in response to environmental stress, including temperature extremes, increased salinity, drought, and phytotoxicity (Bais et al. 2003; Gniazdowska and Bogatek 2005; Ahmad et al. 2008;). In the current study, aqueous extracts from *C. canadensis* induced ROS production in roots of *A. thaliana* (which was positively confirmed by the fluorescence staining, Fig. 2). Surprisingly, we observed that the treatment with the lowest concentration of extract (i.e., the T1 group) showed less fluorescent intensity than the control group (Fig. 2). This result may be because low concentrations of allelochemicals from *C. canadensis* triggered the enzyme protection system in *A. thaliana*, which subsequently eliminated ROS from the roots of the native species (Bailey-Serres and Mittler 2006). Conversely, in highly stressful environments (such as very high concentrations of allelochemicals), excessive levels of ROS are produced that cannot be eliminated from plant tissues and which can damage the antioxidant defense systems and ultimately inhibit root growth (Bais et al. 2003).

Expression of genes related to cell wall metabolism and ROS in *A. thaliana*

Stress from allelochemicals may trigger the expression of genes differentially in plant tissues (Chi et al. 2013). Our results revealed that many genes related to cell wall metabolism (most notably XTH enzymes and EXP genes) were differentially expressed after exposure to extracts from *C. canadensis* (Table S8). Cell wall loosening during plant cell growth is mediated by XTH enzymes (Mcqueen-Mason et al. 1993), while EXP genes are a group of proteins that induce cell wall relaxation and extension (Coleman et al. 1997; Cosgrove 1997). In our study, increased XTH and EXP gene activity suggests that these cell wall-related genes may induce cell enlargement as a response to environmental stresses, thereby protecting the root cells (Christensen et al. 1998; Li et al. 2014).

We found an overall increase in PER activity in *A. thaliana* in response to allelochemicals from *C. canadensis*, which can oxidize and decompose indoleacetic acid (IAA), and ultimately influence cell elongation (Smith et al. 1982; Fujii and Saka 2001). In addition, we found increased activity for many glutathione-S-transferases (GSTs) genes in *A. thaliana* after exposure to extracts from *C. canadensis* (Table S9). GSTs are a family of enzymes commonly associated with detoxification and gene up-regulation in response to many types of stress (Øverby et al. 2015). The up-regulated GSTU4 (AT2G29460), GSTU20 (AT1G78370) and GSTU3 (AT2G29470) (Dixon et al. 2009; Pan et al. 2018) in our study suggest that the application of aqueous extracts from *C. canadensis* induced oxidative stress in roots of *A. thaliana*.

Transporter genes and signalling molecules in *A. thaliana* under allelopathic stress

Secondary transporters are involved in sugar, oligopeptide and nitrate transport (Büttner 2007; Tsay et al. 2007) and

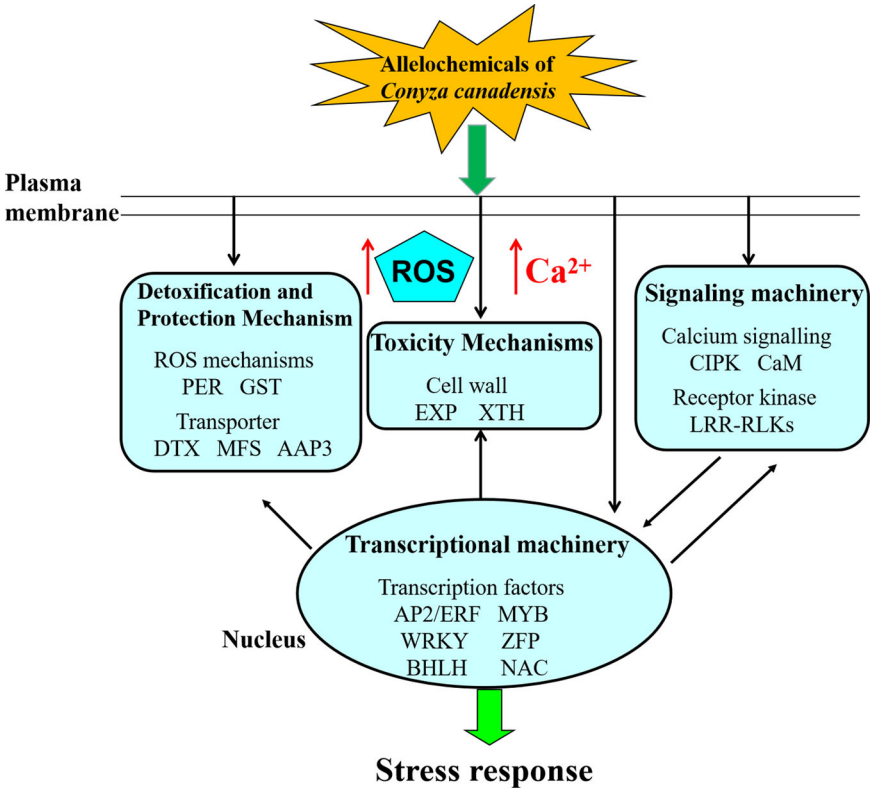

Fig. 5 Molecular model of allelopathic effect of *Conyza canadensis* showing cellular processes and response/regulatory pathways leading to stress response in roots of *Arabidopsis thaliana*

use either ion or solute gradients to translocate substrates across membranes (Wang et al. 2003). Our findings revealed more than half of the secondary transporters in *A. thaliana*, including AAP3 (AT1G77380) and CAT6 (AT5G04770), were down-regulated in response to extracts from *C. canadensis* (Table S10). Given that AAP3 and CAT6 are involved with proton-coupled uptake of structurally diverse amino acids (Okumoto et al. 2004; Su et al. 2004), the down-regulation of these transporters in our study indicates that roots of *A. thaliana* decreased its uptake of amino acids in response to allelopathic stress from *C. canadensis*. In addition, DTX14, a member of the MATE family genes (Upadhyay et al. 2020; Miyauchi et al. 2017) and which is involved with detoxification, was up-regulated indicating that *A. thaliana* likely relies on this transporter to remove xenobiotica and toxins.

Protein kinases are important signaling molecules involved in plant growth and response to environmental stresses (Mizoguchi et al. 1997). For instance, RLK genes are a group of protein kinases that mediate signaling messages at the cell surface and act as key regulators during developmental processes (Gish and Clark 2011). Previous studies have demonstrated that LRR-RLKs (the largest subgroup of RLKs in plants) play important roles in a range of processes during plant growth and development, such as somatic and reproductive cell differentiation (Diévart and Clark 2004; Butenko and Aalen 2012; Li et al. 2017). The LRR-RLKs identified in our study were mostly down-regulated in response to extracts from *C. canadensis* (Table S11). In contrast, most of the CIPK-dependent proteins and calmodulin were up-regulated, as was the gene, HAL1 (AT5G59220) (also known as SAG113) (Table S11). HAL1 is a member of the protein phosphatase 2 C (PP2C) family, and is a negative regulator of abscisic acid (ABA) signaling (Zhang and Gan 2012). ABA is a versatile phytohormone that regulates plant growth and adaptation to environmental stress (Antoni et al. 2012). The upregulation of genes involved in the regulation of ABA signaling in *A. thaliana* in our study suggest that this is an important stress response for this species to allelochemicals from *C. canadensis*.

Transcription factors in *A. thaliana* in response to allelopathy

The production of regulatory proteins, such as transcription factors, can mediate the expression of downstream stress-responsive genes (Pruthvi et al. 2014). In our study, we identified that the transcription factors AP2/ERF, MYB and WRKY were differentially expressed in *A. thaliana* in response to extracts from *C. canadensis* (Table S12). AP2/ERF, MYB and WRKY, have been isolated from many plant species and are important candidates for stress tolerance response (Agarwal et al. 2006; Wang et al. 2007; Ettaki et al. 2018). In fact, the over expression of AP2/ERF, MYB, and WRKY has previously been found to significantly increase tolerance to abiotic stresses in *A. thaliana*

(Yuan et al. 2012; Hsieh et al. 2013; Hyeok et al. 2013). Additionally, we found that aqueous extracts from *C. canadensis* down-regulated ZFPs (Table S12), which have been intensively studied due to the role they play as key regulators of hormone activity in *A. thaliana* (Qu et al. 2014). Specifically, ZFPs play an important role in plant growth regulation via the ABA signal pathway, and cross-talk with the auxin signal pathway (Wang et al. 2011). Finally, we found NAC genes, which are involved in xylem formation and cambial cell division (Kucukoglu 2020), were mostly down-regulated (Table S12). Overall, our findings suggest that the differentially expressed transcription factors identified in this study are likely to be involved in the resistance of *A. thaliana* to allelopathy from *C. canadensis* (see Fig. 5 for a model showing the molecular processes and pathways of stress response in roots of *A. thaliana* to allelochemicals from *C. canadensis*).

Conclusions

Aqueous extracts from *C. canadensis* (in the T3 group, at a concentration of 4.0 mg/mL) inhibited root elongation in *A. thaliana*. Our transcriptome analyses identified 146 DEGs involved in the response of *A. thaliana* to allelopathic stress. EXP and XTH genes were up-regulated following exposure to allelochemicals from *C. canadensis*, while detoxification enzymes, including PER and GST genes, were either up-regulated or down-regulated in response to allelopathic stress. The down-regulated transporter genes, MFS and AAP3, appeared to hinder the transport of nutrients in roots of *A. thaliana* after they were treated with aqueous extracts from *C. canadensis*. Additionally, transcription factors, calcium-regulated proteins, calmodulin and various protein kinases, which are involved in regulatory functions and signal transduction, were differentially expressed when *A. thaliana* was exposed to allelopathic stress. The profiled DEGs provided insights into the molecular pathways utilized by *A. thaliana* when exposed to phytotoxicity from an invasive species. Overall, our findings have implications for the role that allelopathy plays in the invasiveness of *C. canadensis* at the molecular level and could be used in the future to elucidate the mechanisms involved in the invasion success of this species.

Data availability

The sequence data that support the findings of this study have been deposited in the CNSA (https://db.cngb.org/cnsa/) of CNGBdb with accession code CNP0000902.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Consent to publish All authors vouch that the work has not been published elsewhere, completely, in part, or any other form, and that the manuscript has not been submitted to another journal.

Ethical approval Materials used in this study were common plants, and this study does not involve human participants or animals performed by any of the authors.

Plant reproducibility The authors guarantee the reproducibility of experimental results.

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