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Genome-wide identification of the *JAZ* gene family in non-heading Chinese cabbage and the functional verification of *BcJAZ2* in the biosynthesis of β-caryophyllene

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Abstract

JASMONATE ZIM DOMAIN (JAZ) proteins function as negative regulators of the JA signaling pathway and participate in plant development, stress responses, and secondary metabolism. β-caryophyllene is a volatile sesquiterpene compound that contributes to the formation of plant aromas and possesses antibacterial, anti-inflammatory, and antifungal biological activities. In our previous experiments, we found that the *BcJAZ2* was closely related to β-caryophyllene synthesis under low-temperature treatment in non-heading Chinese cabbage (NHCC). To further explore the function of *BcJAZ2*, we characterized *JAZ* gene family in NHCC. In this study, 25 *BcJAZ* genes were discovered in NHCC, and comprehensively analyzed the evolutionary relationships and structural characterizations of BcJAZs. BcMYC2, a positive regulator of terpenoid synthesis, interacted with BcJAZ2 confirmed by yeast two-hybrid and bimolecular fluorescence complementation assays. Overexpression of *BcJAZ2* in *Arabidopsis* and silencing of *BcJAZ2* in NHCC showed that *BcJAZ2* acted as a negative regulator of β-caryophyllene biosynthesis. In addition, three transcription factors BcbHLH137, BcHBI1.1, and BcHBI1.2 were confirmed to be positive regulators of *BcJAZ2* by yeast one-hybrid and LUC assays. The above results enrich our understanding of the regulation of β-caryophyllene synthesis and provide the foundation for in-depth exploration of regulatory mechanisms of *BcJAZs*.

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Introduction

β-caryophyllene is a sesquiterpene commonly found in plants with a variety of pharmacological and biological functions. β-caryophyllene has anti-inflammatory and antifungal properties[1,2]. Simultaneously, β-caryophyllene is capable of mediating signaling between different organisms and plays an important role in plant-induced defense responses. Flowers can attract insect pollination by releasing β-caryophyllene in *Arabidopsis*[3]. β-caryophyllene released from maize roots attracts natural enemies of pests[4]. Two terpene synthesis pathways exist in plants. The mevalonic acid (MVA) pathway located in the cytoplasm and the methyl erythritol-4-phosphate (MEP) pathway located in the plastids. Among them, β-caryophyllene is generated via the MVA pathway and finally catalyzed by the terpene synthase (TPS)[5].

The JASMONATE ZIM DOMAIN (JAZ) proteins originally known as ZIM (zinc finger proteins expressed in inflorescence meristem), work as inhibitors of the JA signaling pathway[6]. *JAZ* subfamily generally contains two highly conserved domains: the ZIM domain (also called TIFY), and the Jas domain (also called CCT_2)[7], which are essential for JAZ to interact with other proteins[8,9]. Under normal growth conditions, the JA-Ile level in plants is low. Then JAZ proteins bind to MYC2 and other transcription factors to inhibit the activity of transcription factors (TFs), thereby suppressing JA signaling[10]. However, under stress conditions, JA signaling molecules are synthesized in plants to form JA-Ile or other amino acid derivatives, prompting the COI1 protein of the SCF^{COI1} complex to interact with JAZ proteins[8]. Subsequently, the JAZ proteins are ubiquitinated and degraded via the 26S proteasome pathway and TFs like MYC2 are released to activate the expression of downstream JA response genes[11]. Research found that when JA response occurred, *JAZ* genes would be significantly induced, which in turn inhibited the activity of MYC2, forming a feedback regulation mechanism, so that the plants would not produce too strong JA response, and avoiding excessive energy consumption within the plants[12,13]. Studies have shown that JAZ is involved in the regulation of plant terpenoid metabolism. In *Artemisia annua*, the AaJAZ8 protein inhibited the artemisinin biosynthesis by repressing the expression of artemisinin synthesis gene[14]. In *Salvia miltiorrhiza*, SmJAZ3 could induce the accumulation of salicylic acid and tanshinone and promote plant growth and development by interacting with SmWD40-170[15]. In *Curcuma wenyujin*, CwJAZ4/9 proteins inhibited the terpene pathway and jasmonic acid response by forming the CwJAZ4/9 - CwMYC2 regulatory cascade, thereby reducing terpene accumulation[16]. Although JAZs play an important regulatory role in plant growth and development, there is currently little known about the regulatory factors upstream of *JAZ*.

Non-heading Chinese cabbage (NHCC) (*Brassica campestris* (syn. *Brassica rapa*) ssp. *chinensis*) is a common leafy vegetable that originated in China and has gradually been cultivated worldwide. 'XQC', one cultivar of NHCC, is named for its unique volatile flavor. β-caryophyllene is one of its volatile components in NHCC. In our previous studies, *BcTPS21* was considered a key gene in NHCC involved in β-caryophyllene biosynthesis. However, there are few knowledge on the regulation of β-caryophyllene synthesis in NHCC.

In this study, the *JAZ* gene family in NHCC have been characterized. The interaction between BcJAZ2 and BcMYC2 has been

confirmed and the role of BcJAZ2 in regulating *β*-caryophyllene synthesis in NHCC was verified through heterologous expression and gene silencing. Additionally, we found that BcbHLH137, BcHBI1.1, and BcHBI1.2 could bind the *BcJAZ2* promoter and activate its expression. These results provide a basis for further exploration of *β*-caryophyllene biosynthesis.

Materials and methods

Identification and characterization of *BcJAZ* gene family in NHCC

The *JAZ* gene sequences of NHCC were retrieved from the NHCC database[17] (http://tbir.njau.edu.cn/NhCCDbHubs/) and TBGR database[18] (www.tbgr.org.cn) and were screened using HMM search and two rounds of BLASTP in TBtools v 2.096[19]. Hidden Markov models (HMMs) of TIFY domain (PF06200) and Jas domain (PF09425) were downloaded from the Pfam database (http://pfam-legacy.xfam.org/). Finally, the screened candidate genes were validated through the NCBI Batch CD-Search (www.ncbi.nlm.nih.gov/) and only selected genes with TIFY and Jas domains. Their physicochemical properties were calculated by the ExPASy (https://web.expasy.org/compute_pi/) and TBtools, respectively. The prediction of subcellular localizations of BcJAZs was conducted using WOLF PSORT website (https://wolfpsort.hgc.jp/).

The conserved motifs of the BcJAZs were predicted using the MEME online website[20] (https://meme-suite.org/meme/) and visualized by TBtools. The Jalview software was performed for multiple sequence alignment of BcJAZ proteins[21]. Based on the NHCC genome GFF file, the chromosomal mapping of *BcJAZ* genes was visualized using TBtools software. Utilizing MCScanX in TBtools, we identified intraspecific gene duplication events and interspecific collinearity relationships of *BcJAZ* family members. Additionally, the cis-elements of *BcJAZ* genes upstream 2,000 bp promoter sequences were predicted via the PlantCARE database[22].

Phylogenetic analysis of *BcJAZ* gene family

The gene sequences of *Arabidopsis* and rice that have been reported were accessed via the TAIR database (www.arabidopsis.org) and Rice Genome Database (http://rice.plantbiology.msu.edu). We utilized the MEGA 11.0 software to align the JAZ protein sequences from *Arabidopsis*, NHCC, and rice, and constructed a phylogenetic tree using the neighbor-joining (NJ) method with 1,000 bootstrap replicates to study the evolution of the *BcJAZ* gene family in NHCC[23].

Plant materials and treatments conditions

The 'XQC' variety of NHCC, *Arabidopsis thaliana* and *Nicotiana benthamiana* mentioned in this experiment were acquired form the Nanjing Agricultural University (Nanjing, China). All plant materials were cultivated in light 16 h/22 °C and dark 8 h/18 °C. Various treatments were carried out with three biological replicates when the seedlings were about one-month-old. The low-temperature treatment conditions were 4 °C for both daytime and nighttime and the hormone treatments were spraying the leaves with 100 μM methyl jasmonate (MeJA) and 100 μM gibberellic acid (GA). Samples without MeJA and GA treatments (taken at 0 h) as control. Plant leaves were taken at 0, 1, 3, 6, 12, 24, and 48 h after treatments. The *BcJAZ2* transgenic lines were created by dipping flowers using *Arabidopsis thaliana* Columbia-0 (Col-0) wild type.

qRT-PCR analysis

Employing the RNA simple Total RNA Kit (Tiangen, Beijing, China), we extracted total RNA of NHCC and *Arabidopsis* and using HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, China) synthesized cDNA. qPCR was carried out with ChamQ SYBR qPCR Master Mix (Vazyme, China) and the relative expression of genes was calculated using $2^{-\Delta\Delta CT}$ method. The reference genes were *BcGAPDH* and *AtACTIN* for NHCC and *Arabidopsis*[24]. All primers used in this study are supplied in Supplementary Table S1.

Plasmid construction

For subcellular localization, *BcJAZ2* was inserted into the PRI101 vector. The *35S:BcJAZ2*-GFP overexpression vector was transformed into *Agrobacterium tumefaciens* strain GV3101 (pSoup) (TOLOBIO, China).

For Y1H and Y2H assays, the *BcJAZ2* promoter segments pAbAi-*proBcJAZ2*-P1 (−1,250 to 0 bp) were integrated into the pAbAi vector. To verify trans-activation activity of transcription factors, the bait proteins BcbHLH137, BcHBI1.1, BcHBI1.2 and BcJAZ2 were recombined into pGBKT7(BD) vectors, while the prey protein BcMYC2 to be validated were integrated into the pGADT7 (AD) vector.

For BiFC assays, the open reading frames (ORF) of *BcJAZ2* and *BcMYC2* were cloned and constructed into BiFC-YC and BiFC-YN vectors, respectively. The created recombinant vectors (YC-*BcJAZ2* and YN-*BcMYC2*) were introduced into *Agrobacterium tumefaciens* strain GV3101 (pSoup) (TOLOBIO, China).

For LUC assays, the CDS without stop codon of *BcHBI1.1*, *BcHBI1.2*, and *BcbHLH137* were integrated into the PRI101 vector to create effector vectors. *BcJAZ2* truncated promoter fragment *proBcJAZ2*-P1 (−1,250 to 0 bp) was constructed into pGreenII-0800-LUC to create a reporter vector. The recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 (pSoup) (TOLOBIO, China). All primers used in this study are supplied in Supplementary Table S1.

Subcellular localization

Agrobacterium tumefaciens strain carrying the GFP vector was mixed with nuclear marker in a 1:1 ratio, and injected into tobacco leaves. The tobacco was cultivated in darkness for 24 h followed by normal cultivation for 24−48 h. Through the confocal laser scanning microscopy (Zeiss, LSM 780, Germany), we detected the position of GFP signals.

Bimolecular fluorescence complementation assays

Agrobacterium tumefaciens strains containing the BiFC-YC and BiFC-YN plasmids were blended in the same ratio and injected into tobacco leaves. Tobacco treatment conditions were the same as for subcellular localization. Using the confocal laser scanning microscope (Zeiss, LSM 780, Germany), distinct fluorescence signals were discovered after 48−72 h.

Virus-induced *BcJAZ2* gene silencing in NHCC

Virus-induced gene silencing (VIGS) is a rapid method used to reveal gene function[25]. Based on the CDS sequence and conserved domains of *BcJAZ2*, a 40 bp specific fragment was designed, which was then reverse-complemented and ligated to the previous 40 bp fragment to form an 80 bp palindromic hairpin structure. The DNA sequence is shown in Supplementary Table S1. Subsequently, the sequences were constructed into PTY-empty vector, and then PTY-empty and PTY-*BcJAZ2* plasmids were transformed into the NHCC variety 'XQC' by the method of gene gun bombardment (Bio-Rad, PDS1000/He), respectively. The phenotypes of the silenced plants were observed after two weeks. Extracted RNA and detected the expression level of *BcJAZ2* by qRT-PCR.

Yeast one-hybrid library screening and yeast two-hybrid analysis

Using the lithium acetate procedure (Takara, Japan), the linearized bait vector pAbAi-*proBcJAZ2*-P1 (−1,250 to 0 bp) was introduced into yeast Y1H Gold strain, which was grown on SD/-Ura

deficient medium containing different doses of Aureobasidin A (AbA) for 2–3 d to find the lowest AbA concentration that repressed self-activation (Supplementary Fig. S1). The One Step Yeast Direct PCR kit (TOLOBIO, China) was used to identify the positive recombinant clones. A cDNA library of 10 μL was added to the 600 μL Y1H Gold cells containing pAbAi bait and was grown on 200 ng/mL AbA SD/-Leu solid medium at 28 °C for 5–7 d. The colonies grown on solid medium were detected by PCR and the sequencing results were compared using the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The prey vectors pGADT7-*BcbHLH137*, pGADT7-*BcHBI1.1*, pGADT7-*BcHBI1.2* and empty pGADT7 were then transferred into yeast strains containing the bait vector, respectively, and cultured for 3–5 d at 28 °C in SD/-Leu deficient medium containing AbA.

For Y2H assays, the bait protein *BcJAZ2*-BD and prey protein *BcMYC2*-AD were co-transformed into yeast Y2H Gold strain by the lithium acetate method (Takara, Japan). Similarly, the bait proteins *BcbHLH137*-BD, *BcHBI1.1*-AD, and *BcHBI1.2*-AD were co-transformed into yeast Y2H Gold strain with AD empty vector, respectively. The co-transformed yeast cells were first cultured on the SD/-Leu-Trp medium and then moved to the SD/-Leu-Trp-Ade-His medium to grow for 3–5 d at 28 °C.

Dual-luciferase reporter assay

Agrobacterium tumefaciens strains containing effector and LUC reporter were blended in the same ratio and injected into tobacco leaves, separately. Tobacco treatment conditions were the same as for subcellular localization. After 48–72 h, the luciferase activity was detected using Dual Luciferase Reporter Assay Kit (Vazyme, China).

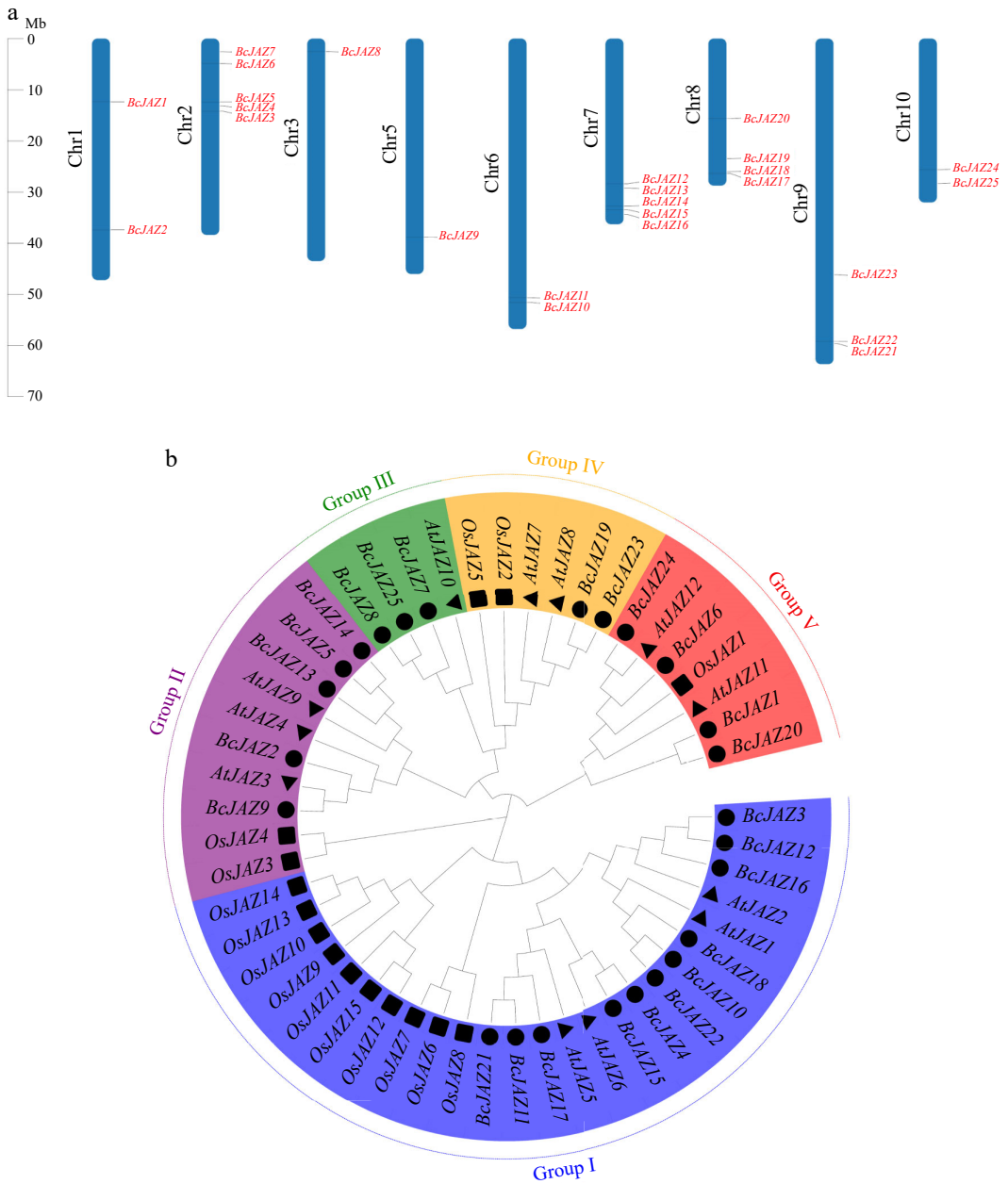

Fig. 1 Chromosomal mapping and phylogenetic tree of *BcJAZ* gene family. (a) Chromosomal mapping of *BcJAZ* family members. (b) Phylogenetic tree of NHCC, *Arabidopsis,* and rice. Subfamilies I, II, III, IV, and V were indicated by different colors. The black circles represent the 25 BcJAZs in NHCC. The black triangles represent the 12 AtJAZs in *Arabidopsis*. The black squares represent the 15 OsJAZs in rice.

GC-MS analysis

Five-week-old *Arabidopsis* inflorescences (0.2~0.5 g) and leaves of *BcJAZ2* silenced plants were collected for *β*-caryophyllene determination. To repress the enzyme reaction, the plant samples were ground into powder and 2 mL NaCl saturated solution was added together and poured into headspace vials (Agilent, CA, USA). A gas chromatograph (TRACE 1310, Thermo Scientific) and a triple quadrupole mass spectrometer were employed for *β*-caryophyllene substance detection. The method of testing was consistent as described by Wang et al.[26].

Statistical analysis

The experiments in this study were set up with three biological and technical replicates and the data in this study were analyzed by Student's t-tests (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$). Error lines represent standard deviations. Statistical significance is indicated by different asterisks.

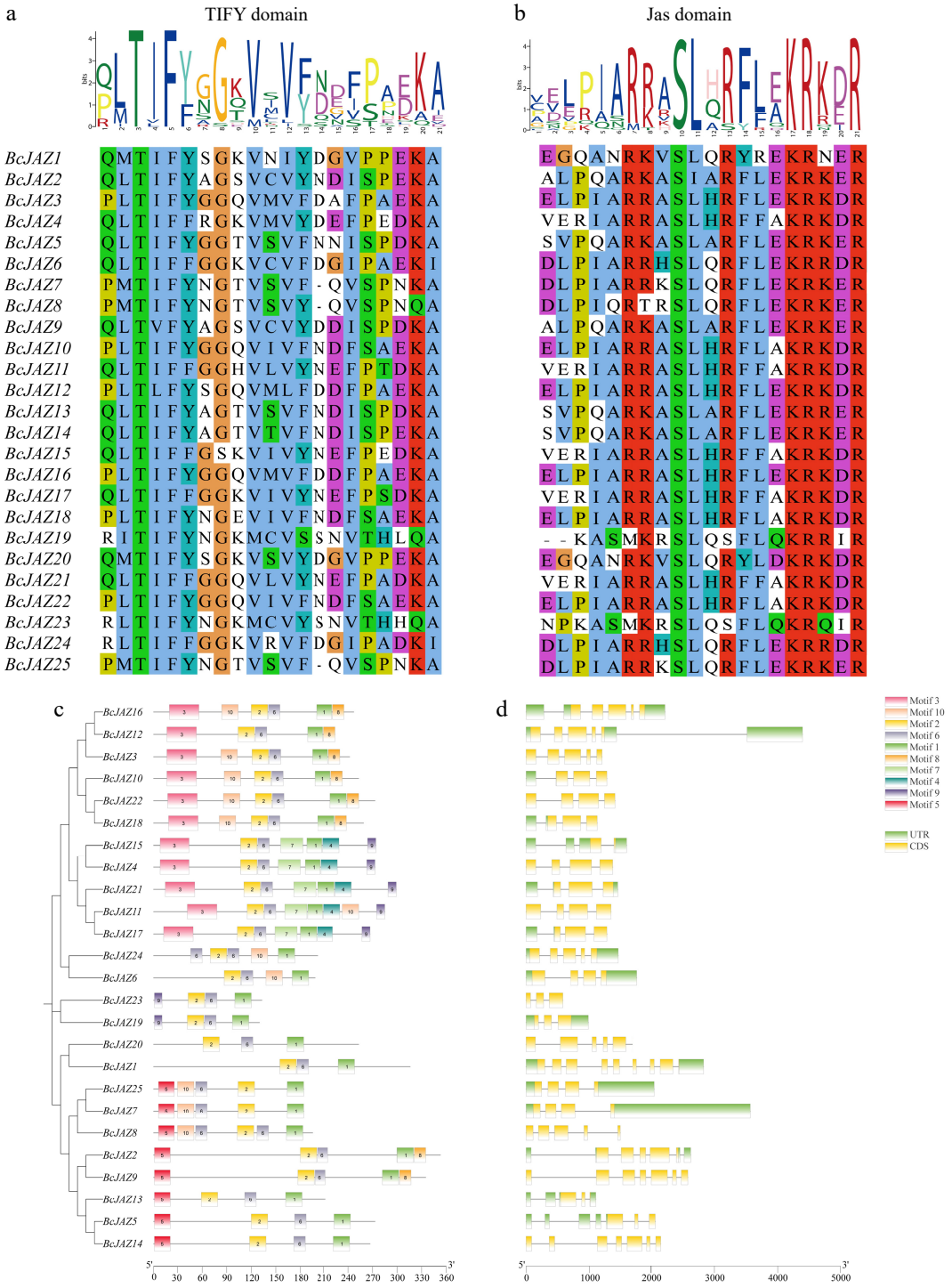

Fig. 2 Multiple sequence alignment, motifs and gene structure of *BcJAZ* genes. (a), (b) Multiple sequence alignment and conserved domains of *BcJAZ*. (c) Conserved motifs of *BcJAZs*. (d) The gene structure of *BcJAZs*; CDS are represented by yellow boxes, introns by grey lines, and UTRs by green boxes.

Results

Identification analysis and phylogenetic relationship of the *BcJAZs*

We used bioinformatics techniques to identify 25 *JAZ* family members in high quality NHCC genomes which were distributed on nine chromosomes (Fig. 1a). The gene sequence lengths of *BcJAZs* ranged from 393 bp (*BcJAZ19*) to 1,062 bp (*BcJAZ2*), and the encoded proteins varied from 130 aa (*BcJAZ19*) to 353 aa (*BcJAZ2*). Their theoretical isoelectric points and molecular masses varied from 5.10 (*BcJAZ6*) to 9.99 (*BcJAZ7*) and 14.97 to 37.58 KDa, respectively. Subcellular localization predictions indicated most BcJAZs showed nuclear distribution (Supplementary Table S2).

Aiming to explore the phylogenetic relationship of JAZ among NHCC, *Arabidopsis,* and rice, we established a phylogenetic tree containing 25 BcJAZs, 12 AtJAZs, and 15 OsJAZs proteins (Fig. 1b). According to the phylogenetic tree, we found that all JAZs were mainly clustered in five groups according to the evolutionary distance. Group I contained 11 *BcJAZ* members which contained most of the *BcJAZ* genes of NHCC. Interestingly, most of the *OsJAZ* genes (10) were also distributed in group I. Group II was comprised of five *BcJAZ* genes, *BcJAZ2, BcJAZ5, BcJAZ9, BcJAZ13*, and *BcJAZ14*. Group III was comprised of three *BcJAZ* genes, *BcJAZ7, BcJAZ8,* and *BcJAZ25*. Group IV was comprised of two *BcJAZ* genes, *BcJAZ19,* and *BcJAZ23*. Group V was comprised of four *BcJAZ* genes, *BcJAZ1, BcJAZ6, BcJAZ20,* and *BcJAZ24*.

Multiple sequence alignment, conserved domain, gene structure of the *BcJAZ* genes

Multiple sequence alignment results showed that BcJAZ proteins had two very obvious conserved domains and were identified: motif 1 corresponded to the Jas domain, while motif 2 corresponded to the TIFY domain (Fig. 2a, b). As shown in Fig. 2c, the majority of the *BcJAZ* genes sharing similar motifs grouped in closer evolutionary branch. The gene structure and predicted motifs of *BcJAZs* were visualized using TBtools. The findings indicated that the *BcJAZ* gene family showed variations in CDS quantities (Fig. 2d).

Collinearity analysis of the *BcJAZ* genes

Intraspecific collinearity analysis of the *BcJAZ* genes revealed 31 gene pairs underwent segmental duplication events (Fig. 3a, Supplementary Table S3), and no tandem duplication clusters were detected, indicating the main gene duplication event for *JAZ* genes in NHCC was segmental duplication. Interspecies collinearity analysis showed that there are 36 collinear gene pairs between NHCC and *Arabidopsis*, and two collinear gene pairs between NHCC and rice (Fig. 3b). The collinearity analysis indicated that the *BcJAZs* of NHCC had closer relationship with the *AtJAZs* of *Arabidopsis*, which was consistent with the present cognition of plant evolutionary history.

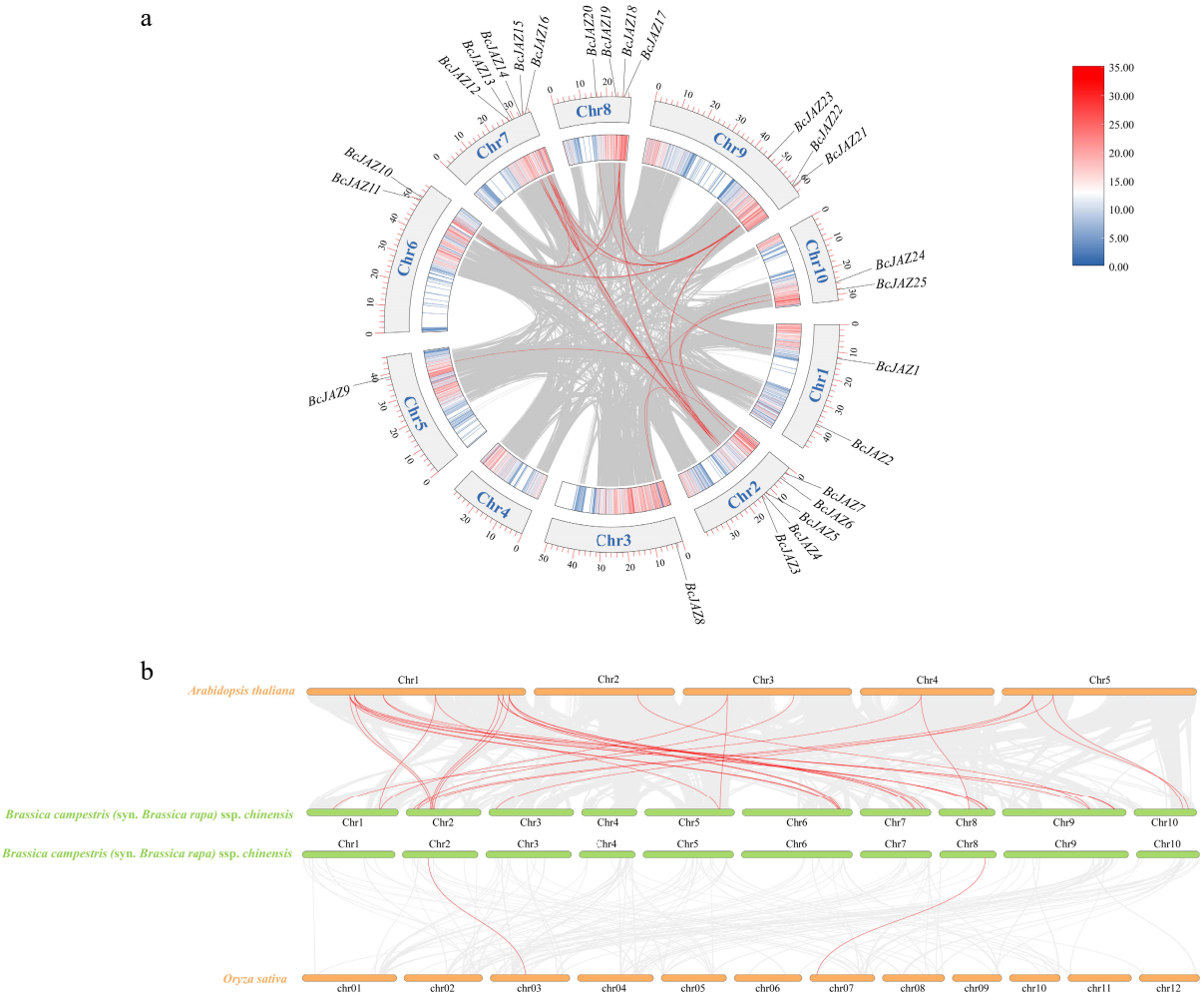

Fig. 3 Collinearity analysis of the *BcJAZs*. (a) Intraspecies collinearity analysis of *BcJAZs*. (b) Interspecific collinearity analysis of *BcJAZs* in NHCC, *Arabidopsis thaliana,* and *Oryza sativa*.

Cis-elements of *BcJAZ* gene family

Gene function can be predicted by analyzing the promoter cis-elements. Based on the predictions of the Plantcare website, we have cis-elements categorized into five groups: core/binding, development, abiotic/biotic elements, light, and hormones (Fig. 4). All 25 *BcJAZs* promoters contained numerous core elements and binding sites, including CAAT-box and TATA-box. Most *BcJAZs* contained certain abiotic-responsive and light-responsive elements, including the drought-responsive element like DRE core, the low temperature-responsive element like LTR, as well as the light-responsive elements BOX4, GT1-motif, and G-box. Some hormone response elements, including SA (TCA-element), IAA (TGA-element), GA (GARE-motif, P-box), MeJA (CGTCA-motif, TGACG-motif), and ABA (ABRE-element), were also detected in most of the *BcJAZs* promoters. These results indicated that *BcJAZs* likely serve in development, abiotic stress, and multiple phytohormone signaling pathways.

Expression patterns of *BcJAZs* in response to abiotic stress and hormone treatments

RNA-Seq analysis showed that under low- and high-temperature stress, ten *BcJAZ* genes (*BcJAZ2*, *BcJAZ3*, *BcJAZ4*, *BcJAZ5*, *BcJAZ9*, *BcJAZ12*, *BcJAZ14*, *BcJAZ16*, *BcJAZ21*, and *BcJAZ24*) were strongly induced (Fig. 5a). Previous research has demonstrated that *BcJAZs* participated in hormone responses, and their expression patterns under different treatments may reflect genes biology function. qRT-PCR data indicated that all genes responded to MeJA treatment (Fig. 5c), among which *BcJAZ2*, *BcJAZ4*, *BcJAZ5*, *BcJAZ9*, *BcJAZ14*, *BcJAZ16,* and *BcJAZ24* showed 'up-down' expression trends after treatment. Under GA treatment (Fig. 5d), the expression levels of *BcJAZ2*, *BcJAZ3*, *BcJAZ5*, *BcJAZ14*, *BcJAZ16,* and *BcJAZ21* showed 'up-down' expression trends after treatment. Although *BcJAZ9* and *BcJAZ12* showed similar expression patterns, the expression of *BcJAZ9* reached its peak at 1 h after GA treatment, while the expression level of *BcJAZ12* was highest at 3 h. Conversely, the expression levels of *BcJAZ16* and *BcJAZ24* continuously decreased until 3 and 12 h after treatment, respectively.

In particular, the expression levels of *BcJAZ2* changed most significantly under long-term low-temperature treatment (Fig. 5a). Meanwhile, we analyzed the expression levels of *JAZs* under short-term (48 h) low-temperature treatment and the expression level of *BcJAZ2* was significantly up-regulated after 6 h of treatment (Fig. 5b). Previous studies showed that low temperatures inhibited *β*-caryophyllene synthesis in NHCC (unpublished data), and *BcJAZ2* was significantly induced by both short- and long-term low temperature treatments. Therefore, we hypothesized that BcJAZ2 may be a key negative regulator of *β*-caryophyllene synthesis.

BcJAZ2 interacts with BcMYC2

The results of website prediction and subcellular localization were consistent and both revealed that BcJAZ2 was located in the nucleus (Fig. 6a). According to predictions of the STRING 12.0 database, *BcJAZ2* appeared to be functionally connected with proteins

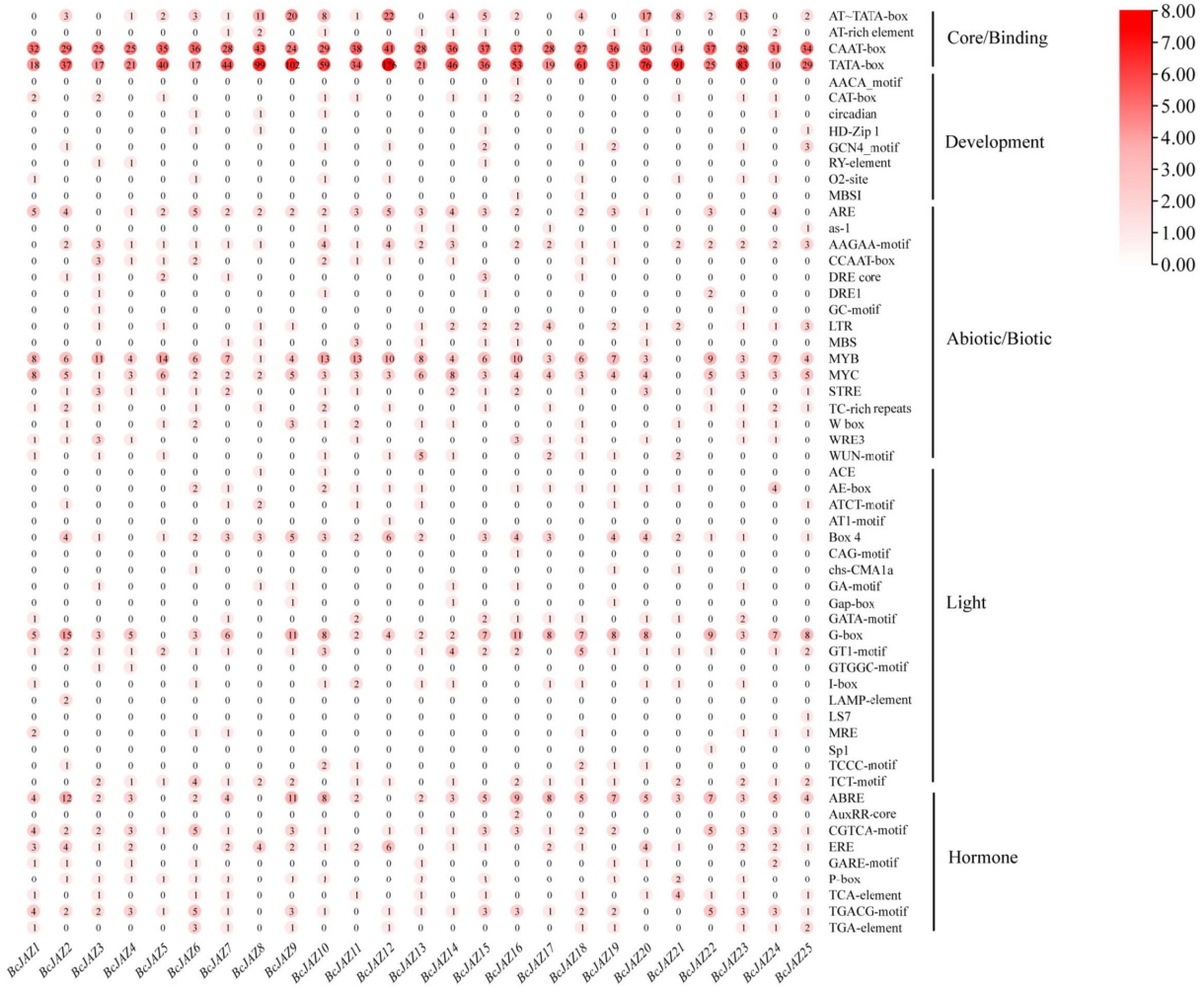

Fig. 4 Cis-elements analysis of *BcJAZs* promoters in NHCC. The numbers within the red circles indicated the quantities of cis-elements.

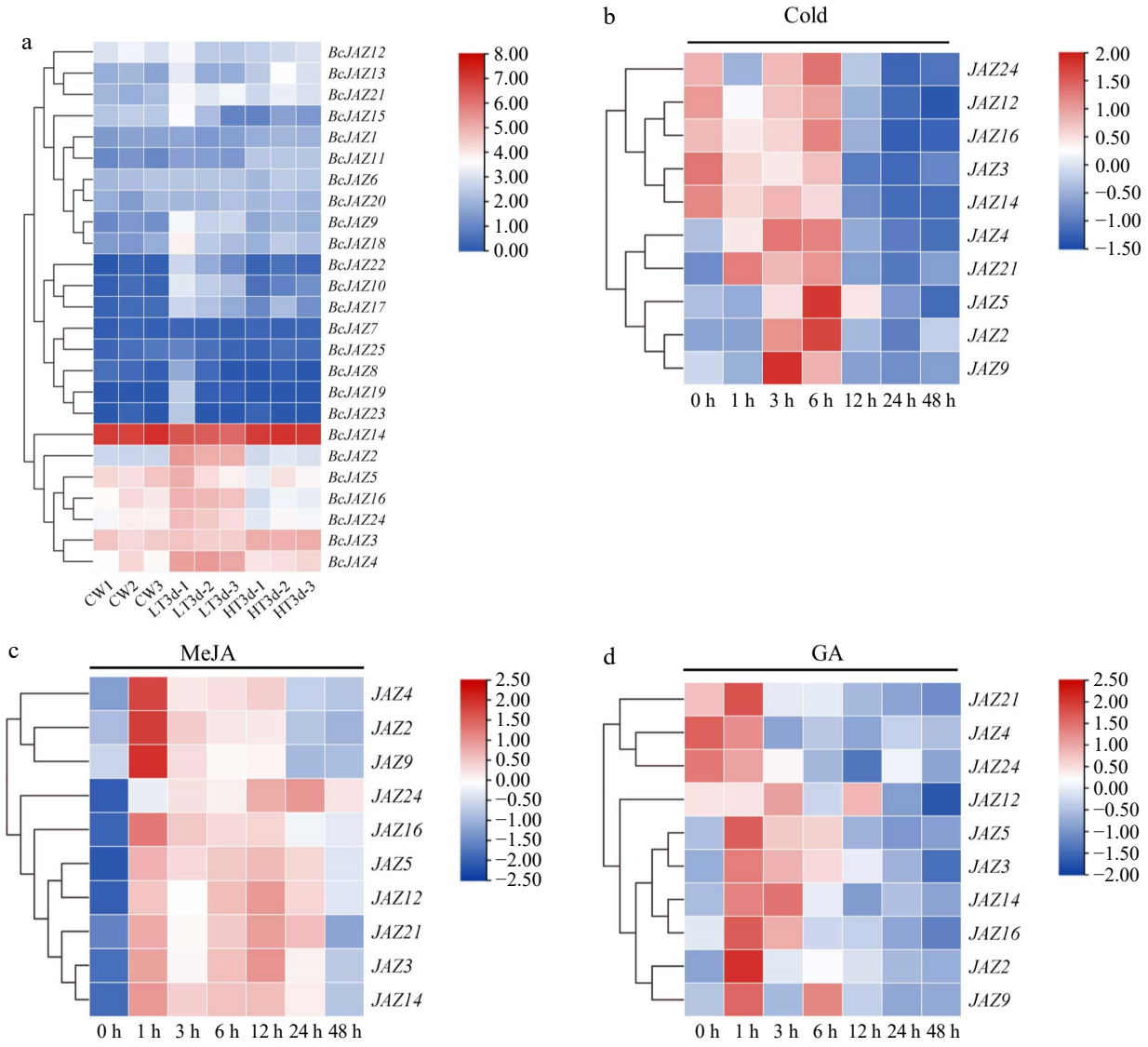

Fig. 5 Expression patterns of *BcJAZ* genes in response to abiotic stresses and hormone treatments. (a) RNA-Seq data of the *BcJAZ* gene family at different temperatures (CW represented normal temperature, LT3d represented low temperature 8/4 °C and HT3d represented high temperature 38/25 °C). The bar graph shows the Log2 (TPM + 1) values, indicating expression levels from low to high. (b) The expression patterns of *BcJAZs* under 4 °C, (c) MeJA, and (d) GA treatment. After being normalized, the expression levels were visualized using TBtools.

including MYC2/MYC3/MYC4, COI1, Ninja family proteins, and JAZ family proteins (Fig. 6b). It has demonstrated that MYC2 in *Arabidopsis* can directly bind to the promoter of the *β*-caryophyllene synthase gene *TPS21* and activate its expression[27]. We verified the relationship between BcMYC2 and BcJAZ2 of NCHH using Y2H (Fig. 6c). Each combination could grow normally on SD/-Trp-Leu mediums, whereas only yeast strains transformed with *BcJAZ2*-BD and *BcMYC2*-AD plasmids and a positive control could grow normally on SD/-Trp-Leu-His-Ade medium, suggesting that BcJAZ2 could interact with BcMYC2 in yeast. To further validate the interactions between BcJAZ2 and BcMYC2, it was verified by BiFC experiments. The results showed that when *BcJAZ2*-cYFP and *BcMYC2*-nYFP were co-injected, yellow fluorescence could be detected, indicating that BcJAZ2 and BcMYC2 could interact in plants (Fig. 6d). These results suggested that BcJAZ2 may repress the expression of the *β*-caryophyllene synthase gene through BcMYC2.

Overexpression of *BcJAZ2* negatively regulates *β*-caryophyllene biosynthesis in *Arabidopsis*

To further explore the function of *BcJAZ2*, we constructed and identified *BcJAZ2* transgenic lines in the background of the wild-type (WT) model plant *Arabidopsis thaliana* (Fig. 7a, b). The formation of almost all floral volatile sesquiterpenes in *Arabidopsis* is attributed to the sesquiterpene synthase genes *TPS21* and *TPS11*, with *TPS21* being accountable for producing of *β*-caryophyllene[27]. In inflorescences of *BcJAZ2* transgenic lines, the expression of *AtTPS21* and *AtTPS11* was significantly repressed compared with WT (Fig. 7c). To determine whether *BcJAZ2* influences the JA signaling, we analyzed the expression levels of JA signaling genes by qRT-PCR. As expected, *AtCOI1* and *AtMYC2* expression levels were suppressed (Fig. 7c). The effects of transcriptional regulation of *BcJAZ2* on *β*-caryophyllene synthesis in overexpression lines were also further monitored by GC-MS (Fig. 7d). Compared with WT, the *β*-caryophyllene content of *BcJAZ2* transgenic lines *Arabidopsis* decreased approximately 45%. Together, these data suggested that *BcJAZ2* negatively regulated the synthesis of *β*-caryophyllene, by inhibiting JA signaling and further suppressing the expression of the sesquiterpene synthase.

Silencing *BcJAZ2* by VIGS promotes sesquiterpene biosynthesis in NHCC

To elucidate the biological function of *BcJAZ2* in NHCC, we obtain PTY-*BcJAZ2* silenced plants using the virus-induced gene silencing

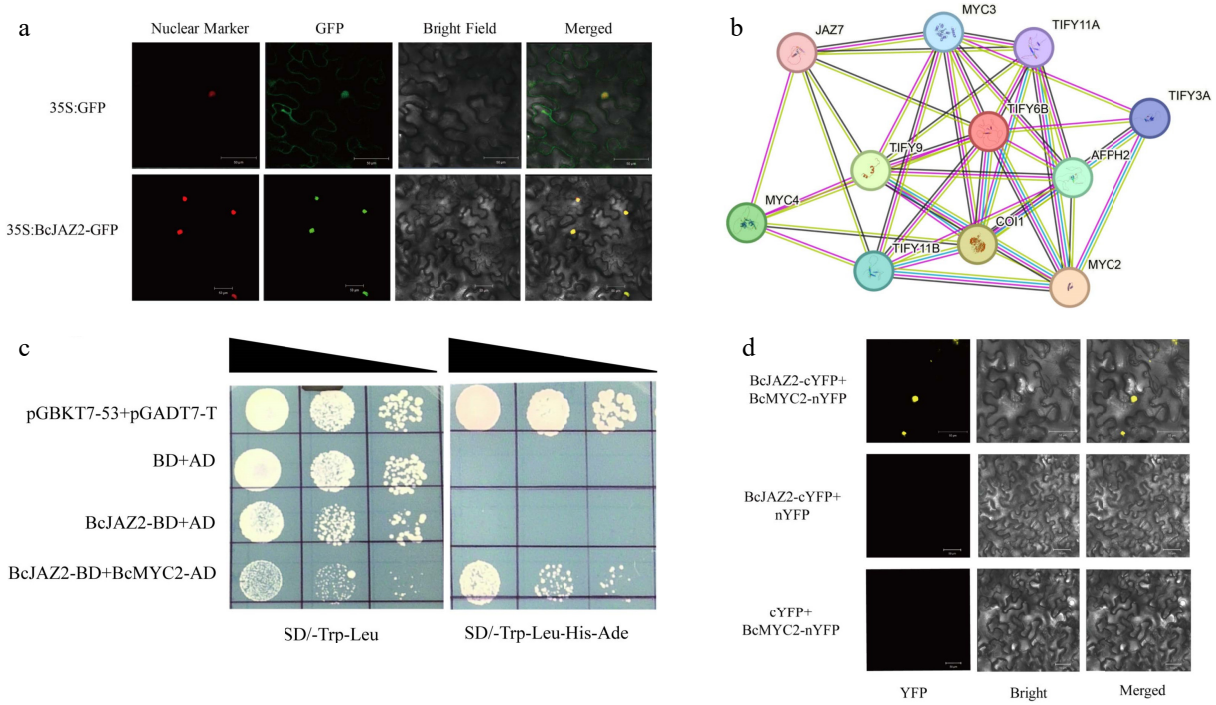

Fig. 6 BcJAZ2 interacts with BcMYC2. (a) Subcellular localization of BcJAZ2. The scale bar was 50 μm. (b) The protein-protein interaction (PPI) network of BcJAZ2 (TIFY6B) from the STRING 12.0 database. (c) Yeast two-hybrid assays. Positive control was yeast co-transformed with pGBKT7-53 and pGADT7-T and yeast co-transformed with pGBKT7 and pGADT7 was negative control. (d) Bimolecular fluorescence complementation assay. The scale bar was 50 μm.

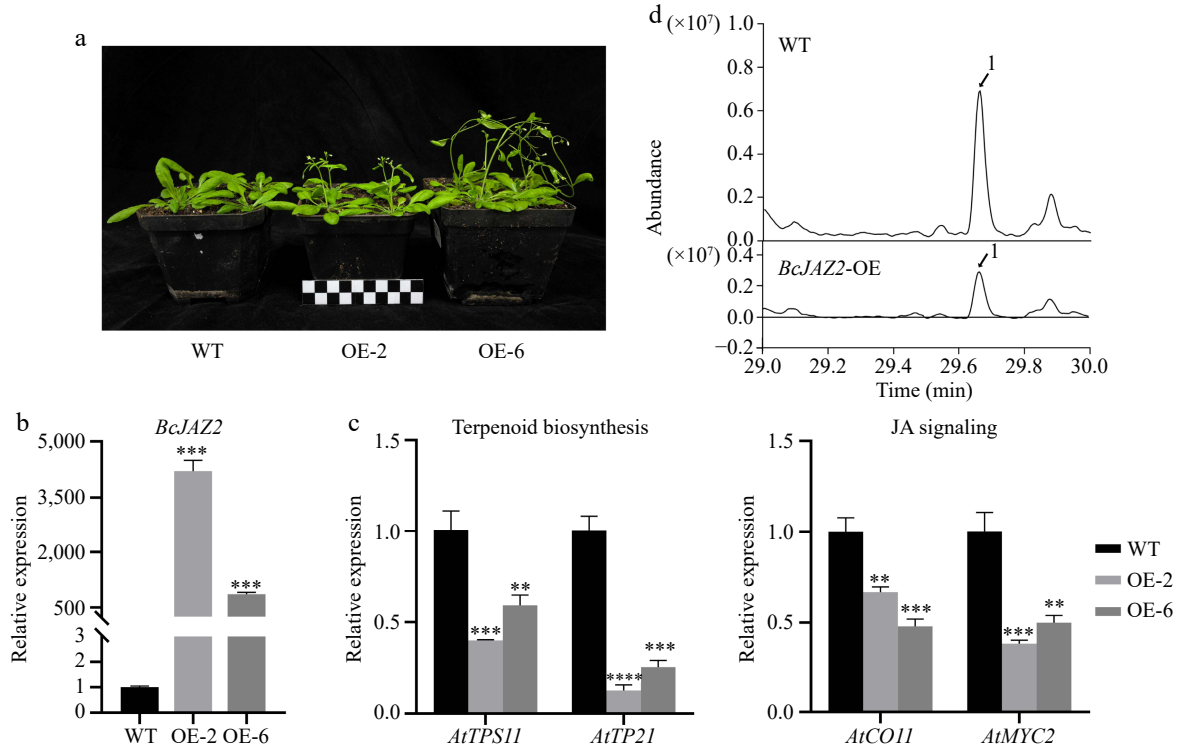

Fig. 7 Overexpression of *BcJAZ2* inhibits *β*-caryophyllene biosynthesis in *Arabidopsis*. (a) The phenotypes of *BcJAZ2* transgenic *Arabidopsis*. The black and white boxes are scale bar and one box represents 1 cm. (b) Relative expression of *BcJAZ2* in *Arabidopsis* transgenic lines inflorescences. (c) Analysis of sesquiterpene synthase and JA signaling genes expression level in inflorescences of *BcJAZ2* transgenic *Arabidopsis*. Student's t-test: $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. Three experiments were repeated and values were means ± SD. (d) GC-MS chromatogram analysis of *β*-caryophyllene emitted by *Arabidopsis* inflorescences in both wild-type (WT) and transgenic lines. Peaks with number represented *β*-caryophyllene.

approach (Fig. 8a). The expression of *BcJAZ2* was significantly reduced in the silenced plants (PTY-*BcJAZ2* #11, #13) compared to the control (PTY), confirming that *BcJAZ2* was successfully silenced (Fig. 8b). qRT-PCR data suggested that the expression levels of *β*-caryophyllene synthase gene *BcTPS21* and key genes for JA signaling in silenced plants were significantly promoted (Fig. 8c).

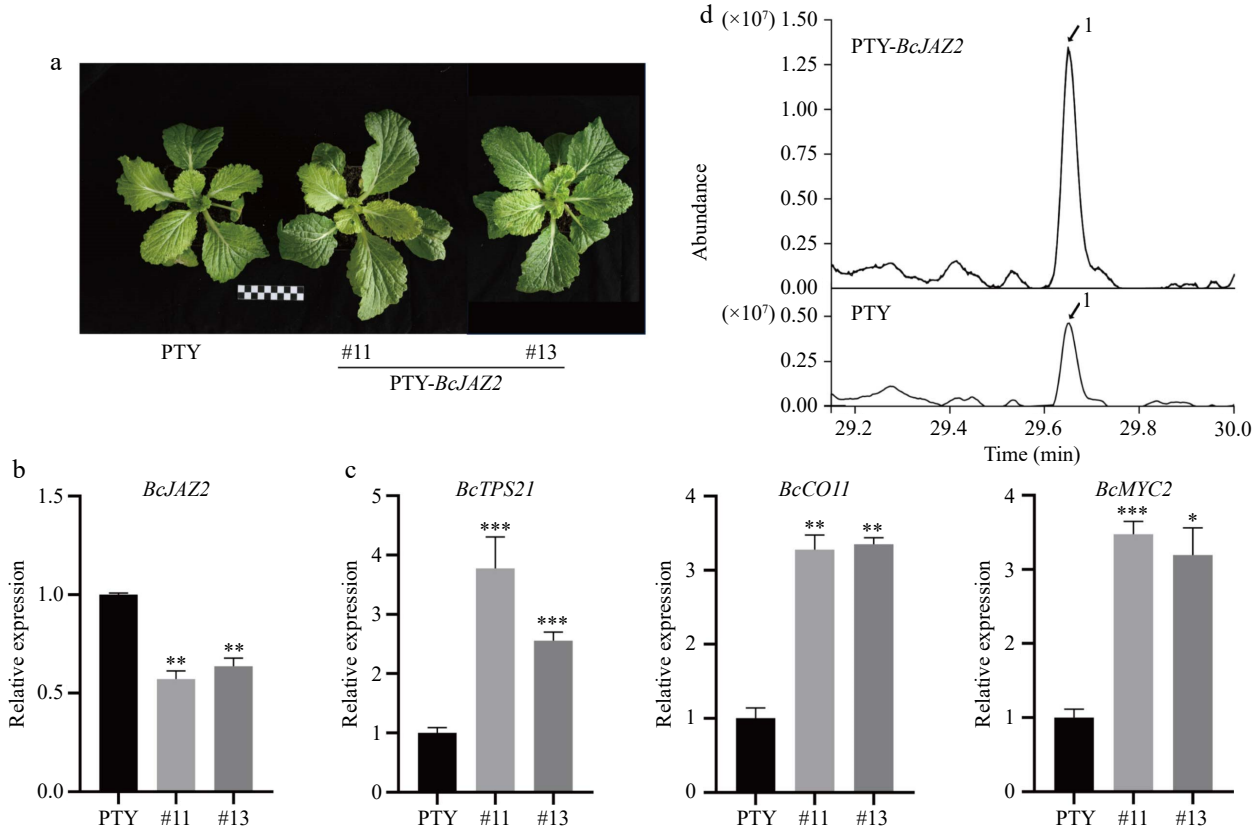

Fig. 8 Silencing *BcJAZ2* by VIGS promotes *β*-caryophyllene biosynthesis in NHCC. (a) The phenotypes of *BcJAZ2* in silenced plants. The black and white boxes are scale bar and one box represents 1 cm. (b) Relative expression of *BcJAZ2* in silenced and control plants. (c) Analysis of sesquiterpene synthase and JA signaling genes expression level of *BcJAZ2* in silenced and control plants. Student's t-test: *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$. Three experiments were repeated and values were means ± SD. (d) GC-MS chromatogram analysis of *β*-caryophyllene in silenced and control plants. Peaks with number represented sesquiterpenes *β*-caryophyllene.

Additionally, further monitoring of the impact of *BcJAZ2* silencing on the *β*-caryophyllene biosynthesis was conducted by GC-MS (Fig. 8d). Consistent with the gene expression patterns, an approximately 3-fold increase in *β*-caryophyllene content was measured in PTY-*BcJAZ2* plants compared to the PTY control.

BcbHLH137, BcHBI1.1, and BcHBI1.2 are upstream regulators of *BcJAZ2*

To search for relevant transcription factors that may regulate *BcJAZ2* expression, we used the yeast one-hybrid screen library technique. Cis-elements analysis of the *BcJAZ2* promoter of upstream 1,250 bp sequence showed that there were 13 kinds of cis-elements in this region (Supplementary Table S4). Finally, three candidate genes (*BcbHLH137*, *BcHB1.1*, *BcHBI1.2*) were screened. Subcellular localization and trans-activation activity assays demonstrated that these three genes are located in the nucleus and all have trans-activation activity capacity, consistent with the characterization of transcription factors (Fig. 9a, b). The Y1H and LUC results showed that BcbHLH137, BcHBI1.1, and BcHBI1.2 all positively regulated *BcJAZ2* (Fig. 9c–e). These results provided candidate genes for upstream regulation of *BcJAZ2* expression, which may provide new clues for exploring more functions of *BcJAZ2*.

Discussion

Plants possess complex biological regulatory networks that control their growth cycles and the synthesis and accumulation of various substances essential for their survival[28]. JAZ is a key negative regulator of the JA signaling pathway and participates in many biological processes as well as complex synergistic and antagonistic interactions between JA and other hormones[29–32]. Although *JAZ* gene family has been identified in many species including *Arabidopsis*[7], rice[33], maize[34], *B. oleracea*[35], and *B. napus*[36], there are few reports on the *JAZ* genes in NHCC. Twenty five *BcJAZ* genes were identified in this study, named *BcJAZ1-25* (Supplementary Table S2). Phylogenetic relationships analysis showed that *BcJAZ* gene family members are categorized into five families (I, II, III, IV, and V) (Fig. 1b). It was shown that 56 and 31 *JAZ* genes were identified in the reference genomes of *B. napus* and *B. oleracea*[37]. Compared with 12 JAZ genes in *Arabidopsis*, this is consistent with a whole-genome triplication (WGT) event that have been demonstrated during the evolution of *Brassica* species. The presence of TIFY and Jas domains in all BcJAZ proteins examined suggested that these domains were highly conserved and may have significant structural characteristics (Fig. 2). Gene duplications confer new functions for the evolution of species, and help them adapt to the environment. Consistent with the findings in *B. napus* and *B. rapa*[37,38], there were no instances of tandem duplications in the *BcJAZ* genes within the NHCC genome (Supplementary Table S3), Intraspecies and interspecies collinearity analyses indicated that segmental duplication was the major duplication event for *BcJAZ* genes, meanwhile, chromosomal and gene amplification events may have occurred during the evolution of NHCC (Fig. 3a, b).

The dynamic expression of genes under different treatments may reflect the gene functions. By analyzing the promoter cis-elements of 25 *BcJAZ* genes, some types of development-related response elements, stress-responsive elements, light-responsive elements, and hormone response elements were detected in most *BcJAZ*

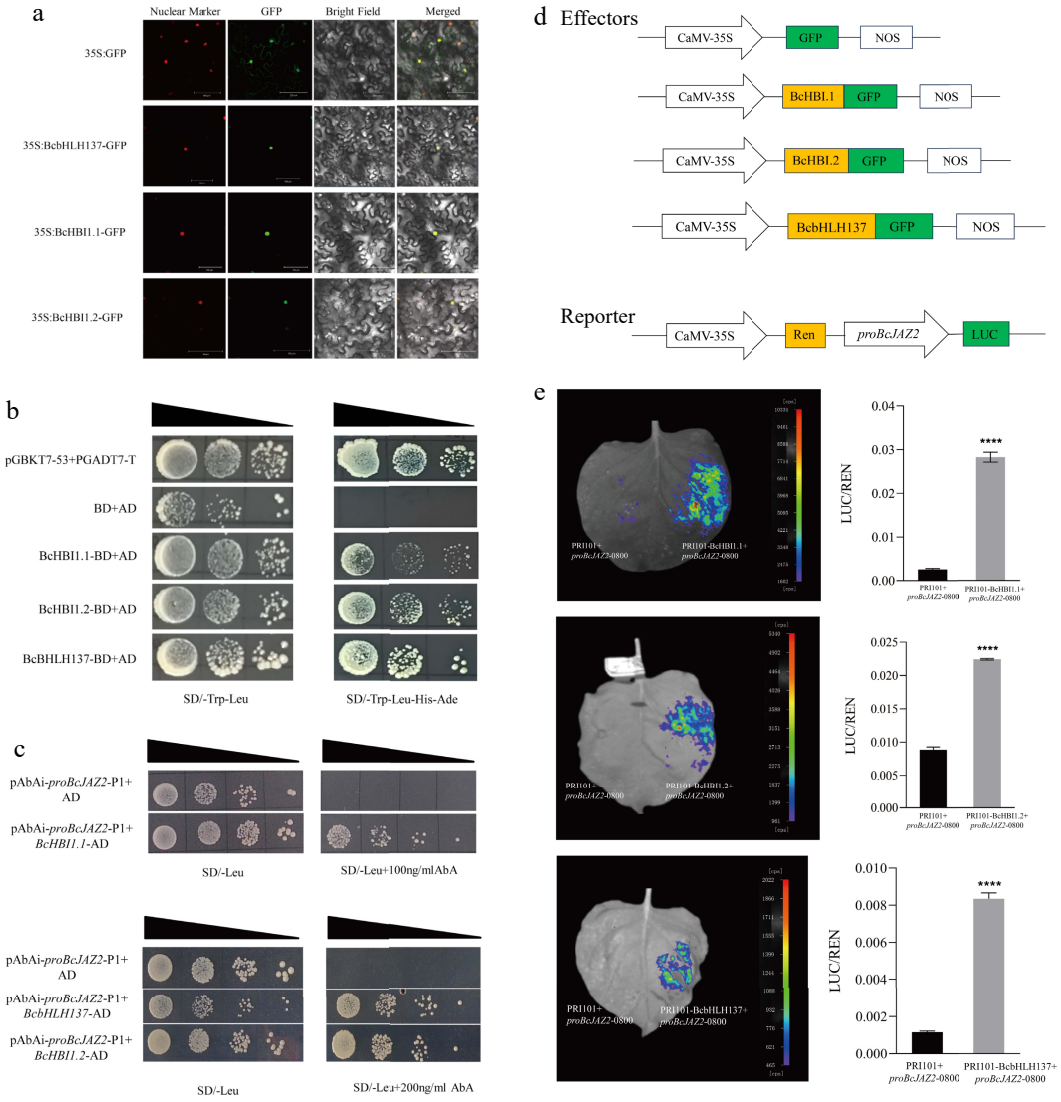

Fig. 9 Screening of upstream regulatory genes of *BcJAZ2*. (a) Subcellular localization of BcbHLH137, BcHBI1.1, and BcHBI1.2. The scale bar was 100 μm. (b) Trans-activation activity of BcHBI1.1, BcHBI1.2, and BcbHLH137 using yeast two-hybrid assay. (c) Yeast one-hybrid assay. (d) Schematic diagram of the effectors and reporter vectors. (e) Dual-luciferase assay showed that the screened transcription factors promoted BcJAZ2 expression. The data were from three experiments replicates and values were means ± SD. Student's t-test: ****$p \leq 0.0001$.

promoters (Fig. 4). qRT-PCR data demonstrated that these *BcJAZ* genes exhibited distinct expression profiles in response to low temperature (4 °C), MeJA, and GA treatments (Fig. 5b–d), which was in accordance with the findings from other *Brassica* crops[37]. The response to different hormone treatments suggested that JAZ proteins were involved in the crosstalk of different hormone signals to maintain normal plant growth. Notably, RNA-seq data revealed that the expression level of *BcJAZ2* was most significantly up-regulated after three days of low temperature (Fig. 5a), while *BcJAZ2* was also induced under short-term low-temperature treatment. However, previous studies showed that low temperatures inhibited *β*-caryophyllene synthesis in NHCC (unpublished data). Therefore, we hypothesized that BcJAZ2 may be a key negative regulator of *β*-caryophyllene synthesis. Similarly, high-temperature treatment significantly induced the expression of the JA signaling pathway repressor *CsJAZ6* in *Camellia sinensis*, which partially mediates secondary metabolite biosynthesis by affecting the formation of the MBW complex[39].

In our study, we further explored the molecular mechanism by which BcJAZ2 was involved in the regulation of *β*-caryophyllene. Subcellular localization confirmed that BcJAZ2 was a nuclear-localized protein (Fig. 6a). As a core component of the JA signaling cascade, MYC2 is responsible for the regulation of secondary metabolism across diverse species[16,27,40]. *AtMYC2* in *Arabidopsis* can directly bind to the promoters of the sesquiterpene synthase genes *AtTPS21* and *AtTPS11* and activate their expression, leading to the release of sesquiterpenes, especially *β*-caryophyllene[27]. Here, we showed that BcJAZ2 interacted with BcMYC2 through Y2H and BiFC assays (Fig. 6c, d). Furthermore, the transcription of sesquiterpene synthase and JA signaling genes were significantly repressed and the content of sesquiterpene *β*-caryophyllene was decreased in *BcJAZ2* overexpression *Arabidopsis* compared with the wild-type (WT) *Arabidopsis* (Fig. 7). As expected, the results in *BcJAZ2* silenced plants were opposite (Fig. 8). Hence, we propose that BcJAZ2 serves the role of a transcriptional repressor within the JA signaling, forming a BcJAZ2-BcMYC2 cascade to regulate the biosynthesis of sesquiterpene in NHCC.

Transcription factors can bind specific DNA sequences and regulate gene expression through activation or repression. Three transcription factors (*BcbHLH137*, *BcHB1.1*, *BcHBI1.2*) were screened

and identified using the yeast one-hybrid (Fig. 9c). LUC results showed that these three TFs all have positive regulation of *BcJAZ2* expression (Fig. 9e). Interestingly, these three TFs all belong to the bHLH family, which regulate cell proliferation and elongation, and their transgenic *Arabidopsis* exhibits an early flowering phenotype[41,42]. Recent studies showed that *JAZ3* transgenic *Arabidopsis* displayed shorter hypocotyls and larger cotyledons under specific light or temperature environments, compared with the wild-type *Arabidopsis*[43]. In this investigation, we also found that *BcJAZ2* transgenic *Arabidopsis* have big cotyledons and early flowering phenotypes. Plants can utilize phytohormones to integrate and transmit various signals in order to adjust their growth and development to adapt to the environment, with various phytohormones involved in this regulatory process by interfering with each other. Among them, some key regulators in the hormone signaling pathway can act on multiple hormone signaling processes. Previous studies revealed that the bHLH transcription factors specifically bind to the E-box element[44] (5'-CANNTG-3'). From this, we suspected that bHLH137 and HBI1 might participate in the transcriptional regulation of plant development and terpene under diverse environments and phytohormone signals, possibly through binding to the *BcJAZ2* promoter E-box element. However, the precise regulatory mechanisms underlying these processes require further investigation.

Conclusions

In conclusion, we performed a genome-wide analysis of the *JAZ* gene family in NHCC. Expression pattern analyses indicated that *BcJAZs* gene were responsive to low temperature, MeJA, and GA. In addition, we confirmed that BcJAZ2 negatively regulates β-caryophyllene synthesis by interacting with BcMYC2. Three transcription factors (BcbHLH137, BcHBI1.1, BcHBI1.2) were screened and positively regulated *BcJAZ2* expression. However, more evidence is needed to determine whether these transcription factors are also involved in the regulation of β-caryophyllene synthesis in NHCC.

Author contributions

The authors confirm contribution to the paper as follows: experiments Design, data analysis, and manuscript writing: Han T, Wang H; experimental guidance: Liang Z, Bai A; manuscript revision: Han T, Wang H, Xu H, Li Y; experimental resources offering: Wang J, Liu T, Hou X, Li Y; experiments supporting: Li Y. All authors reviewed the results and approved the final version of the manuscript.

Data availability

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Conflict of interest

The authors declare that they have no conflict of interest.

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