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Gibberellin Regulates *LBD38-1* Responses to *Xanthomonas arboricola* pv. *juglandis* Infection in Walnut Bacterial Blight Pathogenesis

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Abstract

Background Plant responses to biotic and abiotic stresses are complex processes. Previous studies have shown that the *LBD* gene family plays important roles in plant growth and development as well as in plant defense against biotic and abiotic stresses. The expression of *LBD* genes was investigated in walnuts under biotic and abiotic stresses, revealing that *LBD38-1* may be a key gene in the plant stress response. This study provides new insights into the roles of *LBD* genes in plant responses to biotic stress.

Results Forty-nine members of the *JrLBD* gene family were identified in the walnut genome and classified into six subfamilies. Comparative homology analysis through phylogenetic trees revealed that the presence of Group I-a and Group VI plays an important role in resistance to stressors. The expression of walnut *LBD* genes under cold-temperature, high-temperature, mechanical damage, and biotic stresses was analyzed via transcriptome sequencing, and the expression of *JrLBD38-1* in the Group VI subfamily was particularly prominent. According to transcriptome profile analysis, *JrLBD38-1* is highly expressed in different tissues of walnuts, suggesting that it plays a regulatory role in the growth and development of different tissues. The function of the Gibberellin (GA) response element in the *JrLBD38-1* promoter was further analyzed and verified. These findings confirmed that GA regulated *JrLBD38-1* expression changes during *Xanthomonas arboricola* pv. *juglandis* infestation of walnut leaves.

Conclusion Forty-nine walnut *JrLBDs* were identified and classified into six subfamilies. *JrLBD38-1* has GA-inducible expression, is regulated by GA under pathogenic bacterial stress, and is involved in the response to biotic stress. This function of *JrLBD38-1* provides new insights into walnut disease resistance mechanisms.

Keywords Walnut, *LBD*, Stress, Gibberellin, Blight, Transcriptome sequencing

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Background

Walnuts (*Juglans regia* L.), also known as English and Persian walnuts, are deciduous trees of the family Juglandaceae. Walnuts are rich in protein, fiber, polyphenols, alpha-linolenic acid, and many other nutrients that are important for human health [1, 2]. The cultivation history of walnuts in China dates back more than 2000 years [3]. In recent years, the frequent climate anomalies, drought, heat, waterlogging, and cold damage has increased the attention of researchers on the response mechanisms of walnuts to biotic and abiotic stresses [4–6]. On the other hand, China's promotion of varietalization of walnuts and large-scale planting of precocious cultivars have led to the rapid expansion of precocious cultivars with poor resistance, resulting in the occurrence and gradual aggravation of diseases such as blight and anthracnose, which have severely affected the quality and yield of walnuts [7, 8].Walnut blight, caused by *Xanthomonas arboricola* pv. *juglandis* (*Xaj.*), is one of the most common infectious diseases of walnuts, causing severe economic losses. Therefore, considerable effort should be invested in understanding the innate resistance mechanisms in plants.

Lateral organ boundary (LBD) genes are a class of transcription factors that regulate the development and morphogenesis of plant lateral tissues and organs, and as the *LBD* gene family is present only in plant genomes, it may be involved in plant-specific regulation [9]. The LOB domain contains three motifs: the CX2CX6CX3C motif consists of four fully conserved cysteine residues separated by several conserved amino acid residues; the Gly-Ala-Ser motif consists of glycine, alanine, and serine; and the leucine-like zipper-like motif (LX6LX3LX6L) [10, 11]. Members of the *LBD* gene family are divided into two subfamilies depending on the completeness of the LOB domain that they contain: Class I genes contain the complete LOB domain, and Class II genes lack the Gly-Ala-Ser motif [11].

The *LBD* family of genes is widely distributed in many plants, such as *Arabidopsis* [12], *Zea mays* [13], *Triticum aestivum* [14, 15], *Solanum tuberosum* [16], and *Vitis vinifera* [17]. In recent years, the *LBD* gene family has been reported to be closely associated with plant growth and developmental regulatory processes, such as secondary growth, fruit growth and development, the environmental stress signal response, and the pathogen response [18–23]. ARF7/ARF19-LBD16/LBD18, via AUX1/LAX3 auxin influx carriers, plays a pivotal role in lateral root formation and is involved in AR formation in *Arabidopsis* [24]. In *Vitis vinifera* [17] and *Solanum lycopersicum* [25], LBD transcription factors are involved in fruit development, berry ripening, and hormone response [26]. Specifically, LBD plays a regulatory role in the response to biotic and abiotic stresses [27]. *SlLBD40* is involved in JA signaling and is a negative regulator of drought resistance in tomato [28], and *SlLBD40* knockout enhances drought resistance in tomato. On the other hand, *LBD* genes are involved in regulatory processes in response to biotic stress. *LBD20* may function in jasmonate (JA) signaling, and disruption of the *LBD20* gene leads to increased resistance to the root-infecting vascular wilt pathogen [29]. PthA4, the effector gene of the *Xanthomonas* citri subspecies, enters the citrus genome, where it specifically recognizes and binds to the *CsLBD1* gene promoter, regulates the expression of *CsLBD1* in the host, and increases susceptibility to citrus bacterial canker disease [30]. Genes in the *LBD* family are also affected by GAs during the regulation of plant growth, development, and resistance to stress. *ZmLBD5* is involved in the regulation of maize (*Zea mays*) by affecting the synthesis of GAs and abscisic acid (ABA) through the regulation of maize growth and the drought response by affecting the synthesis of GAs and ABA [31]. In transgenic rice plants heterologously overexpressing *PheLBD12*, GA_3 bioactivity was reduced, and the internodes were shortened [32].

The central role of the GA class of growth hormones in the response to abiotic and biotic stress is becoming increasingly evident [33, 34]. Genes and regulatory proteins involved in GA synthesis and signaling pathways are closely related to plant stress tolerance [33, 35]. Plants can adapt to unfavorable external conditions by regulating the expression of GA-related metabolic genes, such as *GA20oxs*, *GA3oxs*, and *GA2oxs*, and GA signaling-related proteins, such as DELLAs [36]. In addition, plants can improve salt tolerance by reducing active GAs [37]. The overexpression of *GA2oxs* in rice enhances salinity tolerance, which is lost when GA_3 is applied externally [38]. Similarly, GAs play an important role in the plant response to biotic stress. Exogenous GA_3 treatment of *Nicotiana benthamiana* plants increased their susceptibility to the infectious pathogens *Ralstonia solanacearum* and *Rhizoctonia solani* [39]. In particular, the exogenous application of GA_3 and an inhibitor of GA synthesis (uniconazole) can increase disease susceptibility and resistance to bacterial blight, respectively [40].

In this study, we performed a systematic analysis of *LBD* family genes at the genomic level, including chromosomal localization, phylogenetic tree construction, gene structure analysis, conserved motif detection, and promoter element analysis, via published walnut genomic data. Changes in the expression of walnut *LBD* family genes in response to cold-temperature, high-temperature, mechanical damage, and biotic stresses were investigated, and the stress-sensitive response gene *JrLBD38-1* was screened by combining the expression differences of *LBD* genes in different tissues. Furthermore, expression

analysis of *JrLBD38-1* and functional validation of promoter cis-acting elements confirmed that GA participates in the response to biotic stress by regulating the *LBD*. This study clarifies the relationship between GA and *LBD* and provides new insights into the role of GA and *LBD* genes in plant responses to biotic and abiotic stresses.

Results

Identification and analysis of LBD genes in the walnut genome

In this study, 49 *JrLBD* genes were identified from the walnut 2.0 genome, and these 49 family members were named according to their covariance with *Arabidopsis* (Supplementary file 1). Among all the *JrLBD* genes, the longest gene length was 27,203 bp (*JrLBD21*), the shortest length was 390 bp (*JrLBD22-4*), the length of the cds sequences formed after coding was the longest at 1266 bp (*JrLBD36-4*) and the shortest at 390 bp (*JrLBD22-4*), the translated length of the protein was between 129 and 421 aa, and the theoretical molecular weight was between 14.84 kDa and 46.80 kDa. There were 29 proteins with an isoelectric point (pI) < 7, of which the smallest was 4.97 (JrLBD33), and 20 proteins had an isoelectric point (pI) > 7, of which the largest was 9.88 (JrLBD22-4). Subcellular localization prediction revealed that all *JrLBD* family genes were localized in the nucleus, indicating that LBD transcription factors can function in the nucleus.

Phylogenetic trees are widely used to show evolutionary relationships among genetic families. To study the evolutionary relationships between *LBD* family genes in walnuts and *Arabidopsis thaliana*, a phylogenetic tree was constructed via the neighbor–joining (NJ) method in the MEGA 11 program with the full-length proteins of 49 genes in walnut and 42 genes in *Arabidopsis thaliana*. According to the phylogenetic tree, the 49 members of the walnut LBD family were divided into six groups, Groups I-VI, of which Group I was divided into Group I-a and Group I-b. Among them, subfamily II was the largest, with significant gene duplication and extension. Group II was further divided into groups II-a, II-b, II-c, and II-d (Fig. 1).

Chromosomal analysis and collinearity analysis of walnut JrLBD members

Chromosomal localization analysis revealed that the *JrLBD* family genes presented a random and uneven distribution on the remaining 15 chromosomes, except of chromosome 16 (Supplementary file 2). The chromosome numbers with the highest to lowest numbers of genes were, in descending order, chromosomes 7, 10, 12, 1, 14, 2, 11, 8, 5, 3, 15, 13, 9, 6, and 4, with a total of 10 genes on chromosome 7, which had the highest number of genes in the distribution, and only one gene on chromosomes 15, 13, 9, 6, and 4. Among the 49 *JrLBD* family members identified, more than half were present in gene clusters.

The within-genome duplication events of the *JrLBD* genes were analyzed via MCScan. The results indicated that six genes located on chromosomes 1, 7, and 10 were involved in tandem duplication events, resulting in the formation of three gene pairs. We also performed collinearity analysis of *the LBDs* to explore the evolutionary relationships of *the JrLBDs*. The results revealed that 11 syntenic *JrLBD* gene pairs derived from whole-genome duplication or segmental duplication were distributed on 13 of the 16 chromosomes (Fig. 2).

Conserved motif and gene structure analyses of the walnut LBD gene family

To determine the genetic structure and evolutionary trajectory of the *JrLBD* gene in walnut, the exon–intron compositions of 49 *JrLBDs* were studied. The gene with the greatest number of introns was *JrLBD36-4*, with five, and there were no introns in *JrLBD2-1* or *JrLBD22-4*. In addition, the genes forming the subgroups presented similar gene structures (Fig. 3a, b).

The conserved motifs of JrLBD proteins were predicted. Ten conserved motifs, named 1–10, were identified in this study (Supplementary file 3). All the proteins encoded by the *JrLBD* family genes contained the predicted motif 1 and motif 3, and the number and type of conserved motifs in the protein sequences were relatively consistent across proteins. The class I members had motif 1 (CX2CX6CX3C), motif 3 (Gly-Ala-Ser), and motif 4 (LX6LX3LX6L), whereas the class II members lacked motif 4 (LX6LX3LX6L), a result that is consistent with the findings of previous studies.

Expression patterns of *JrLBD* genes under biotic and abiotic stress

The transcriptome data of walnut were used to analyze the gene transcription and expression patterns of the *JrLBD* family in the 'Qingxiang' cultivar under cold-temperature stress for 48 h (Fig. 4a). The results revealed that the transcription levels of *JrLBD38-1* and *JrLBD38-4* significantly increased after 0.5 °C low-temperature stress treatment for 0 h and 48 h, and the expression level of *JrLBD38-1* significantly increased, making it the most highly expressed gene of the LBD transcription factor family.

The transcriptome data of walnut were used to analyze the gene transcription and expression patterns of the *JrLBD* family in the 'Qingxiang' cultivar under heat stress for 6 h (Fig. 4b). The results revealed that the expression of *JrLBD38-1* changed the most after 0, 2, and 6 h of high-temperature stress in the potted walnut seedlings.

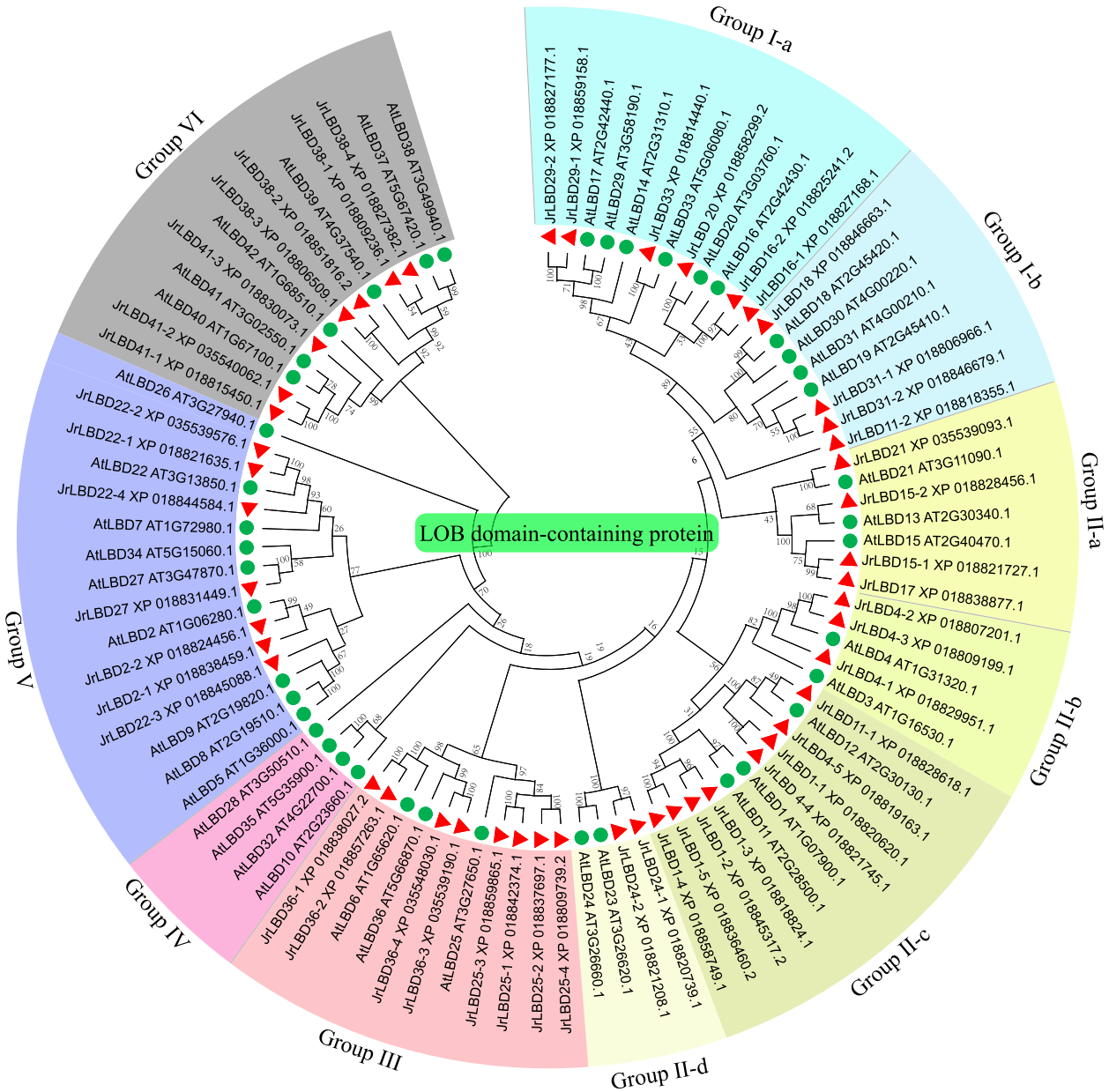

Fig. 1 Phylogenetic analysis of LBD proteins in *Arabidopsis thaliana* and *Juglans regia*. Areas of different colors represent different subfamilies. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches

The expression of *JrLBD38-1* significantly decreased after 2 h of high-temperature stress.

The transcriptome data of the walnuts were used to analyze the gene transcription of the *JrLBD* family under mechanical damage stress (Fig. 4c). The results revealed that the expression levels of *JrLBD38-1*, *JrLBD41-1*, *JrLBD41-2*, and *JrLBD41-3* increased to different degrees after 12 h of injury stress, with the expression level of *JrLBD38-1* being the highest. The expression levels of *JrLBD38-1*, *JrLBD41-1*, and *JrLBD41-3* were lower than those at 12 h after 24 h of injury but were still higher than those at 0 h, and the expression levels of *JrLBD41-2* increased. The expression of *JrLBD38-1* was the highest 24 h after injury.

The transcriptome data of walnuts were used to analyze the transcription patterns of *JrLBD* family genes under biological stress (Fig. 4d). The transcript levels of *JrLBD38-1* tended to decrease but then increase, whereas those of *JrLBD1-3* tended to increase but then decreased.

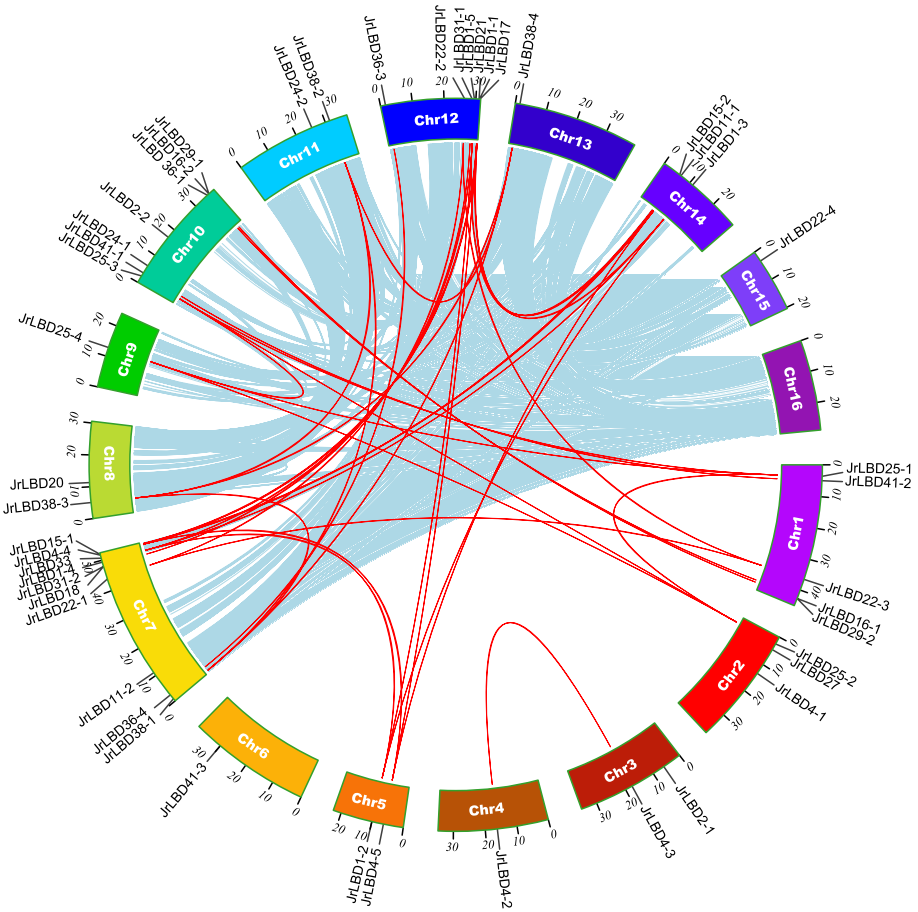

Fig. 2 Distribution of *LBD* genes derived from segment replication in the walnut genome. The red lines represent the pairing of the *JrLBD* gene, whereas the gray lines represent the pairing of all the walnut genes. The outer ring represents the position of *JrLBDs* on each chromosome. Boxes of different colors represent the 16 chromosomes of walnuts

Expression analysis of *JrLBDs* in different walnut tissues

The transcriptome data of seven different tissues of 'Qingxiang' plants (pistils, catkins, roots, mature leaves, young leaves, young stems, and buds) revealed differences in *JrLBD* expression (Fig. 5). *JrLBD38-1* presented the highest expression in roots, catkins, and young leaves. *JrLBD38-4* was highly expressed in mature leaves, and *JrLBD41-1* was highly expressed in roots. Multiple genes belonging to the Group VI subfamily presented high expression levels in different tissues (Supplementary file 4). The expression of genes from different subfamilies also significantly differed across different tissues. These findings suggest that *JrLBDs* play important roles in tissue formation and differentiation. Therefore, the present study was further conducted on *JrLBD38-1* on the basis of its differential expression in different tissues and under different stresses.

Changes in the GA content and expression of *JrLBD38-1* during bacterial stress treatment of leaves

Since the expression of *JrLBD38-1* changed significantly under different stress treatments, to further determine its bacterial stress response, RNA was extracted from the leaves at different times after inoculation (Fig. 6a), and cDNA was obtained via reverse transcription. qRT–PCR was performed on the *JrLBD38-1* gene, and the results revealed that the expression of *JrLBD38-1* tended to decrease from 0–24 h, after which the expression increased over time, which was generally consistent with the transcriptome sequencing results. (Fig. 6b).

Hormone levels play an important role in plant responses to pathogen stress. Generally, gene function

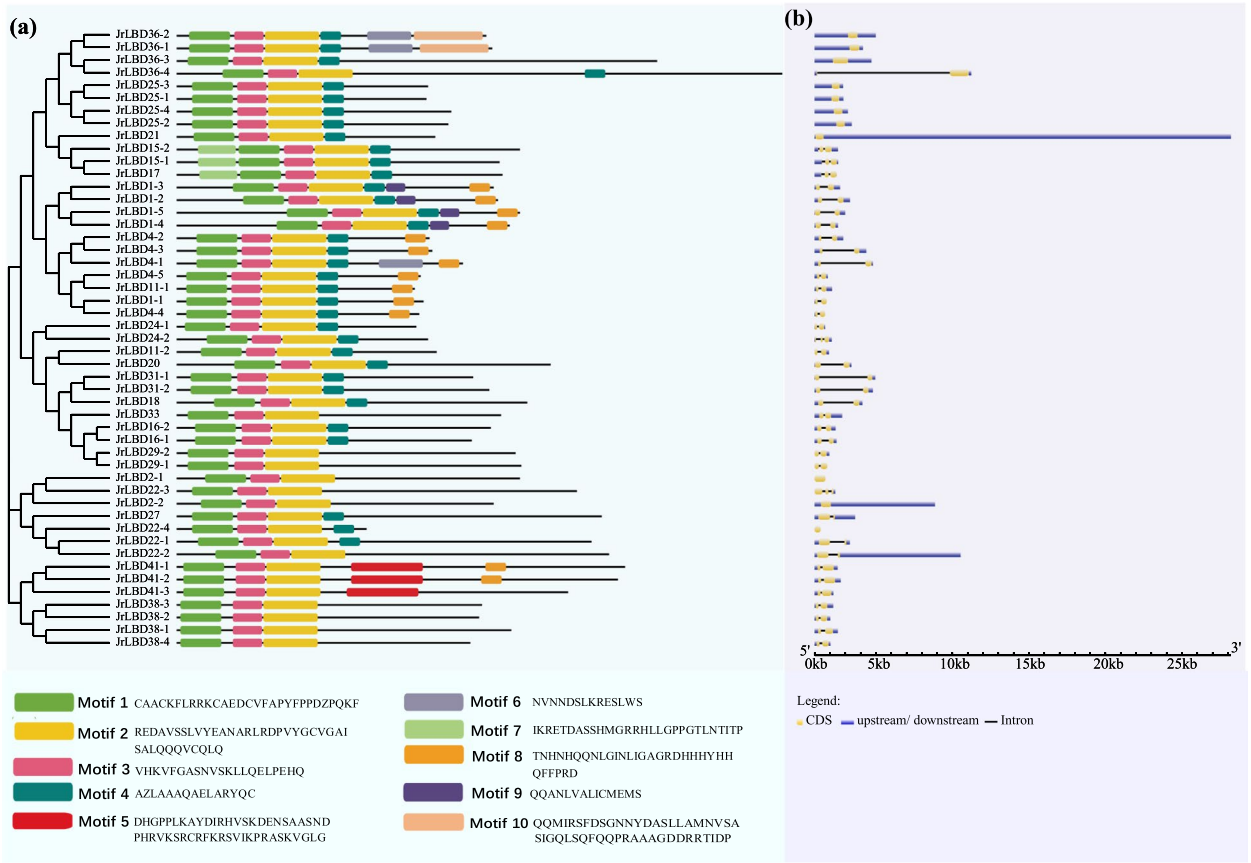

Fig. 3 Conserved motif analysis and gene structure analysis of walnut *LBD* members. **a** Gene structure analysis. **b** Conservative motif analysis. The number represents the branch series

is affected by the distribution of cis-acting elements in the promoter region. According to the predicted gene structure of the members of the *JrLBD* gene family, the upstream 2000 bp of the CDSs were extracted as the promoter sequence. Analysis of the promoter cis-acting elements of *JrLBD38-1* revealed that among the hormone-related cis-acting elements, the number of GA-responsive elements was the greatest (Fig. 6c). Thus, the content of GAs in the inoculated leaves was determined, and the results revealed that the GA content in the inoculated leaves gradually increased from 0 to 48 h with increasing treatment time and then decreased to the initial level after 72 h of treatment. Quantitative analysis of *JrLBD38-1* revealed that both *JrLBD38-1* expression and the GA content peaked at 48 h after inoculation (Fig. 6d). Therefore, we suggest that the expression of *JrLBD38-1* is regulated by GA and that the GA response element in the *JrLBD38-1* promoter may be responsible for this regulation.

Analysis of the locus of GA affecting *LBD* expression

To further explore the role of GA response elements in the *JrLBD38-1* promoter, a recombinant vector of *the JrLBD38-1* promoter-pGREEN II 0800-LUC was constructed, and luciferase was instantly transformed into tobacco (Fig. 7a). The fluorescence results revealed that the fluorescence intensity of the Q0 segment of the *JrLBD38-1* promoter significantly increased after GA treatment. The fluorescence intensity of the promoter Q1 segment also significantly increased after GA treatment. The fluorescence intensity of the Q2 segment of the *JrLBD38-1* promoter did not change significantly after GA treatment (Fig. 7b and c). After the GA element in the *JrLBD38-1* promoter fragment was removed (Fig. 7d), the fluorescence results revealed that the fluorescence intensity of the Q1* and Q2* segments of the *JrLBD38-1* promoter did not change significantly after GA treatment. The fluorescence self-activation of the Q2* segment of the *JrLBD38-1* promoter was

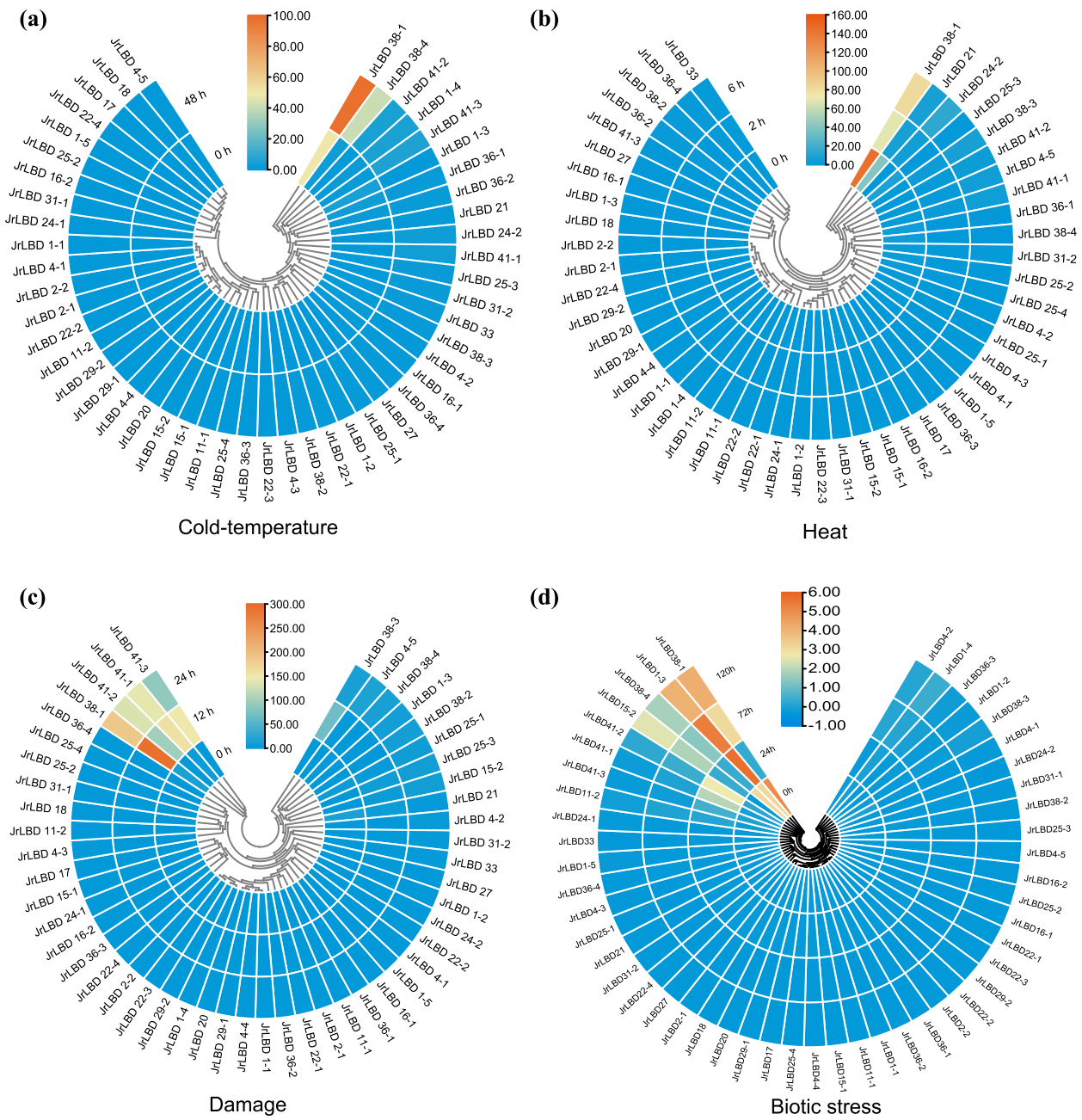

Fig. 4 Expression patterns of *JrLBD* genes under biotic and abiotic stresses (**a**). Changes in *JrLBD* expression during cold stress. **b** Changes in *JrLBD* expression during heat stress. **c** Changes in *JrLBD* expression during mechanical injury stress. **d** Changes in *JrLBD* expression during biotic stress

stronger than that of the Q2* segment of the *JrLBD38-1* promoter (Fig. 7e and f). On the basis of the fluorescence invariant results of different segments, we determined that the *JrLBD38-1* promoter was responsive to GA treatment and that the responsive segment was Q1. In summary, *JrLBD38-1* exhibits GA-induced expression, is regulated by GA under pathogen stress, and is involved in the response to biotic stress.

Discussion

As plant-specific transcription factors, LBD family members have been extensively studied in plants. To date, the *LBD* gene family has been identified and characterized in many plants, including 56 *LBDs* in *Solanum lycopersicum* [41], 55 in *Phyllostachys edulis* [42], 67 in *Malus domestica* [43] and 41 in *Rosa rugosa* [44]. In this study, 49 LBD family members were identified and analyzed

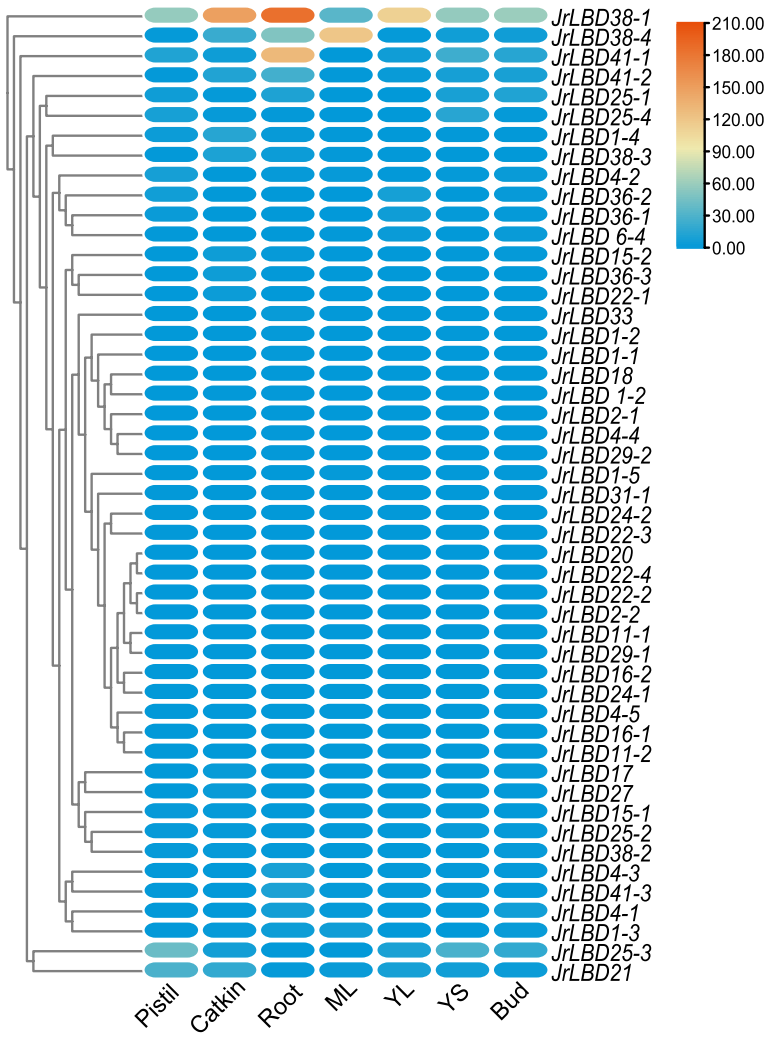

Fig. 5 Transcriptional expression of JrLBD gene family members in different walnut tissues (young stems, buds, young leaves, mature leaves, pistils, catkins, and roots)

on the basis of their protein properties. In addition, a similar genetic structure was observed for each small branch, with high subfamily conservation. Fragmentary and tandem replication of duplicated genes are the two main causes of gene family expansion in plants [45, 46]. There are 24 pairs of *LBD* gene replication sequences in the Ginkgo genome, including 22 pairs with fragment replications and only two pairs with tandem replications [47]. In contrast, there were 11 groups of collinear genes among the members of the walnut *LBD* gene family, and analysis of the collinear regions revealed that most of the genes probably originated from whole-genome duplication or gene fragment duplication.

A study of wheat *LBD* family genes revealed that the expression of some genes was affected by temperature, and the expression of two genes, *Ta-2B-LBD73* and *Ta-4D-LBD52*, was greater at 4 °C than at 23 °C [48]. This study also confirmed that low- and high-temperature stress are involved in regulating the expression of LBD family genes. However, cold-temperature and high-temperature treatments had opposite effects on *JrLBD38-1* expression, with cold-temperature treatment increasing *JrLBD38-1* expression and high-temperature treatment decreasing *JrLBD38-1* expression, suggesting that *JrLBD38-1* expression is strictly regulated by temperature and that *JrLBD38-1* may function under low- or high-temperature stress. This may be related to the role of the low-temperature response element in the *JrLBD38-1* promoter.

Further expression analysis and promoter function verification of the *LBD38-1* gene revealed that more important promoter elements, including the GA response, drought response, and ABA response elements, exist in the promoter region of the gene. The presence of these

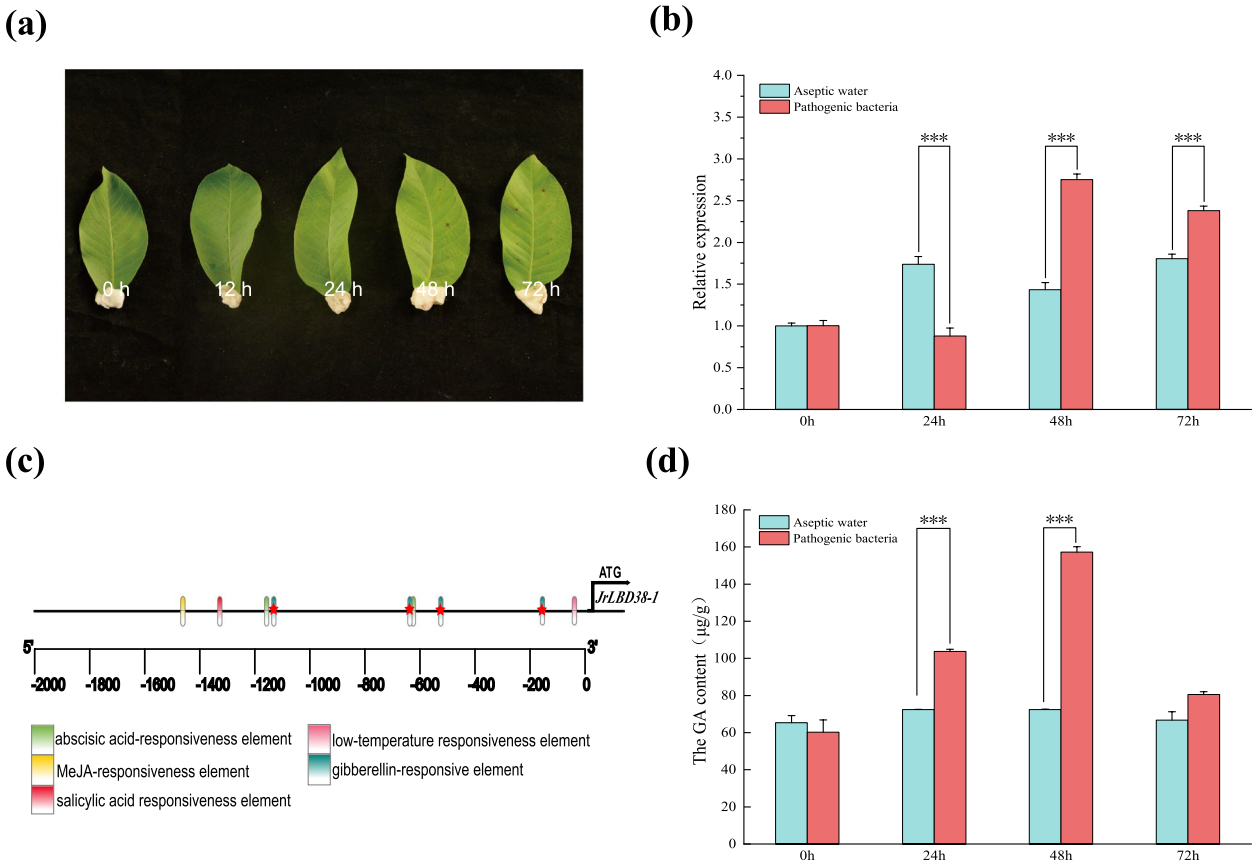

Fig. 6 **a** Leaf changes under different inoculation treatment times. **b** Relative expression of *JrLBD38–1*. The bars represent the means ± SDs ($n = 3$). Significant differences between treatments were determined by Student's t test: * < 0.05. ** $P < 0.01$. *** $P < 0.001$, Student's t-test. The same as below. **c** Analysis of the promoter cis-acting element analysis of *JrLBD38-1*. Different colors represent different components. The red star marks the original response element of gibberellin. **d** Variation in the GA content

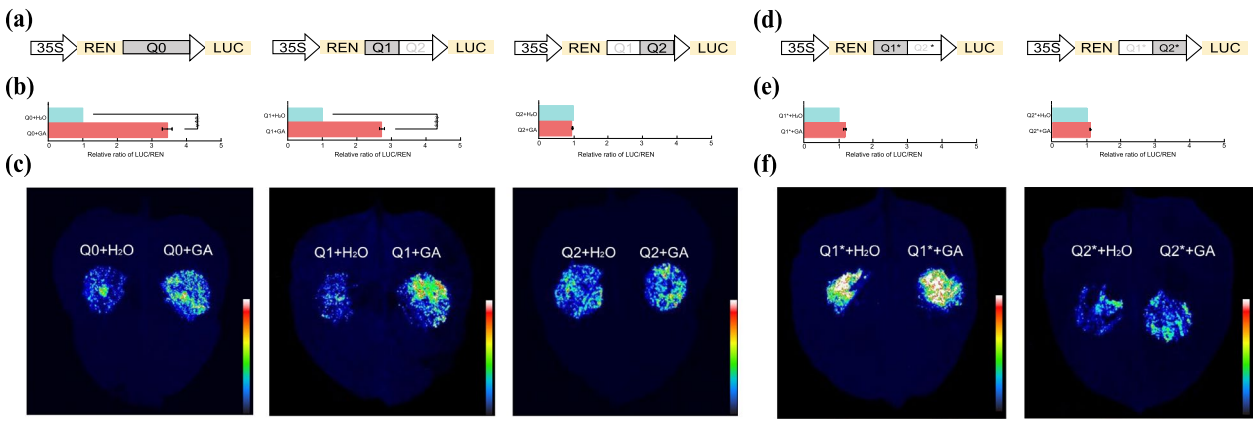

Fig. 7 Analysis of the cis-regulatory elements of the promoter. **a** Schematic representation of the reporter plasmids used in the dual LUC assay (retaining the gibberellin-responsive originals Q0, Q1 and Q2). **b** Relative activity levels of LUC (Q0, Q1 and Q2). Each bar indicates the mean ± SD from three biological replicates (*** $P < 0.001$). **c** Analysis of the Dual Luciferase Assay System (Q0, Q1, Q2, CK: sterile water application treatment as the control group; GA: 100 mg/L GA solution-coated treatment as the experimental group.) **d** Schematic representation of the reporter plasmids used in the dual LUC assay (removing the gibberellin-responsive originals Q1*, Q2*). **e** Relative activity levels of LUC (Q1*, Q2*). Each bar indicates the mean ± SD from three biological replicates (*** $P < 0.001$). **f** Analysis of the Dual Luciferase Assay System (Q1*, Q2*, CK: sterile water application treatment as the control group; GA: 100 mg/L GA solution-coated treatment as the experimental group.) Note: Q0 is the promoter fragment −1,150 bp to −125 bp, Q1 is the promoter fragment −1150 bp to −584 bp, and Q2 is the promoter fragment −604 bp to −125 bp

important response elements suggests a role for the LBD family in the response to hormonal signaling. The promoter of the citrus stress resistance gene *CcGASA4* contains a GA response element (GARE), and exogenous GA_3 induces the expression of this gene in the leaves, suggesting that GA can respond to stress responses by regulating functional genes [49]. *CsLBD38* and *CsLBD39-2* expression is upregulated in tomato after methyl jasmonate (MeJA) treatment, *SlLBD40* is involved in the JA signaling pathway, which enhances drought resistance in tomato, and the ABA signaling pathway has been shown to regulate *AtLBD40* in response to salt stress [50]. Therefore, we hypothesized that *JrLBD38-1* is regulated by hormones through a response element in the promoter region, which in turn responds to biotic stress.

GAs play signaling roles in plant responses to biotic and abiotic stresses [33]. Drought-induced GA deactivation in guard cells contributes to stomatal closure in the early stages of soil dehydration, whereas the inhibition of GA synthesis in leaves suppresses canopy growth and restricts area [33]. GA also plays a key role in root-to-shoot communication to induce stomatal closure under water deficit conditions [35]. Exogenous GA_3 treatment increases the susceptibility of tobacco to the pathogens *Ralstonia solanacearum* and *Rhizoctonia solani* [39]. In this study, significant changes in the GA content occurred after *Xaj.* infestation of walnut leaves, which was synchronized with the changes in the relative expression of *JrLBD38-1*. We also found that the promoter of *JrLBD38-1* contained GA-responsive elements and was regulated by GA. In turn, changes in the GA content are affected by pathogen stress, and the GA content increases at the beginning of pathogen infection. To date, studies on the hormonal responses of *LBD* family genes have revealed that the *Zea mays LBD* family genes *LBD5* and *LBD33* regulate GA synthesis [31]. Therefore, we suggest that the expression of *JrLBD38−1* increases in response to pathogenic bacterial stress by regulating the GA signaling pathway at an early stage of pathogen infection. In addition, we are also concerned that ABA- and SA-related response elements are also included in the cis-acting elements of the *JrLBD38-1* promoter, and whether ABA and SA regulate *JrLBD38-1* to cope with pathogenic bacterial infestation has become the focus of future studies.

Conclusion

Forty-nine walnut *JrLBDs* were identified and classified into six subfamilies. *Xaj.* infestation resulted in changes in the GA content of the walnut leaves. *JrLBD38-1* has GA-inducible expression, is regulated by GA under pathogenic bacterial stress, and is involved in the response to biotic stress. The mechanism of the LBD family in response to disease resistance should be further elucidated. In conclusion, the discovery of *JrLBD38-1* provides new ideas for the genetic improvement of disease resistance in walnuts.

Methods

Plant materials

The plant materials used in this study were 'Qingxiang' walnut (*Juglans regia*) and tobacco (*Nicotiana benthamiana*). The potted seedlings used for the low-temperature treatment were 'Qingxiang' two-year-old seedlings cultured in an incubation room at constant temperature and humidity (22 ± 2 °C, 70 ± 5%). Walnut explants were used for mechanical damage treatment (taken from the annual branches of 'Qingxiang' trees grown in the Experimental Field of Hebei Agricultural University, sterilized and cultured). Walnut tissues (young stems, mature stems, young leaves, mature leaves, pistils, catkins, roots and shoots) were collected from different seasons. For biotic stress, uniformly sized leaves were collected from potted seedlings of ' Qingxiang ' walnut (Supplementary file 5).

Stress treatments

To investigate the gene expression patterns of 'Qingxiang' walnut (*Juglans regia*) under various biotic and abiotic stresses across different tissues, we conducted stress treatments on potted walnut seedlings. On the basis of previous findings demonstrating significant physiological changes in walnut seedlings exposed to low temperature stress, we implemented a controlled low-temperature treatment (0.5 °C for 48 h) in a constant-temperature incubator. This treatment was applied to entire potted walnut seedlings to simulate cold stress conditions.

In parallel, considering the well-documented response of *Arabidopsis thaliana* to high-temperature stress [51], we designed a heat stress experiment in which walnut tissue cultures were used. Stem tip tissues of 'Qingxiang' walnut were cultured in DKW media for 28 days, followed by heat treatment at 42 °C for various durations (0 h, 2 h, and 6 h). This experimental design allowed us to examine the temporal effects of heat stress on walnut tissue cultures while maintaining controlled environmental conditions.

Walnut explants were cut and cultured in walnut agar media, and those subjected to mechanical damage treatments were sampled at 0, 12 and 24 h. The main causal agent of walnut black spot disease, *Xanthomonas arboricola* pv. *juglandis*, was isolated from walnut fruits in a diseased state, cultured and inoculated into walnut leaves (5 inoculations along the left and right sides of the main leaf veins by the needle-puncture method), and 1 cm^2 of leaves around the diseased spots were collected at 0, 24, 72, and 120 h. All the plant materials were set up in

triplicate, frozen in liquid nitrogen, and stored at −80 °C for testing.

Identification of the LBD gene family and bioinformatic analysis

The walnut genome used for genome-wide identification of the LBD transcription factor family in this study was derived from the NCBI database (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_001411555.2/). The *Arabidopsis* LBD TF protein sequences (Supplementary file 6) were downloaded from the UniProt database (http://www.uniprot.org), and HMMER 3.1b software was used to construct a model with the *Arabidopsis* LBD TF protein sequences via the hmmbuild command to preliminarily screen for protein sequences containing LBD structural domains in walnuts (e-value < 1e-20). Moreover, via BLASTP, the walnut LBD TF sequences were screened using the *Arabidopsis* LBD TF protein sequences as seed sequences, the sequences obtained from the intersection of the two screenings were taken, and the structural domain information was viewed via the CDD-search tool and Pfam database (http://pfam.org/). After the sequences with redundancy and structural domain mutations were manually removed, the final walnut LBD transcription factor family was obtained.

The CDSs of the genes encoding the *JrLBD* family were submitted to GSDS 2.0 (https://gsds.gao-lab.org/) to analyze the gene structures. Two thousand bp upstream of the CDSs were used as the promoter region, and the CDSs were analyzed via Plant-CARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to predict their cis-acting element situation. TBtools software [52] was used to map the distribution of the cis-acting elements of the *JrLBD38-1* promoter. The MEME website (https://meme-suite.org/meme/tools/meme) was used to predict the conserved motifs. The Cell-PLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) online tool was used to predict the subcellular localization of family members. Phylogenetic analysis was performed via MEGA 11 (neighbor joining, bootstrap value of 1000), and covariance analysis within species of the *JrLBD* family was performed via MCScanX. The figures were processed via Adobe Illustrator.

Gene expression analysis

Total RNA was extracted from leaves treated with bacteria for different durations via the RNAprep Pure Plant Kit (Tiangen Biotech, China). cDNA was obtained via reverse transcription via the PrimeScript™ RT reagent Kit with gDNA Eraser Perfect Real Time (Tiangen Biotech, China). cDNA was used as a template, and *JrActin* [53] was used as an internal control. qRT–PCR was performed on *JrLBD38-1* via the specific primer (Supplementary file 7), and three independent experiments were performed to ensure reproducibility of the qRT–PCR results (Supplementary file 8). The changes in the relative expression of genes under different treatments and treatment times were determined via the $2^{-\Delta\Delta Ct}$ method.

Extraction and content determination of GA

To detect the content of GA in leaves at different times after inoculation, 0.5 g of plant samples at different times (0 h, 24 h, 48 h, and 72 h) were weighed and placed in a 10 mL centrifuge tube. After rapid freezing in liquid nitrogen, the samples were crushed via a fully automatic sample grinder. Sample powder (0.5 g) and 80% precooled (< 0 °C) methanol (4 mL) were added to 200 μL of the antioxidant BHT and mixed well at 4 °C, avoiding light extraction for 12 h, low-temperature ultrasonication without light for 1 h, and low-temperature centrifugation at 12,000 rpm/min at 4 °C for 15 min. The supernatant was transferred, and nitrogen was blown to the aqueous phase (approximately 0.8–1 mL; the aqueous phase was adjusted to pH 8 with 1 mol/L sodium dihydrogen phosphate (30–50 μL)) (excess was adjusted back with 1% acetic acid). The aqueous phase was adjusted to pH 8 with 1 molar of sodium dihydrogen phosphate (30–50 μL) (excess was adjusted back with 1% acetic acid). A total of 0.1 g of cross-linked polyvinylpyrrolidone (PVPP) was added to remove phenolic impurities. The mixture was ultrasonicated at low temperature in the dark for 0.5 h and then centrifuged at 12,000 rpm at 4 °C for 5 min at low temperature, after which the supernatant was transferred. The aqueous phase was adjusted with 1 mol/L citric acid to pH 3 (excess of which can be adjusted back with 1% sodium hydroxide) and added to 3 mL of the ethyl acetate extract. The mixture was then ultrasonicated for 10 min at a low temperature, protected from light, and allowed to stand for 2 h, after which the upper liquid nitrogen was transferred to the upper liquid nitrogen. The upper liquid nitrogen was transferred, the mixture was blown with ethyl acetate, 2 mL of 20% methanol was added to the activated C18 column, and 4 mL of 10% methanol was rinsed with 1 mL of 100% methanol (pH 8) to rinse the samples into the bottle. Three independent experiments were performed to ensure reproducibility of the results.

Gene cloning and vector construction

To construct the luciferase reporter gene vector of the *LBD38-1* promoter as follows: from −1150 bp to −125 bp, segment 0 was named and amplified by primers F1 and

R1 (Supplementary file 7). The −1150 bp to −584 bp segment was named the Q1 segment and amplified via primers F1 and R2. The −604 to −125 bp segment was named the Q2 segment, which was amplified by F2 and R1. The 20 bp segment of the GA element was deleted from the Q1 segment, which was named the Q1* segment and amplified by F3 and R3. The segment 20 bp after the GA element was deleted in Q2 was named the Q2* segment and amplified by F4 and R4.

Specific primers were used to amplify the five promoter fragments (Q0, Q1, Q1*, Q2, and Q2*), and the restriction sites were SalI and BamHI, which were ligated to the pGREEN II 0800-LUC vector.

Functional analysis of the cis-acting elements of the *LBD38-1* promoter via a luciferase reporter gene validation assay

The mixture was subsequently transferred to Agrobacterium, which was subsequently used to infect tobacco (*Nicotiana benthamiana*, 4 weeks of age). Tobacco leaves were treated with 100 mg/L GA_3 for GA treatment on one side and untreated with GA on the other side as a control. The fluorescence was observed after 2 h of incubation. The activities of LUC and REN were determined via a dual-luciferase assay system (APE×BIO Technology LLC, Houston, USA), and the relative ratio of LUC/REN was calculated. Each experiment included three biological replicates for each treatment group.

Statistical analysis

The experiments were performed in accordance with a completely randomized design. The data are shown as the means ± standard errors (SEs) of three or six independent biological replicates. The data were analyzed via SPSS (version 20.0; IBM Corp., Armonk, NY, USA) and Excel 2010 software (Microsoft Corp., Redmond, WA, USA).

Abbreviations

LBD	Lateral organ boundary domain
Xaj.	*Xanthomonas arboricola* pv. *Juglandis*
ML	Mature leaves
YL	Young leaves
YS	Young stems
GA	Gibberellin
NJ	Neighbor-joining method

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11518-9.

Supplementary Material 1. Characteristic of JrLBDs family genes from walnut (*J. regia* L.).

Supplementary Material 2. Chromosome distribution of LBD genes in walnut.

Supplementary Material 3. Conserved motif.

Supplementary Material 4. Differential expression of LBD subfamily in different tissues of walnut.

Supplementary Material 5. Potted walnut seedlings.

Supplementary Material 6.

Supplementary Material 7. Primers for the vector of *LBD38-1* promoter luciferase Reporter gene.

Supplementary Material 8. Illustration of primer specificity and PCR efficiency.

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Authors' contributions

S.Z, W.D and Y.L designed the research. S.Z, W.D, Y.L, and Z.C performed the experiments, analyzed the data and wrote the paper. H.W, J.P, F.H, K.L, H.W and X.A participated in the data analysis. All authors read and approved the final the manuscript.

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Data availability

The RNA-Seq datasets are available from the GSA (submission number: CRA011411; https://bigd.big.ac.cn/gsa/browse/CRA011411; submission number: CRA011462; https://bigd.big.ac.cn/gsa/browse/CRA011462; submission number: CRA004704; https://bigd.big.ac.cn/gsa/browse/CRA004704; submission number:CRA017929; https://ngdc.cncb.ac.cn/gsa/browse/CRA017929) and NCBI (https://www.ncbi.nlm.nih.gov/biosample/SAMN25872099).
The CDS and genome sequences of LBDs in walnut were retrieved from the whole walnut genome database (accession GCA_001411555.2) of the NCBI. All the data and materials are presented in the main paper and additional files.

Declarations

Ethics approval and consent to participate

Walnut is a widespread fruit tree in China and is not an endangered species. Walnut trees were obtained from the Experimental Field of Hebei Agricultural University. No specific permits were required for sample collection from the walnuts.
All the plant materials used followed national and international standards and local laws and regulations. The use of plant materials does not pose any risk to other species. No specific permission was required to collect all the samples described in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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