CwJAZ4/9 negatively regulates jasmonate-mediated biosynthesis of terpenoids through interacting with CwMYC2 and confers salt tolerance in Curcuma wenyujin

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1 INTRODUCTION

Autotrophic plants are sessile and often optimize fitness under harsh environments by producing specialized metabolites (SMs) to reshape the metabolic interface between growth and defense (Weng et al., 2021). Defense terpenoids, as SMs, comprise a large class of compounds widespread throughout the plant kingdom and play crucial roles in the interaction of plants with stresses (Zhou & Pichersky, 2020). Curcuma wenyujin Y. H. Chen et C. Ling (CW), belonging to the family of Zingiberaceae, can produce more than 160...
terpenoids to confront biotic and abiotic stresses (Li et al., 2021; Liu et al., 2012). More importantly, these stress-induced terpenoids in CW represent copious structural varieties and functionalized activities and have been widely exploited as pharmaceuticals (Zhai et al., 2019; Zhang et al., 2020). Given the huge pharmacological properties of terpenoids, their biosynthetic pathway has been recently exploited using high-throughput sequencing platforms (Chen et al., 2022; Jiang et al., 2021; Lu et al., 2020). It is well known that stress-inducible SMs biosynthesis is mediated by concerted transcriptional activation with multiple transcription factors (TFs) (Colinas & Goossens, 2018), in which jasmonates (JAs) play a crucial role. Our previous study revealed that multiple MYC2 TFs, known as master regulators of the JA signaling pathway, are highly co-regulated with 26 ‘signature’ genes of terpenoid biosynthesis (TBS) in CW plants (Jiang et al., 2021). However, the regulatory mechanism related to TBS in CW remains to be uncovered via the JA signaling pathway.

JAs, including jasmonate-l-isoleucine (JA-Ile) and derivative (methyl-jasmonate, MeJA), known as defense signals, play a central part in linking stress perception to large-scale transcriptional responses that permit plant growth and development, as well as producing SMs under harsh environments (Guo et al., 2018a; Kazan & Manners, 2008). In recent decades, tremendous progress has been made in understanding how JA alters hormone-dependent gene expression via the JA signaling framework (Chini et al., 2007; Zhang et al., 2015). JASMONATE ZIM-DOMAIN (JAZ) protein is among the most important components of the JA signaling pathway and represses the activity of downstream bHLH-type MYC TFs or other TFs through their interactions in low JA-Ile level cell circumstances (Chini et al., 2007; Pauwels & Goossens, 2011). When levels of JA-Ile raise a concentration threshold under stress environments, JA-Ile immediately promotes the binding of JAZ proteins and the CORONATINE INSENSITIVE1 (COI1) receptor (Xie et al., 1998). JAZ proteins tagged with the E3 ubiquitin ligase Skp1-Cullin-F-box protein (SCF) COI1 are subsequently ubiquitinated and targeted to the 26S proteasome for degradation (Thines et al., 2007), thereby relieving the repression of MYC TFs and allowing them to activate downstream responses.

JAZ proteins belong to the plant-specific TIFY family and have two canonical domains, ZIM and jas. The ZIM domain controls homo- and heterodimerization between JAZ proteins and interaction with the adaptor protein NINJA (Pauwels et al., 2010). On the other hand, the jas domain with a conserved motif (SLX2FX2KRX2RX5PY), stimulates the COI1-JAZ and a wide range of JAZ-TFs interactions (Fernández-Calvo et al., 2011; Song et al., 2011; Zhang et al., 2015), such as JAZ-MYC2, JAZ-MYB, JAZ-bHLH family TFs. It was reported that JAZ proteins reprogram the SMs’ biosynthesis to produce diverse defense or bioactive compounds (Guo et al., 2018b). Recently, in Arabidopsis thaliana, JAZ6/8 recruits a novel adaptor protein of ECAP to form a co-repressor and suppress JA-responsive anthocyanin accumulation by repressing the WD-repeat/bHLH/MYB complex (Li et al., 2020). SmJAZ8, as a repressor, controls the JA-induced biosynthesis of salvianolic acids and tanshinone in Danshen hairy roots (Pei et al., 2018). In addition, GhJAZ2 from cotton negatively regulates a-linolenic acid metabolism and JA signaling by repressing the transcriptional activity of GhMYC2 (Sun et al., 2017). In comparison with well-characterized MYC2, an understanding of JAZs regulating JA-responsive metabolic reprogramming lags due to multiple restrictions, although JAZ proteins have been widely identified in higher plants (Garrido-Bigotes et al., 2019). Firstly, the JAZ gene family evolves multi-membered JAZ copies, many of which are alternatively spliced to generate diverse JAZ isoforms and play both redundant and specific roles in response to JA (Chini et al., 2016). Secondly, the combinatorial complexity of JAZ-TFs results in the specificity and diversity of JA response (Pauwels & Goossens, 2011). Thirdly, no strong phenotypes in jaz single mutants were observed to explain the functional redundancy of JAZ members. Recent studies showed that the genome/transcriptome-wide data set allows us to identify the sole member of the JAZ family regulating JA-responsive plant growth and metabolic reprogramming (Monte et al., 2019; Oblessuc et al., 2020; Pei et al., 2018; Yang et al., 2015).

In this study, we report the identification, classification, and structural analysis of 62 CwJAZ proteins through transcriptome-wide bioinformatics analysis, and identify two JAZ candidates (CwJAZ4/9) involved in regulating TBS via the JAZ-MYC2 transcriptional module. Transgenic experiments in hairy roots showed that overexpression of CwJAZ4/9 genes significantly decreases the contents of bioactive terpenoids in CW hairy roots, whereas suppression of CwJAZ4/9 enhanced the MeJA-induced terpenoid accumulation. Both CwJAZ4/9 can alter the JA-induced expression of multiple TBS pathway genes. Meanwhile, we establish a putative model that CwJAZ4/9 confers salinity tolerance via the JA, stress signaling, and responses. Our results provide a genetic framework to understand how CwJAZ4/9 controls the TBS reprogramming and the enhanced mechanism of salt adaptability, which makes it feasible to increase the production of high-value terpenoids and, improve resistant CW varieties by a genetic approach.

2 MATERIALS AND METHODS

2.1 Plant material and treatments

Curtcula wenyujin (CW) plants were grown in pots containing commercial soil (Stanley Fertilize, China) using dissected rhizomes (100 g per pot) under a glasshouse with a day/night temperature of 24/18°C. Samples of flower, leaf rhizome, and tuber were collected at 0 (Control), 1, 4, 6, and 10 h after MeJA treatment.

CW hairy roots were developed from the infection of sterile rhizomes with Agrobacterium rhizogenes K599 (For details, see Method S1 and Figure S1). The developed hairy roots from different lines were subcultured for 3 weeks in the MS liquid medium with a...
shaker (110 rpm) at 26°C and then used for further analyses. The 3-week-old hairy roots from CwJAZ4/9 transgenic lines and the control were transferred to a shaking flask with MS liquid medium containing 100 μM MeJA and collected 48 h post-MeJA treatment for RNA-sequencing and RT-qPCR analyses. Nicotiana benthamiana seeds were grown in soil, and 4-week-old seedlings were used for transient expression assays.

2.2 In silico analysis of putative JAZ repressors in C. wenyujin

Two typical Hidden Markov Models (HMMs) of JAZ protein-containing TIFY domain (PF06200) and Jas motifs (PF09425) were used to annotate JAZ family members using HMMER V3.1 with an e-value cutoff of <1e−6. The CW protein data set was derived from amino acid sequences encoded by a full-length transcriptome obtained based on the Pacbio platform (Jiang et al., 2021). The resulting sequences containing TIFY and Jas domains were further confirmed using the SMART program (http://smart.embl.ac.uk/) and NCBI CDD search tool (https://www.ncbi.nlm.nih.gov/cdd). The complete amino acid sequences of all CwJAZ proteins were further analyzed with MEME (https://meme-suite.org/index.html) to discover their conserved motifs. Amino acid sequences of CwJAZ proteins and known JAZs from other species were aligned with Clustal X2 software. Phylogenetic trees were constructed to classify CwJAZ proteins using MEGA-X software with the maximum-likelihood method and 1000 replicates bootstrap test. The generated tree was rendered using Evolview v3 (Subramanian et al., 2019).

Previously reported transcriptome data from four different tissues (flower, leaf, rhizome, tuber) were obtained from the Sequence Read Archive (SRA) database (BioProject accession: PRJNA481732). The tissue-specific (TS) expression profile of CwJAZ genes was analyzed using our previously developed TS scoring algorithm (Wu et al., 2020). To further analyze the correlation between CwJAZ gene expression and terpenoid accumulations, we performed a Pearson correlation analysis based on transcriptomic and metabolomics data set. and metabolome from CW samples has been detected by GC-MS from parallel samples with transcriptome analysis (Jiang et al., 2021). In this analysis, putative CwJAZ candidates involved in regulating TBS were considered to be typical structure JAZs that show higher expression levels relative to other JAZ members and negatively correlated (Student’s t-test for both types of data, p values < 0.05) with main terpenoids. The heatmap of correlations was performed by the pheatmap package in R (version 4.1.1).

2.3 Plasmid construction and generation of transgenic hairy roots

Full-length coding sequences (CDSs) of CwJAZ4/9 were amplified into the pTOPO-TA vector by PCR from the CW leaf cDNA template and subcloned into a modified pCAMBIA2304 vector using the Nimble Cloning method (Yan et al., 2020), containing the CaMV35S promoter and the nptII terminator, to generate overexpression plasmids. To construct the RNA interference (RNAi) vector, 200 bp (for CwJAZ4) and 270 bp (for CwJAZ9) interference fragments in their coding regions were subcloned into the modified pCAMBIA2304 vector, as described above. The resulting plasmids were mobilized into Agrobacterium rhizogenes K599 through the freeze-thaw method and then transferred into hairy roots to generate transgenic lines using the protocol as described for wild hairy root development. Transgenic hairy roots were verified by PCR amplification of rolB, and interest genes. Two independent hairy root lines for each genotype were selected for downstream analyses.

2.4 Yeast two-hybrid (Y2H) assay

The CDSs of CwJAZ4/9 were first fused with the GAL4 DNA-binding domain in the pGBK7T vector to ensure that there were no auto-activation and toxicity through self-activation tests in yeast. In addition, the CwMYC2 CDS was amplified by PCR and subcloned into the pGADT7 vector. The resulting plasmids were co-transformed into the yeast strain AH10 using the Matchmaker GAL4 Two-Hybrid System (Clontech). Successfully transformed colonies were identified on SD medium lacking Leu and Trp. The positive clones were transferred to and grown on SD-Trp/-Leu and SD-Trp/-Leu/-His/-Ade plates. Plates were photographed after incubation at 28°C for 4 d. The empty vector pGBK7T (bait control) and pGADT7 (prey control) were co-transformed as the negative control.

2.5 Luciferase complementation imaging (LCI) assays

The in vivo interaction between CwJAZ4/9 and CwMYC2 was detected using LCI assays in tobacco (N. benthamiana) leaves as described previously (Chen et al., 2008). In brief, the CDSs of CwJAZ4/9 were subcloned into the p1300-35S-nLUC vector. The CwMYC2 CDS was obtained by PCR and ligated into the p1300-35S-cLUC vector. The constructed plasmids were separately transferred into A. tumefaciens K599 through the freeze-thaw method and subcloned into the p1300-35S-nLUC vector. The CW protein data set was derived from RNA-sequencing and RT-qPCR analyses. Nicotiana benthamiana seeds were grown in soil, and 4-week-old seedlings were used for transient expression assays.

2.6 Pull-down assays

The CDSs of CwJAZ4/9 and CwMYC2 were cloned into the pET30a and pGEX-6p-1 vectors, respectively (specific primers listed in Table S1), and then introduced into Rosetta (DE3) cells to yield...
GST or His tag fusion proteins. The His-CwJAZ4/9 and GST-CwMYC2 fusion proteins were expressed by using 0.1 mm isopropyl-β-thiogalactopyranoside induction at 20°C for 16 h and purified using GST Bind Resin (Millipore) or Ni-IDA Resin (Clontech). For in vitro pull-down assay, purified GST fusion proteins (GST-CwMYC2 and the negative control GST) were further incubated with GSTagarose beads (Solarbio) in PBS buffer for 4 h at 4°C, and then His-CwJAZ4/9 fusion proteins were added and incubated for another 2 h at 4°C. Next, the resin was washed five times with PBS buffer and resuspended in SDS sample buffer. The collected proteins were detected by western-blot analysis using anti-His (Proteintech) and Anti-GST(YEASEN) antibodies.

### 2.9 JA and JA-Ile measurements

The hairy roots 0, 2, 4, 6, and 12 h post-salt treatment were sampled and ground to powder in liquid nitrogen for hormone detection. Approximately 200 mg for each treatment was extracted twice with 2 mL of ethyl acetate (0.5% acetic acid) containing a mixture of D2-JA and [13C6]-JA-Ile as internal standards. The combined extracts were dried under a stream of nitrogen gas, and the pellet was reconstituted in 0.2 mL of 70% methanol. The resulting extract was centrifuged at 18000 × g for 30 min at 4°C. Ten microlitres of the supernatant was analyzed using a quadrupole time-of-flight mass spectrometer (MS) (TripleTOF 6600; AB SCIEX) combined with a UPLC system (Agilent 1290 Infinity LC system) with the HSS T3 column (1.8 μm, 2.1 mm × 100 mm, Waters). Analysis and quantification of JA, JA-Ile were performed as described previously (Yan et al., 2016). The final concentrations of JA and JA-Ile were calculated by comparing their abundances with those of internal standards.

### 2.10 Gas chromatography (GC)-MS analysis

The 3-week-old transgenic hairy roots and the control (EV) were collected and ground to powder in liquid nitrogen. Terpenoid extraction and measurements were performed using a GC-MS system (Agilent7980 GC-5977B Mass spectrometer) equipped with a DB-Wax mass spectrometry column (30 m × 250 μm × 0.25 μm, Agilent), as previously described (Cao et al., 2006). Known standards (Germacrone, Curcumol, Curdione, Furanodiene, Curzerene, β-elemene) (Yuanye Bio-Technology) were run to identify elution times and mass fragments. All experiments were performed with three independent replicates for each sample.

### 2.11 Quantitative real time-PCR assay

Total RNA from frozen samples of collected CW tissues above and hairy roots were isolated with the RNAprep Pure kit including DNase treatment (DP441/412; TIANGEN Biotech). The cDNA template was prepared with 1 μg of total RNA using a PrimeScriptTM RT reagent kit with a gDNA Eraser (TaKaRa). Quantitative RT-qPCR assays were performed using the ABI7500 real-time PCR system (Applied Biosystems) with the RealMasterMix (SYBR Green I; Takara) as described previously (Jiang et al., 2021). The experimentally optimized CW 18S gene was used as an internal control. All RT-qPCR experiments were performed in triplicate and repeated at least twice.

### 2.12 RNA-seq and data analysis

Two independent lines of CwJAZ4_Ox, CwJAZ9_Ox, CwJAZ4_Ri, and CwJAZ9_Ri subjected to 48 h MeJA treatment (see Plant material and treatments) were separately merged and collected
for RNA extraction. The MeJA-treated EV line was used as the control. Three independent RNA samples per line were sequenced on Illumina HiSeq 2500 sequencer (Illumina Inc.) with paired-end mode (PE150) as our previously described methods (Jiang et al., 2021). The expression level was normalized into fragments per kilobase of exon per million fragments mapped (FPKM) using Cufflinks (Trapnell et al., 2012). DESeq. 2 was used to assess differentially expressed genes (DEGs) relative to EV, according to Benjamini & Hochberg’s false discovery rate (FDR) correction p value < 0.01 and |fold change| ≥ 2. Gene ontology (GO) enrichment analysis was performed using the clusterProfiler package (Yu et al., 2012), with FDR < 0.01.

3 | RESULTS

3.1 | Identification of putative JAZ proteins in CW

Given the highly conserved ZIM and Jas domains in the JAZ family, we predicted a total of 62 full-length CwJAZ proteins using the HMM search program (Dataset S1). These sequences were referred to as CwJAZ1-CwJAZ62 afterward. To determine the classification and the evolutionary history of CwJAZ proteins, we constructed an unrooted phylogenetic tree based on amino acid sequences of 62 CwJAZ proteins and known JAZs from other species. As shown in Figure 1, all JAZ sequences were grouped into five major groups.

**FIGURE 1** Phylogenetic analysis for JAZ proteins from C. wenyujin and other species. At, Arabidopsis thaliana; Cw, C. wenyujin; Mp, Marchantia polymorpha; Os, Oryza sativa; Pp, Physcomitrella patens; Vv, Vitis vinifera. A bootstrap maximum-likelihood phylogenetic tree was constructed to reveal the relationship between Cw and other species using MEGA-X software. The numbers on the branch represent bootstrap values from 1000 replicates. JAZ proteins with consistent background color indicate that they can be clustered into the same group. JAZ proteins marked in red represent the JAZs from lower lower bryophyte plants. JAZ proteins in the tree are available in Dataset S1. [Color figure can be viewed at wileyonlinelibrary.com]
LPIAR(R/K) sequences (Figure S3) that can make up the core region of 23 CwJAZ proteins displays the canonical degron 29 TS end of the C terminus using the MEME web server (Figure S2). Similar to the topology of the unrooted tree constructed above, CwJAZ proteins were classified into five major groups. The variation of amino acid residues in the Jas domain results in JAZ functional divergence (Melotto et al., 2008). To characterize this variation, we performed an alignment analysis of multiple sequences and found that the Jas region of 23 CwJAZ proteins displays the canonical degron LPIAR(R/K) sequences (Figure S3) that can make up the core-receptor COI1-JAZ complex by binding to the F-box protein COI1 protein in the presence of JA-Ile (Xie et al., 1998). The degron variants occurred frequently at the first and third residues. In addition, the most conserved sequence of the Jas motif was recognized as HSLQRFLEKRKDR and represented 42.4% (14 proteins). Compared with the degron and Jas motif, NLS in CwJAZs was the most variable motif and shared the basic XXXXPY sequences. In this study, 56 members of 62 CwJAZ proteins had NLS motifs (Figure S3), except for CwJAZ26/30/31/42/43/49. Of them, the prevailing NLS motif is recognized as V(H)S(P) KAPY residues (12 proteins). Interestingly, we could not find the LxLxL-type ethylene-responsive factor associated with the amphiphilic repression (EAR) domain in CwJAZs, indicating that these CwJAZ members may not recruit the TOPLESS to mediate the transcriptional repression of jasmonate responses (Kagale et al., 2010; Pauwels et al., 2010).

3.2 Tissue-specific profiles of CwJAZ transcripts and their correlations with terpenoid metabolites

Considering that plant-specialized metabolites are often produced in a tissue-special manner (Colinas & Goossens, 2018), we, therefore, analyzed CwJAZ transcript profiles to link the spatial accumulation of terpenoids using our previously reported transcriptomic and metabolomics datasets from different CW tissues (flower, leaf, rhizome, and tuber) (Jiang et al., 2021). Among 62 CwJAZs identified above, 55 transcripts were expressed in at least one tissue, with varying expression patterns in different tissues. Here, we used a tissuespecifically (TS) expressed scoring algorithm, as previously characterized (Wu et al., 2020), to identify TS gene sets. This analysis yielded 29 TS-expressed CwJAZs with a TS score > 0.5. Clustering analysis showed that these TS CwJAZ transcripts exhibit strong tissue specificity and peak expressions in their corresponding tissues (Figure 2a). In particular, we noted that TS CwJAZ transcripts were abundant and highly expressed in flower (11 TS genes) and leaf (10 TS genes), in which terpenoids are accumulated at a lower level than in rhizome and tuber (Jiang et al., 2021). Furthermore, we randomly selected 10 TS JAZs (CwJAZ1/4/6/7/9/14/15/39/40/48) and re-verified their tissue-specific expression profiles using RT-qPCR assays (Figure S4).

To better understand the contribution of CwJAZs to TBS, we performed a correlation analysis across the expression levels of TS CwJAZ transcripts and the abundance of terpenoids in CW different tissues. As shown in Figure 2b, the expressions of 25 CwJAZs (most as TS genes) were negatively correlated with the accumulations of 15 CW major terpenoids. Notably, several CwJAZs, including CwJAZ22/3/4/7/9/14/15/16/21/23/26/38/48, showed significantly negative relationships with some main bioactive constituents (curdione, furanodiene, cumenol, germacrone and others) in CW, probably implying their critical roles in negatively regulating the TBS. Together, these narrowed gene sets of CwJAZs were potential candidates for controlling terpenoid production.

3.3 Identification of putative CwJAZ genes involved in the JA signaling

JAZ acts as a repressor of downstream core regulator MYC2 via the JAZ-MYC signaling cascade (Goossens et al., 2015). Therefore, relevant candidate JAZ genes involved in the JA pathway would be those that repress the expression of MYC2. Previous studies reported that a cryptic MYC interacting domain (CIDM) at the N-terminal of some JAZ proteins (AtJAZ1/10) has been considered to be specific for interaction with MYC TFs (Goossens et al., 2015; Zhang et al., 2017), and the basic FxxxCxLxxY motif is highly conserved amino acids of CMID in higher plants (Garrido-Bigotes et al., 2019). Based on this speculation, we aligned the fragments of these CwJAZ candidates obtained above with AtJAZ1 as a reference and found that only CwJAZ4/9/15/16/21/23/48 have a typical CMID region at the N-terminal (Figure 3a, Figure S5).

To further determine whether these CwJAZs genes containing CMID respond to JA signals, we detected their expression changes in MeJA-treated leaves using RT-qPCR assays. The expression levels of all analyzed CwJAZ genes were upregulated to varying magnitudes by durative MeJA treatment. In particular, CwJAZ4/9 transcript levels were rapidly elevated by more than 109-fold and 113-fold at 1 h post-MeJA treatment (Figure 3b), respectively, and then decreased sharply. By contrast, CwJAZ15/16//21/23/48 levels were upregulated gradually, reaching their maximums at 4 h or 6 h, with relatively lower expression changes than that of CwJAZ4/9. In addition, CwMYC2 displayed the same expression pattern with CwJAZ4/9 (Figure 3b), whose abundance was increased by more than 167-fold after 1 h MeJA treatment relative to the control (0 h), demonstrating their huge responses to JA treatment at the early stage. Interestingly,
CwJAZ4/9 exhibited highly leaf-specific expressed profiles that largely inverse bioactive terpenoid accumulation (Figure 2b, Figure S4). We, therefore, speculated that CwJAZ4/9 may play a prominent role in repressing the biosynthesis of terpenoids in CW by JA signaling.

### 3.4 CwJAZ4/9 forms heterodimers and directly interacts with CwMYC2

Since leaf-specific CwJAZ4/9 exhibited the highest expression changes at the early stage of MeJA treatment and had typical CMID domains, they were thus chosen for functional experiments. To determine whether CwJAZ4/9 are involved in the JA signaling by the interaction with CwMYC2, we expressed CwJAZ4/9 genes in the pGBKT7 vector, and CwMYC2 in the pGADT7 vector. Self-activation tests of all investigated proteins confirmed that they have no self-activation properties in the Y2H system (Figure S6). Y2H screening showed that the yeast could grow on the selective synthetic medium (SD-Trp/-Leu/-His/-Ade) when co-transformed with CwMYC2 and CwJAZ4/9 vectors, suggesting that CwJAZ4/9 interacts with CwMYC2 protein in yeast cells (Figure 3c). Similarly, LCI assays showed that the co-transformation of CwJAZ4/9-cLUC and CwMYC2-nLUC resulted in the LUC signals in tobacco leaves when the fusion proteins had the same localization and direct interaction (Figure 3d). To substantiate the above observations, in vitro pull-down experiments were further performed, in which both His-CwJAZ4 and His-CwJAZ9 recombinant proteins were pulled down by GST-CwMYC2 (Figure 3e). In contrast, the His-CwJAZ4/9 proteins were not captured in the control (without GST-CwMYC2 as bait). These results demonstrated that CwJAZ4/9 proteins physically interact with CwMYC2 in vitro and in vivo, indicating that both
**FIGURE 3** (See caption on next page).
CwJAZ4/9 proteins act as upstream co-repressors of CwMYC2. On the other hand, interactions between CwJAZ4 and CwJAZ9 were also detected by Y2H and LCI assays. Results revealed that CwJAZ4/9 interacts with each other in yeast and tobacco leaves (Figure 3c,d), strongly indicating that they form heterodimers to exercise biological functions.

On the other hand, MYC2 may influence JAZ protein localization (Withers et al., 2012). To determine the subcellular localization of CwJAZ4/9, the CwJAZ4/9-GFP fusion constructs and 35S: GFP control vector were separately co-introduced into tobacco protoplasts with the nuclear NLS-mKate marker. As shown in Figure 3f, the GFP signals of CwJAZ4/9-GFP fusion proteins were exclusively observed in cell nuclei and completely co-localized with the RFP signals of NLS-mKate, whereas the control 35S: GFP showed fluorescence in both cytoplasm and nuclei. These results strongly indicated that CwJAZ4/9 proteins act as targets to the cell nucleus as regulators in the transcriptional system. Based on these findings, we believe that CwJAZ4/9 genes are elite candidates involved in the JA signaling and regulate the TBS by repressing CwMYC2.

3.5 | CwJAZ4/9 inhibits the biosynthesis of terpenoids by modulating the expression of JA-induced genes

To determine whether CwJAZ4/9 regulates the TBS through the JA signaling, we generated overexpression and RNA interference (RNAi) hairy root lines (CwJAZ4_Ox, CwJAZ9_Ox, CwJAZ4_Ri and CwJAZ9_Ri) under the control of the CaMV35S promoter, respectively, with the empty vector (EV) control. The positive transgenic lines were verified by specific primers and two independent lines were selected for further experiments (Figure S7). Consequently, the abundances of CwJAZ4/9 were significantly raised in overexpression lines (CwJAZ4/9_Ox) relative to the control (EV), with an increase of over 50-fold, whereas their expressions were distinctly reduced by more than 15-fold downregulation in RNAi lines (CwJAZ4/9_Ri) (Figure 4a), indicating the effectiveness of transgenic manipulation.

To determine whether CwJAZ4/9 influences the JA signaling induced by MeJA, the expression levels of marker genes involved in JA biosynthesis (CwAOS1, CwJAR1) and signaling (CwMYC2, CwCOI1) in transgenic lines were quantified using RT-qPCR assays. As expected, in response to MeJA, all investigated genes were significantly repressed in CwJAZ4/9_Ox lines and increased in CwJAZ4/9_Ri lines (Figure 4b). This suggested that CwJAZ4/9 reduced the JA sensitivity and may be components of a negative regulatory loop that controls the JA signaling pathway. Furthermore, we analyzed the expression levels of our previously identified TBS pathway genes in transgenic lines (Jiang et al., 2021). Under MeJA treatment, overexpression of CwJAZ4/9 significantly repressed the expression of CwFPS2, CwTPS5 and CwTPS10, whereas silencing of CwJAZ4/9 substantially enhanced the transcript levels of these genes (Figure 4b). The effects of transcriptional regulation of CwJAZ4/9 on terpenoid profiles in overexpression- and silencing- hairy root lines were further monitored by GC-MS (Figure 4c). Accordingly, the concentrations of several pharmacologically important metabolites that were negatively correlated with CwJAZ4/9 (Figure 2b), including germacrone, curcumol, curdiene, furanodiene, curzerene, β-elemene, were significantly downregulated by 30%-70% reductions in MeJA-induced Ox lines and upregulated by 2-3.5 fold increases in MeJA-induced RNAi lines, compared with control (EV). This accumulation pattern of terpenoids was consistent with the expression changes of TBS pathway genes. Taken together, these results suggested that CwJAZ4/9 negatively regulated the TBS by repressing the JA signaling.

**FIGURE 3** CwJAZ4/9 proteins are involved in the JA signaling pathway through interaction with CwMYC2. (a) Sequence logos and various CMID of the N-terminal region for typical CwJAZ proteins. The complete alignment of these sequences is available in Figure S5. The arrowheads indicate highly conserved amino acids (FxxxCxxLxxY) of CMID for JAZ proteins from monocots and dicots, created by Garrido et al. (2019). CMID, cryptic MYC2-interacting domain. (b) Time course of the MeJA induced expressions of CwJAZs and CwMYC2 in CW leaves, demonstrating that CwJAZ4/9 genes are rapidly induced by MeJA. Expression levels were normalized to the CW 18s expression level by RT-qPCR. Data are means ± standard deviation from three biological replicates. (c) Y2H assays of the CwJAZ4/9-OX lines and upregulated by 2-3.5 fold increases in MeJA-induced RNAi lines, compared with control (EV). This accumulation pattern of terpenoids was consistent with the expression changes of TBS pathway genes. Taken together, these results suggested that CwJAZ4/9 negatively regulated the TBS by repressing the JA signaling.
FIGURE 4  (See caption on next page).
3.6 | CwJAZ4/9 genes repress the salt-mediated JA responses

JAZ proteins often play important roles in regulating plant development and defense processes by the JA signaling when subjected to various abiotic stresses (Oblessuc et al., 2020; Valenzuela et al., 2016). In this study, we treated the control hairy roots (EV) with salt (100 mM NaCl) to investigate whether CwJAZ4/9 genes regulate the stress-induced responses. In line with the expression profiles induced by MeJA (Figure 3b), the expressions of CwJAZ4/9 genes were highly induced by salt at the early stage, with >40-fold increases at 2 h post-salt treatment and thereafter a rapid reduction (Figure 5a). Meanwhile, we analyzed the time course of JA and JA-Ile accumulation in response to salt stress (Figure 5b). The levels of JA and JA-Ile increased sharply by 8.4-fold and 5.6-fold, respectively, within 2 h after salt treatment. Subsequently, these levels decreased slowly with the extension of treatment (from 2 to 12 h) but remained relatively higher than that of the control (0 h). This indicated that the salt-induced rapid activation of CwJAZ4/9 is tightly correlated with JA and JA-Ile accumulation.

To deeply understand the contribution that CwJAZ4/9 genes influence salt-mediated responses by the JA signaling, RT-qPCR analyses were further performed to reveal the expression of genes involved in JA responses during salt stress. Expressions of CwMYC2, CwCOI1, CwJAR1 and CwAOS1 in all lines were induced after 4 d salt treatment; however, the magnitudes of induction were significantly different among transgenic lines. Compared to the normal environment (MS), salt stress slightly mediated the expressions of JA-responsive genes in CwJAZ4/9_Ox lines, or with no significant changes. Conversely, such salt-mediated expression of JA-related genes was significantly increased in CwJAZ4/9_Ri lines with the largest rising alterations (Figure 5c). On the other hand, the expressions of all investigated genes in EV were moderately induced by salt. These results suggested that CwJAZ4/9 repressed the salt-mediated effects downstream of the JA signaling pathway.

3.7 | CwJAZ4/9 confers salt tolerance in CW hairy roots

To further explore the effect of CwJAZ4/9 genes in regulating hairy root growth under salt stress, we measured the length of transgenic CW hairy roots subjected to salt treatment. As shown in Figure 6a, the growth length of hairy roots of transgenic and EV lines had no obvious changes under normal circumstances but was distinctly inhibited after 4 d salt treatment. Interestingly, the length of EV and RNAi lines was inhibited more severely than that of overexpression lines. In addition, we detected the accumulation of proline, as a key signaling molecule activating defense and survival mechanisms (El Moukhtari et al., 2020), as well as the total antioxidant capacity in all investigated lines. Salt significantly mediated the proline accumulation in overexpression and EV lines, but CwJAZ4/9_Ox lines had higher relative increasing doses of proline than that of EV. Surprisingly, the salt-mediated proline accumulation was not observed in CwJAZ4/9_Ri lines, the content of proline significantly decreased (Figure 6b). Under normal conditions, CwJAZ4/9_Ox lines resulted in 1.4–2.0-fold increases in TEAC levels compared with that of EV, whereas RNAi lines showed a 43%–77% reduction (Figure 6c). After 4 d salt treatment, the TEAC levels in EV and RNAi lines were strongly decreased. However, the significant reduction induced by salt was not expectedly achieved in CwJAZ4/9_Ox lines, implying that CwJAZ4/9 genes maintain high antioxidant capacity even under salt stress. These results suggested that CwJAZ4/9 reduces stress-mediated sensitivity and confers salt tolerance in CW hairy roots.

3.8 | Global transcript profiling identifies CwJAZ4/9-‐dependent gene expression

To further understand the MeJA-mediated gene expression changes under the control of CwJAZ4/9, we performed RNA-sequencing to compare transcript profiles of EV (the control), CwJAZ4/9_Ox, and CwJAZ4/9_Ri hairy roots grown in the presence of MeJA. We here used a stringent statistical criterion to define CwJAZ4/9-‐dependent genes. Firstly, candidate genes showed a reverse expression pattern between overexpression lines and RNAi lines and were differentially expressed both in overexpression lines versus EV, and RNAi lines versus EV. Secondly, of these DEGs identified, we selected overlapped candidates between CwJAZ4 and CwJAZ9 transgenic lines as the final gene lists. Based on this analysis, we identified 1201 CwJAZ4/9-‐dependent genes that were differentially expressed between CwJAZ4/9_Ox lines and the EV, and 994 genes showing significant expression differences between CwJAZ4/9_Ri lines and the EV (Figure 7a).

GO analysis of 1201 DEGs in CwJAZ4/9_Ox lines showed that GO terms, including defense response, response to oxidative

**FIGURE 4** CwJAZ4/9 alters the expression of genes involved in JA signaling and terpenoid biosynthesis and regulates the production of terpenoids. (a) Relative expression of CwJAZ4/9 in CwJAZ4/9 transgenic and the control (EV) hairy root lines. (b) Relative expression of genes related to the JA signaling and terpenoid biosynthesis in transgenic and EV lines with 100 µM MeJA treatment for 48 h. All gene levels were determined by RT-‐qPCR and normalized to the CW 18S expression levels. (c) Measurement of representative terpenoids in transgenic hairy root lines with 100 µM MeJA treatment for 48 h. Terpenoids in all investigated lines were analyzed by GC-‐MS, and the concentrations of terpenoids were estimated based on peak area compared to standards. Values presented here represent means ± standard deviation from three biological replicates. Statistical significance was calculated using Dunnett’s multiple comparisons tests. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. GC-‐MS, gas chromatography-‐mass spectrometer.
stress, cell wall, ATP metabolic process, etc, were significantly enriched biological processes (Figure 7a). Likewise, the CwJAZ4/9-dependent 994 gene set in RNAi lines were associated with GO terms, including jasmonic acid biosynthetic process, response to hormone stimulus, response to ethylene stimulus, and terpenoid biosynthetic process. Next, we focused on over-represented genes involved in these enriched biological processes. We found that most of the genes related to stress signaling and defense
response were significantly upregulated in CwJAZ4/9_Ox lines and downregulated in CwJAZ4/9_Ri lines, including known genes encoding plasma membrane Na⁺/H⁺ antiporter (SOS1), CBL-interacting serine/threonine-protein kinase (SOS2), mitogen-activated protein kinase (MAPK6), aquaporin protein (PIP), K⁺ efflux antiporter (KEA), glutathione synthetase (GSH), betaine aldehyde dehydrogenase (BADH), proline biosynthetic enzymes (P5CS, P5CR, OAT), stress-responsive proteins (proline-rich...
protein, PEK; heat shock protein, HSP), pathogenesis-related protein (PR) (Figure 7b, Table S2). In addition, most genes involved in JA biosynthesis and response, TBS and regulation, were repressed in CwJAZ4/9 Ox lines and enhanced in CwJAZ4/9 Ri lines (Figure 7b, Figures S8–9), which was consistent with the RT-qPCR confirmed results (Figure 4b). Notably, multiple genes encoding ethylene-responsive transcription factor1 (ERF1), AP2 domain transcription factor PLETHORA1 (PLT1) that integrate JA and ethylene signals to regulate various defense responses and SM biosynthesis (Chen et al., 2011; Cheng et al., 2013), were highly expressed in CwJAZ4/9 Ox lines and suppressed in CwJAZ4/9 Ri lines (Figure 7b). Importantly, ABA stress-related processes were also activated in CwJAZ4/9 Ox lines by upregulation of ABA-responsive genes encoding zeaxanthin epoxidase (ZEP), PYL2/6/8, SNF1-Related Protein Kinases type 2 (SnRK2), etc. (Dataset S2). We also observed that several DELLA repressors of the gibberelin (GA) signaling pathway were strongly repressed in CwJAZ4/9 Ox lines. These findings suggest a potential role for CwJAZ4/9 as negative regulators of MeJA-induced TBS in CW and as positive regulators of stress responses and further highlight the complex crosstalks between JA signaling and other phytohormone signalings (Song et al., 2014).

4 | DISCUSSION

4.1 | CwJAZ4/9-MYC2 transcriptional cascade regulates the biosynthesis of terpenoids in C. wenyujin

Although bioactive JA has long been recognized as an elicitor of SMs through altering a multitude of transcriptional programs (Wang et al., 2019; Zhou & Memelink, 2016), the underlying transcriptional mechanisms that control SMs pathways in different stress environments and specific cell types still need to be explored. Recent progress has come from studies on JA signaling showing that JAZ proteins repress JA responses through their interaction with a series of TFs to control the biosynthesis of SMs (Guo et al., 2018b; Major et al., 2017; Xie et al., 2016; Zhu et al., 2022). For medical plants, the identification of sole JAZ that influences SMs, especially for defensive metabolites with huge pharmacological effects, is deeply interesting for genetically improving their traits. However, it is difficult to find which individual JAZs integrate JA signaling to regulate SMs, because of the existence of diverse JAZ gene copies. Consistent with previous studies (Chao et al., 2019; Song et al., 2022), we also observed redundant JAZ members (62 individuals) and divergent domain (Tify, Jas) sequences in CW. A high number of JAZs...
members results in functional redundancy, such as that reported in A. thaliana (Liu et al., 2021) and S. miltiorrhiza (Ma et al., 2022). Here, we used a selection strategy that candidate genes are negatively correlated between their expressions and terpenoid accumulations in the same tissues, and quickly identified CwJAZ2/3/4/7/9/14/15/16/21/23/26/38/48 genes that potentially influence the TBS. The presence of CMS, md, and degron sequences has been proposed as the mechanism to explain which JAZ proteins behave as degradable constitutive repressors of JA signaling (Zhang et al., 2017). For instance, the COI1-interacting degron of JAZs includes a conserved LPIARR(K) loop region that encloses JA-Ile in its binding pocket (Sheard et al., 2010). Among these CwJAZs, only CwJAZ4/9/15/16/21/23/48 proteins bear a CMID and degron region (Figure 3a, Figure S5), of which CwJAZ4/9 expression levels were sharply upregulated at 1 h post-MeJA treatment in CW leaves (Figure 3b). These results strongly support that CwJAZ4/9 operates the JA signaling activated by MeJA.

JAZ proteins bind directly to and repress the activity of an expanding range of TFs involved in specific aspects of JA-induced responses, such as MYC2/3/4, MYB21/24, AaTCP14, ABI5, and bHLH proteins (Cheng et al., 2011; Fernández-Calvo et al., 2011; Ju et al., 2019; Ma et al., 2018; Niu et al., 2011; Song et al., 2011). Among these JAZ targets, JAZ proteins particularly regulate a specific MYC-dependent transcriptional cascade to modulate SM biosynthesis and various defense responses. Consistent with previous findings (Major et al., 2017; Zhang et al., 2012), our results from Y2H, LCI, and in vitro pull-down assays, and transgenic experiments in hairy roots established a direct link between CwJAZ4/9 and their interacting CwMYC2 in controlling terpenoid production. Compared with the control (EV), silencing and overexpression of CwJAZ4/9 significantly increased and decreased the MeJA-mediated terpenoid accumulation in CW hairy roots, respectively. Meanwhile, silencing and overexpression of CwJAZ4/9 enhanced and repressed the MeJA-mediated expression of multitudinous genes involved in JA biosynthesis and responses (Figure 4b; Figure S8; Dataset S2), respectively, which directly resulted in the enlargement and reduction of the MeJA-induced JA responses. This result coincides with a mechanistic explanation that exogenously applied JA in JAZ knockdown lines accelerates the degradation of JAZ proteins and releases the JAZ-targeted MYC2/3/4 to regulate the expression of JA-responsive genes (Chini et al., 2007; Fernández-Calvo et al., 2011). In addition, CwJAZ4/9 can form heterodimers (Figure 3c,d), thus allowing them to regulate each other at the protein level. Based on these findings, we propose that CwJAZ4/9 acts as transcriptional repressors of JA signaling, and forms CwJAZ4/9-MYC2 cascade to regulate the TBS in CW.

Our transcriptome results in CwJAZ4/9 transgenic lines also provide new insight into processes underlying the expression of pathway genes related to TBS. We found that genes upregulated in CwJAZ4/9_Ri lines are enriched in the categories related to JA biosynthesis and response, as well as secondary metabolism, including TBS (Figure 7a, Dataset S2). In particular, a previously characterized gene module containing CwFPS2, CwTPS5/10, CwCYP2/4/6 (Jiang et al., 2021), and multiple pathway genes from the MVA and MEP routes, are coordinately upregulated in CwJAZ4/9 RNAi lines and downregulated in CwJAZ4/9_Ox lines (Figure 4b, Figure S9, Dataset S2), which is consistent with the role of JA elicitor to mediate CW terpenoid production (Wei et al., 2022). MYC2 can activate the expression of SM pathway genes by binding the G-box elements present in their promoters. For example, AaMYC2 promotes artemisinin biosynthesis by upregulating the expression of CYP71AV1 and DBR2 (Shen et al., 2016); Arabidopsis MYC2 directly targets the TPS11/21 promoters and promotes sesquiterpene production (Hong et al., 2012). In this study, CwMYC2 and terpenoid pathway genes showed consistent expression patterns in both CwJAZ4/9 transgenic hairy roots, implying that these pathway genes are likely targeted by CwMYC2. Compared with pathway genes involved in multiple enzymatic steps, metabolic engineering of CwJAZ4/9, and CwMYC2 is potentially an effective tool in regulating the production of target terpenoids in CW.

4.2 CwJAZ4/9 genes confer salinity tolerance by weakening the salt-mediated JA responses

Increasing evidence has documented that plant JAZ can alleviate salt-mediated responses and improve root cell growth during salt stress via the JA signaling pathway (Toda et al., 2013). In line with previously reported results (Liu et al., 2017; Peethambaran et al., 2018), the expressions of CwJAZ4/9 were swiftly raised in CW hairy roots at the early stages of salt stress (2 h) (Figure 5a). Strikingly, this rapid activation of CwJAZ4/9 was well coincident with a large rise in JA (8.4-fold) and JA-Ile (5.6-fold) levels at 2 h post-salt treatment (Figure 5b), which is in agreement with previous studies (Chung et al., 2008; Koo et al., 2009). A previous study showed that even low amounts of biologically active JA-Ile under salt stress are sufficient to mediate JA-responsive genes (Thurow et al., 2020). Consistent with this observation, our results reflected that the JA signaling is operated in hairy roots at the early stages of the salt stress response.

Salt triggers the upregulation of JA biosynthesis and signaling genes like AOC1, AOC2, COI1, and MYC2/3/4 in Arabidopsis root cells (Hazman et al., 2015; Valenzuela et al., 2016). Similarly, we found that the expressions of CwMYC2, CwCOI1, CwJAR1, and CwAOS1 are significantly upregulated by salt in all CwJAZ4/9 hairy root lines (Figure 5c). However, compared with the control (EV), overexpression of CwJAZ4/9 could hold down partially the salt-mediated upregulation of JA-responsive genes but, in contrast, silencing of CwJAZ4/9 could amplify the salt-mediated upregulation. We, therefore, conclude that CwJAZ4/9 facilitates the repression of JA responses induced by salt, which coincides with the fact that salt adaptation requires efficient fine-tuning of JA signaling (Ismail et al., 2014). Previous studies showed that overexpressing OsJAZ8/9 in rice (Peethambaran et al., 2018; Wu et al., 2015), and overexpressing PnJAZ1, and MdJAZ2 in Arabidopsis enhances root growth by reducing the salt-mediated JA-sensitivity (An et al., 2017; Liu et al., 2019). This action is well exemplified by rice salt-sensitive 3 (RSS3).
encoding a JAZ-stabilized protein (Toda et al., 2013), which represses JA-responsive genes and thus regulates root cell elongation during the adaptation to salinity stress. Consistent with these results, we observed that overexpression of CwJAZ4/9 directly confers salinity tolerance and sustains hairy root growth during salt stress, whereas silencing of CwJAZ4/9 inhibits hairy root growth compared with the control (Figure 6a). These results, together with the known roles of JAs in root growth regulation (Zhou et al., 2019), suggest that CwJAZ4/9 plays a crucial role in sustaining CW hairy root growth under salt stress by weakening the salt-mediated JA responses.

4.3 | CwJAZ4/9 regulates the transcriptional reprogramming of stress signaling and responses

In addition to influencing the JA response, plant JAZs also regulate the growth-defense balance by directly mediating stress biological processes (Major et al., 2017). It was reported that all down-regulated genes in the Mpjaz-1 mutant cause a defect in abiotic stress responses, cell wall organization, and photosynthesis (Monte et al., 2019). In this study, GO analysis based on CwJAZ4/9-dependent gene sets in CwJAZ4/9_Ox lines (vs the control line) showed a general redundancy in defense response, response to oxidative stress, and cell wall organization (Figure 7a, Dataset S2). This strongly suggests that CwJAZ4/9 affects gene reprogramming involved in these biological processes. The SOS pathway was the first abiotic stress-signaling pathway established in plants (Zhu, 2016). We found that multiple SOS1/2 genes related to ionics stress-signaling are activated in CwJAZ4/9_Ox lines. Meanwhile, the MAP kinase (MAPK6) pathway was rapidly upregulated in CwJAZ4/9_Ox lines and downregulated in CwJAZ4/9_Ri lines, which has been implicated in osmotic signals from abiotic stimuli such as salt, drought, cold, heat, and wounding (de Zelicourt et al., 2016). This supports that CwJAZ4/9 has the potential capacity to enhance the threshold of stress responses.

For various abiotic stresses, accumulating effective antioxidants and osmotic protectants, such as glutathione, proline, and betaine, is one of the important strategies to enhance plant stress tolerance. Several lines of evidence showed that transgenic upregulation of glutathione synthesis scavenging stress-mediated ROS accumulation improves abiotic stress tolerance in Arabidopsis (Cheng et al., 2015). Stress also induces the expression of genes related to proline biosynthesis. Genetic evidence in rice shows that a P5CSF129A gene accumulates proline and further enhances salt stress tolerance (Kumar et al., 2009). Conversely, knockout of P5CS1 in Arabidopsis leads to hypersensitivity to salt (Székely et al., 2008). Similar to these results, multiple genes encoding enzymes related to glutathione metabolism (GSH, GST), and proline biosynthesis (P5CS, P5CR) were upregulated in CwJAZ4/9_Ox lines (Dataset S2), which directly resulted in higher accumulation levels of proline and TEAC in CwJAZ4/9_Ox lines than that of other lines (Figure 6b,c). A previous study showed that BADH genes associated with betaine biosynthesis also increase the tolerance to various stress stimuli and protect cell membrane integrity (Niazian et al., 2021). Similarly, we found that six BADH transcripts are upregulated in CwJAZ4/9_Ox lines (Dataset S2). This suggests that the mechanism underlying the CwJAZ4/9-mediated salt tolerance appears to be related to the increased ROS-scavenging activities and production of defense metabolites.

Various transporters across the membranes are critical for regulating ionic and water homeostasis in the cells under stress conditions. For instance, K+∕Na+ antiporters can pump the stress-induced toxic K+∕Na+ out of the cell, thus restoring ion homeostasis (Assaha et al., 2017; Zhu, 2016). It was reported that OsJAZ9 in rice confers salt tolerance by regulating K+∕Na+ homeostasis (Wu et al., 2015). In addition, plant plasma membrane P- and V-type H+-ATPase (AHA, VHA) also play a major role in stress adaption by modulating the activity of H+-ATPases (Li et al., 2022). Aquaporin (PIP) is another important transporter with a critical role in water transport and osmotic stress adjustment, overexpression of aquaporin genes (TaAQ8 in tobacco, HvPIP2/5 in barley) enhances salt stress tolerance (Alavilli et al., 2016; Hu et al., 2012). In line with these findings, our transcriptome results revealed that a large number of known genes encoding transporters (PIP, KEA, AHA, VHA) are overrepresented and upregulated in CwJAZ4/9_Ox lines (Dataset S2).

Plant hormones also play crucial roles in regulating the growth-defense balance via the sophisticated hormone cross-talks. For instance, JA-activated MYC2 can interact with ETHYLENE INSENSITIVE 3 (EIN3), a core ET signaling component, and represses its activity to antagonize ET-promoted defense responses and plant growth (Zhang et al., 2014). JAZ proteins also directly interact with and repress EIN3 activity (Zhu et al., 2011). On the other hand, the released MYC2, owing to the degradation of JAZ proteins, directly represses the PLT expression and inhibits root growth during JA modulation (Chen et al., 2011). In addition, ET signaling can also independently promote salt tolerance by upregulating the expression of EIN3-dependent defense-related genes, including ERF1, and PR1 (Yu et al., 2020). The crosstalk between JA and ABA is in part dependent on the interaction of MYC2 and ABA receptor PYL (Aleman et al., 2016), which can enhance plant tolerance under salt and drought (Chen et al., 2017). It was reported that PnJAZ1 protein promotes salinity tolerance by ABA signaling (Liu et al., 2019). Previous studies showed that ABA binds to the PYL receptor, allowing the activation of SnRK2s by auto-phosphorylation, the ABA-activated SnRK2 can phosphorylate downstream effectors, and directly regulate ROS and osmotic homeostasis to promote root growth (Zhu, 2016). In this study, we observed that many genes related to ET signaling (EIN3, ERF1), ABA biosynthesis (ZET, NEDC), and signaling (PYL2/6/8, SnRK2.1/SnRK2.2) are activated in CwJAZ4/9 lines (Dataset S2), implying that the JA, ET signalings, and crosstalks among them are potentially required for regulating the CwJAZ4/9-mediated salt tolerance in hairy roots.
Based on these findings, we conclude that CwJAZ4/9 negatively regulates the MeJA-induced TBS by the interaction with CwMYC2, and confers salt tolerance for hairy root growth by altering the expressions of genes related to stress signaling and responses, hormone signaling. We propose a putative molecular framework to reveal these biological processes (Figure 8). Under MeJA treatment, CwJAZ4/9 is rapidly induced and then degraded, which further releases CwMYC2 to promote terpenoid production. Evidence in CwJAZ4/9 transgenic lines (Figures 3 and 4, Figures S8–9) demonstrates that CwJAZ4/9 strongly represses the expression of numerous genes involved in the MVA and MEP pathways, and JA responses. On the other hand, CwJAZ4/9 confers salt tolerance and sustains hairy root growth via the crosstalks among multiple hormone signals, in which the salt-mediated JA responses can be weakened. In addition, the CwJAZ4/9-mediated repression of CwMYC2 promotes PLT expression and further stabilizes EIN3 activity, leading to the activation of downstream defense genes. The CwJAZ4/9 inhibited-CwMYC2 also interacts with ABA receptor PYL, thus increasing ABA responses and SnRK2 activity. Meanwhile, CwJAZ4/9 plays crucial roles in activating stress signaling, osmotic homeostasis, and antioxidant biosynthesis. See the ‘Conclusion’ section for details. Arrows represent positive regulation, and blunt ends represent negative regulation. The detailed information on genes in the map is seen in Dataset S2. [Color figure can be viewed at wileyonlinelibrary.com]

5 | CONCLUSION

For salt tolerance, CwJAZ4/9 functions as upstream core regulators to regulate stress defense by the crosstalks of multiple hormones (JA, ET and ABA). This mechanism can be described as follows: (i) CwJAZ4/9 inhibits JA responses and triggers the suppression of CwMYC2, which further eliminates the MYC2-inhibited response of PLT to promote hairy root growth. (ii) CwJAZ4/9 induces the ABA-activated SnRK2, which further controls ROS and osmotic homeostasis. (iii) CwJAZ4/9 interacts with and represses CwMYC2, the inhibited CwMYC2 further promotes the EIN3 activity and consequently enhances the expression of ERF1; while CwJAZ4/9 can also repress EIN3 expression. Meanwhile, the CwJAZ4/9 mediates salt tolerance, at least in part, by upregulation of genes related to the SOS1/2 and MAPK6 stress signaling, cell wall osmotic adjustment, antioxidant biosynthesis, and stress-responsive proteins. Overall, our results provide a genetic framework to understand how the CwJAZ4/9-MYC2 cascade controls TBS, and regulates salt tolerance. However, a challenge for future studies will be to identify other specific TFs interacting with CwJAZ4/9 to uncover the links between CwJAZ proteins and downstream physiological responses in CW.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.
DATA AVAILABILITY STATEMENT
The datasets supporting the results of this article are included in this published article and its supplementary information files.

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REFERENCES


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to jasmonate signalling. *Nature*, 464(7289), 788–791. Available from: https://doi.org/10.1038/nature08854


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