

Fine mapping of BhDDL4.1, a major gene controlling the regulation of the deeply lobed leaf trait in wax gourd (*Benincasa hispida*)

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Abstract

Lobed leaves play a vital role in high-density cultivation and breeding of wax gourd. Thus, determining the molecular mechanisms underlying the development of lobed leaves is important. To this end, in this study, we aimed to resequence 105 recombinant inbred lines, constructed using the parental lines, GX-7 and my-1, to elucidate the molecular mechanisms underlying leaf development in wax gourd (*Benincasa hispida*). Genes associated with lobed leaves in wax gourds were first evaluated via quantitative trait loci (QTL) mapping. Next, the F₂ population was expanded to 2,000 plants for fine mapping and candidate gene analyses. Thus, the candidate area is reduced to 1.129 Mb, located between the markers InDel980 and InDel853. Functional analyses of candidate genes were performed using gene functional annotation, coding sequence analyses, and expression analyses. Among 48 genes in the candidate region, only *Bch04G012650* (termed *BhDDL4.1*) showed differences in expression between two parents. Using sequence differences of previously screened candidate genes, an InDel marker (InDel623) was developed in *BhDDL4.1* for molecular marker-assisted breeding of wax gourd, and the accuracy rate was 74.03%. Our results indicate that *BhDDL4.1* may play a key role in regulation of the lobed leaf trait; thereby, we provided a theoretical basis for further exploration of the molecular mechanisms underlying the lobed leaf trait in wax gourds.

Introduction

Wax gourd (*Benincasa hispida* (Thunb.) Cogn.) ($2n = 2x = 24$), also known as pillow melon, broad melon, or big gourd, belongs to the family Cucurbitaceae. It is an annual herb originating in southern China and eastern India. It has a wide planting range and a long planting history of cultivation. It is one of the most important vegetables transported from southern to northern China (Hu et al. 2022). The leaves are responsible for photosynthesis, nutrient distribution, water transport, and gas exchange and are, therefore, considered economically important nutritional organs. Phenotypic differences in leaf shape occur within and between species (Nicotra et al. 2011); (Andres et al. 2014); (Rodriguez et al. 2014); (Jiao et al. 2016)). Leaf shape in wax gourd can either be non-cracked, serrated, or deeply lobed (Ni et al. 2015). Additionally, the leaf shape is affected by genetic factors as well as external conditions, such as temperature and light (Tsukaya 2005); (Walter and Schurr 2005). It may affect plant growth, development, structure, and function as well as the final quality of fruits (Heng et al. 2020); (Karamat et al. 2021)). Generally, only the upper leaves of those densely planted plants with larger leaves are fully exposed to sunlight and effectively engage in photosynthesis. The leaves of plants with small leaf areas are not obstructed by adjacent leaves and are, therefore, able to fully absorb sunlight and effectively utilize light energy to improve photosynthetic efficiency. This reduces transpiration in plants (Yates et al. 2010); (Wilf 1997). Additionally, leaf shape is associated with plant stress resistance (Lopez-Iglesias et al. 2014). In practice, crops with deeply lobed leaves are more suitable for high-density cultivation (Tu et al. 2013). Therefore, studies focused on investigation of the regulatory genes linked to wax gourd-lobed leaves may positively impact the efforts aimed at improving germplasm resources, increasing yield, and enhancing economic benefits.

In recent years, many genes and gene families associated with the development of leaf lobes in plants, such as *Arabidopsis thaliana*, have been discovered. The class I homeodomain leucine zipper transcription factor LMI1 plays an important role in the formation of serrated lobes in *Arabidopsis* (Saddic et al. 2006a). The LMI1HD-ZIP transcription factor contributes to variation in deeply lobed leaves in various crops. Members of the HD-ZipI family participate in many biological processes, including the plant leaf growth and development, leaf polarity establishment, and light signal transduction (Merelo et al. 2016); (Zhou et al. 2019); (Whitewoods et al. 2020). A comparison of leaf morphology in *A. thaliana* and related plants has shown that leaflet development depends on the homology domain protein REDUCED COMPLEXITY (RCO) (Vlad et al. 2014). Furthermore, the HD-Zip transcription factor GhLMI1-D1b, which is a homolog of LMI1-like genes, was associated with different phenotypic traits in cotton leaf (Andres et al. 2017). Polymorphic LMI1-like genes regulate the growth and development of watermelon and rapeseed (Wang et al. 2021). Lobed leaves are modulated by the incompletely dominant gene *BnLLA10* in rapeseed and the homologous gene *LOBED-LEAF1* (*BnLL1*) in cabbage (Ni et al. 2015); (Hu et al. 2018). At the same time, both of these genes can regulate the inheritance of deep lobed leaf in kale (Ni et al. 2017); (Hu et al. 2020). Feather leaf traits in ornamental cabbage are modulated by the semi-dominant gene *BoFL*, which encodes an alpha-1,2-glucosyltransferase in *Arabidopsis* (Feng et al. 2020). BrLMI1 is an incompletely dominant positive regulator of lobed leaf formation in non-heading cabbage (Li et al. 2023). As a meristem identity regulator, LMI1 plays an important role in regulating the growth and development of meristems, leaf, or bract (Saddic et al. 2006b). In cotton, genes that regulate lobed leaves belong to *LMI1/RCO* and *KNOX1* (Chang et al. 2019). RCO regulates the activity of cytokinins, thereby affecting leaf shape parameters (Hajheidari et al. 2019), while *KnoX1* and *RCO* synergistically regulate the plant leaflet growth (Wang et al. 2022).

Genes that regulate lobed leaves have been evaluated in Cucurbitaceae. The deeply lobed leaves of Cucurbitaceae are regulated by a single incompletely dominant gene, *CpDII*, which encodes an HD-Zip I transcription factor; in particular, its high expression may be related to the formation of deeply lobed leaves (Bo et al. 2022b). Moreover, the HD-Zip transcription factor plays a major role in the presence of lobed leaves in watermelon (Xu et al. 2023). A single dominant gene, *CILL1*, regulates the growth and development of leaf lobes in watermelon (Wei et al. 2017). The lobed leaf trait in melon is regulated by a single recessive gene, *PLL*, and only one putative gene (*MELO3C010784*) has been predicted in the genomic region (Gao et al. 2014). However, genetic studies identifying genes that regulate lobed leaves in wax gourds are lacking. Therefore, to fill in this knowledge gap, the present study aimed to elucidate the molecular basis of deeply lobed leaves in wax gourd (*Benincasa hispida*) using quantitative trait loci (QTL) mapping, in addition to functional, phylogenetic, and expression analyses.

Materials and methods

2.1 Plant materials, phenotyping, and data processing

The parental (P) wax gourds, GX-7 and my-1, were characterized by shallow and deeply lobed leaves, respectively. Planting was completed in May 2023 in a Yong'an wax gourd experimental field (Nanning,

Guangxi, China), involving 107 genealogies and including the parents. A field-based randomized block design with three replicates was used. Conventional open-field cultivation and management practices were followed. All materials used in this study were provided by the Nanning Kenong Seedling Corporation (Guangxi, China).

The plants were periodically observed; 10 complete leaves were randomly selected from each of the biparental plants, and the growth and development of nodes 15–17 were monitored on days 0, 3, 6, 9, 12, 15, 18, 21, 26, and 31. A leaf sample was collected using a Vernier caliper, and the split depth and lateral vein length, as well as the ratio of the split depth to the lateral vein length (L-S/L), were calculated (Fig. 1). Phenotypic data were analyzed using Microsoft Excel 2019 for descriptive statistics; *t*-tests and analysis of variance were performed using SPSS V27.0 (SPSS Inc, Chicago, IL, USA). GraphPad Prism 8.0.2 was used to perform a frequency distribution analysis of plant data to determine whether the differences between parents were significant (Supplementary Fig. S1).

2.2 Histological analysis

Leaf samples were used to compare cytological characteristics of lobed leaves belonging to GX-7 and my-1. These samples were taken from leaves with the same growth stage at the mature stage of both parents (Fig. 2) to ensure that the maximum cross-sections were obtained. Sections were stained with toluidine blue. Tissue section images were observed using Case Viewer (<https://www.3dhistech.com/solutions/caseviewer/>). ImageJ (<https://imagej.nih.gov/ij/>) was used to observe cell sizes in both parents and to estimate the number of cells.

2.3 DNA extraction

After two weeks, fresh young leaves of the parents and F₂ plants were collected in 2-mL centrifuge tubes and stored at – 80°C for DNA extraction. DNA was extracted using the improved hexadecyl trimethyl ammonium bromide (CTAB) method (An et al., 2011), and the concentration and purity of DNA were detected using a K5800 ultra-differential photometer (Kaiao, Beijing, China). The quality of DNA was assessed via 1.2% agarose gel electrophoresis.

2.4 QTL mapping

We resequenced the parents and 105 inbred lines. Sequences were compared with reference genomes of wax gourds to detect the mutations, and a high-density genetic linkage map was constructed. High-density genetic maps and detailed phenotypic data were used for QTL mapping. The composite interval mapping (CIM) method and QTL Cartographer (version 1.17j) software were used for QTL mapping. The threshold limit of detection (LOD) value was determined using 1,000 permutation tests.

2.5 Fine mapping of candidate genes

Based on the QTL sequencing results, InDel markers were designed and developed in the candidate regions. The polymerase chain reaction (PCR) products were scanned and genotyped via PCR. The PCR-

amplified products were separated using 8% polyacrylamide gel electrophoresis and genotyped. The primer sequences for the InDel markers are shown in Table S1.

2.6 Expression analysis of candidate genes

Samples were obtained from the mature leaves of wax gourds. RNA was extracted from seven different parts of each leaf (Fig. 3); four were located at the leaf tip (#1–4) and the remaining three were located at the bottom of each sinus (#5–7) at the position of the lobe. Primers were designed using Premier5.0 for reference and candidate genes (Table S1). Candidate gene expression levels were detected using qPCR and analyzed using QuantStudio6 Flex software (ThermoFisher, Waltham, MA, USA), with actin as the internal control. Relative expression levels were measured using the $2^{-\Delta\Delta CT}$ method, with *RG* as the internal reference gene. GraphPad Prism 8.0.2 was used to analyze significant differences between relative expression levels.

2.7 Cloning and sequence analysis of candidate genes

Cloning primers were designed using Premier5.0 software according to the gene coding sequence (Table S1). Total RNA was extracted from the tender leaves of two parents, GX-7 and my-1, using an Eastern® Super Total RNA Extraction Kit (Promega, Beijing, China) according to the manufacturer's instructions. The sequencing was performed by Shenggong Biotechnology Co., Ltd. (Shanghai, China) and alignments were generated using DNAMANV.6 based on DNA and amino acid sequences.

2.8 Homologous gene identification and phylogenetic analysis of Bch04G012650

The protein sequence encoded by *BhDDL4.1* was analyzed using NCBI BLAST (NCBI, Bethesda, MD, USA). Additionally, the protein sequences of homologs in *Cucurbitaceae*, yam, and turnip were downloaded from NCBI in the FASTA format (Table S2). We constructed a phylogenetic tree using the neighbor-joining method (NJM) and performed 1000 bootstrap analyses (MEGA7.0 software, <https://www.megasoftware.net/>).

2.9 Molecular marker validation

Molecular marker-assisted selection experiments were conducted using the parents, F₁ generation plants, and 60 extreme wax gourd germplasm resources (50 with shallowly lobed leaves and 10 with deeply lobed leaves). Insertion or deletion markers (InDels) were developed based on coding sequence (CDS) differences between the candidate genes of parents. Premier5.0 (Premier, Canada) was used to design primers (Table S1).

Results

3.1 Phenotypic analysis

The split depth to lateral vein length (L-S/L) ratios differed significantly between GX-7 and my-1 (Fig. 4a). Therefore, GX-7 and my-1 could be considered suitable parents for constructing recombinant inbred line populations for QTL mapping and mining in relation to lobed leaves. GX-7 exhibited a lobed leaf, whereas my-1 exhibited a deeply lobed leaf. During leaf growth and development, the growth trends of GX-7 and my-1 were essentially the same (Fig. 4b); however, during the early stage, growth was more rapid in the male parent, GX-7, than in the female parent, my-1. After 3 d, the ratio of development of lobed leaves in GX-7 was less than that in my-1. A slight difference was observed in growth rates when the L-S/L ratios of GX-7 and my-1 were compared during the early stages of leaf growth (0–9 d). The development of lobed leaves of GX-7 and my-1 tended to stabilize in GX-7 and my-1 at 15–31 d. As shown in Fig. 4, lobed leaves formed rapidly during the early stages of leaf growth and development in wax gourds. The development of deep and shallow lobed leaves was stable during the middle and later stages of leaf growth and development, with the degree of lobed depth tending to stabilize.

3.2 Histological analysis

Paraffin sections of the GX-7 and my-1 transverse leaf planes with evident leaf lobes (nos. 6 and 7) were studied at 30 d. The cross-section of the leaf included the cuticle, upper epidermis, palisade tissue, parenchyma cells, stomata, gas channels, veins, and lower epidermis. The size and number of parenchyma cells in transverse sections 6 and 7 differed significantly between GX-7 and my-1. Furthermore, my-1 had a larger number of round parenchymal cells (Fig. 5a, b), while GX-7 had small, round parenchyma with fewer cells (Fig. 5c, d). The parents showed significant differences in the number of cells in leaf tissue Nos. 6 and 7 (Fig. 5e).

3.3 QTL mapping

Based on the constructed high-density genetic map (total length 1357.145 cM, average distance between adjacent markers 0.002 cM, and maximum interval between adjacent markers 19.647 cM) and phenotypic values, QTL mapping of the leaf lobes of phenotypic traits, i.e., depth, was performed via the CIM method. On Chr04, one peak exceeded the correlation threshold (Fig. 6a), revealing a single region (38,779,807–41,241,642) with a total length of 2.46 Mb. The maximum LOD was 4.653, and the phenotypic variation explained (PVE) was 13.60% (> 10%). Therefore, the QTL for lobed leaf was stable.

3.4 Fine mapping of candidate genes

QTL mapping showed that the candidate genes regulating lobed leaves were located within the Chr04 interval (Fig. 6a). Using the reference genome GX-19 and parental resequencing data, the candidate genes were sequenced and annotated. Seven InDel markers were developed for the candidate intervals (Table S1), and fine mapping between two of these markers, InDel980 and InDel853, narrowed the candidate region to 1.129 Mb (Fig. 6b). The results showed that 48 candidate genes were localized to the region; functional annotations were obtained for 44 genes, and 11 genes had nonsynonymous mutations (Table 1). Real-time fluorescence quantitative analysis indicated that a significant difference in expression levels in the lobed leaf sites between the parents was only observed for BhDDL4.1, which

was annotated as a calcium- and calmodulin-dependent serine and threonine protein kinase that regulates kinase activity and participates in many key intracellular signaling pathways. It also promotes cell metabolism, cell polarity, and DNA damage. The preliminary results of functional annotation and sequence alignment indicated that BhDDL4.1 may play a key role in the regulation of lobed leaves in wax gourds.

Table 1
List of candidate genes within the precise localization region of Chr04

Genes	Location	Nonsynonymous Mutations in Coding Sequences	annotation
Bch04G012560	Chr04:40,135,450 – 40,142,061 (-)	Yes	putative CCA tRNA nucleotidyltransferase 2
Bch04G012570	Chr04:40,146,296 – 40,149,834 (-)	No	alpha-(1,4)-fucosyltransferase
Bch04G012580	Chr04:40,153,408 – 40,160,839 (-)	Yes	uncharacterized protein LOC103496554 isoform X2
Bch04G012590	Chr04:40,213,209 – 40,218,827 (+)	Yes	receptor protein-tyrosine kinase CEPR2
Bch04G012600	Chr04:40,220,051 – 40,225,413 (-)	Yes	adenylate kinase, chloroplastic
Bch04G012610	Chr04:40,227,371 – 40,228,877 (+)	Yes	protein phosphatase 1 regulatory subunit INH3-like
Bch04G012620	Chr04:40,229,048 – 40,230,468 (-)	Yes	protein EPIDERMAL PATTERNING FACTOR 1
Bch04G012630	Chr04:40,234,655 – 40,234,937 (-)	Yes	ATP synthase CF1 beta subunit
Bch04G012640	Chr04:40,277,451 – 40,281,723 (+)	Yes	uncharacterized protein LOC101215032
Bch04G012650	Chr04:40,284,809 – 40,288,848 (-)	Yes	calcium and calcium/calmodulin-dependent serine/threonine-protein kinase-like
Bch04G012660	Chr04:40,293,414 – 40,295,250 (-)	Yes	ETHYLENE INSENSITIVE 3-like 1 protein isoform X1
Bch04G012680	Chr04:40,319,049 – 40,320,020 (-)	Yes	uncharacterized protein LOC105435754
Bch04G012690	Chr04:40,320,035 – 40,324,314 (-)	No	type I inositol polyphosphate 5-phosphatase 10 isoform X1
Bch04G012700	Chr04:40,333,129 – 40,341,884 (-)	No	protein SAD1/UNC-84 domain protein 1
Bch04G012710	Chr04:40,347,421 – 40,349,045 (+)	No	cleavage stimulation factor subunit 50 isoform X1
Bch04G012720	Chr04:40,349,652 – 40,355,953 (-)	No	DNA polymerase alpha subunit B
Bch04G012730	Chr04:40,392,152 – 40,398,099 (-)	No	ABSCISIC ACID-INSENSITIVE 5-like protein 2

Genes	Location	Nonsynonymous Mutations in Coding Sequences	annotation
Bch04G012740	Chr04:40,465,769 – 40,469,061 (-)	No	uncharacterized protein LOC103496536 isoform X1
Bch04G012750	Chr04:40,472,870 – 40,476,877 (-)	No	transcription factor GAMYB
Bch04G012770	Chr04:40,526,402 – 40,532,998 (+)	No	uncharacterized protein LOC103496534 isoform X2
Bch04G012780	Chr04:40,561,581 – 40,561,897 (+)	No	hypothetical protein Csa_005338
Bch04G012790	Chr04:40,562,149 – 40,566,622 (+)	No	phospholipid-transporting ATPase 1
Bch04G012800	Chr04:40,569,769 – 40,574,508 (+)	No	probable mitochondrial import inner membrane translocase subunit TIM21 isoform X1
Bch04G012810	Chr04:40,576,718 – 40,581,899 (-)	No	probable inactive leucine-rich repeat receptor-like protein kinase At3g03770 isoform X2
Bch04G012830	Chr04:40,584,452 – 40,584,770 (-)	No	hypothetical protein FNV43_RR08242
Bch04G012840	Chr04:40,588,295 – 40,600,524 (-)	No	chlorophyll(ide) b reductase NOL, chloroplastic isoform X1
Bch04G012850	Chr04:40,639,011 – 40,643,724 (-)	No	MAP7 domain-containing protein 1-like
Bch04G012860	Chr04:40,647,775 – 40,648,785 (-)	No	hypothetical protein Csa_005322
Bch04G012870	Chr04:40,784,746 – 40,793,265 (+)	No	nuclear pore complex protein NUP1-like
Bch04G012880	Chr04:40,820,754 – 40,822,939 (+)	No	uncharacterized protein At2g39795, mitochondrial
Bch04G012890	Chr04:40,825,902 – 40,826,979 (-)	No	uncharacterized protein At3g28850
Bch04G012910	Chr04:40,846,472 – 40,847,543 (+)	No	uncharacterized protein LOC103496524
Bch04G012920	Chr04:40,983,374 – 40,986,936 (-)	No	protein SMAX1-LIKE 3-like
Bch04G012930	Chr04:40,990,180 – 40,990,975 (-)	No	hypothetical protein Csa_005443

Genes	Location	Nonsynonymous Mutations in Coding Sequences	annotation
Bch04G012940	Chr04:40,996,277 – 41,001,266 (-)	No	uncharacterized protein E5676_scaffold304G001060
Bch04G012950	Chr04:41,012,533 – 41,013,029 (-)	No	hypothetical protein E6C27_scaffold24G001560
Bch04G012960	Chr04:41,049,074 – 41,050,610 (-)	No	40S ribosomal protein S17-3
Bch04G012980	Chr04:41,067,711 – 41,069,639 (-)	No	pentatricopeptide repeat-containing protein At5g04780, mitochondrial
Bch04G012990	Chr04:41,106,333 – 41,110,776 (-)	No	cationic amino acid transporter 6, chloroplastic
Bch04G013000	Chr04:41,157,122 – 41,158,799 (+)	No	transcription factor DIVARICATA
Bch04G013010	Chr04:41,182,633 – 41,185,045 (-)	No	uncharacterized protein LOC103496516
Bch04G013020	Chr04:41,224,854 – 41,228,790 (+)	No	multiple myeloma tumor-associated protein 2 homolog
Bch04G013030	Chr04:41,233,167 – 41,241,111 (+)	No	uncharacterized protein At2g38710-like
Bch04G013040	Chr04:41,253,437 – 41,256,706 (+)	No	vesicle-associated membrane protein 727

3.5 qPCR analysis of candidate genes

To further identify candidate genes, qPCR was used to detect the spatiotemporal expression of GX-7 and my-1 at distinct positions during the mature stage, characterized by deeply lobed leaves. Among 48 candidate genes, only *BhDDL4.1* showed a significant expression difference among distinct positions on lobed leaves, whereas the other genes showed no significant differences. The expression levels across seven parts of the leaves are shown in Fig. 7b. Significant differences were observed between the parent lines in the position seven, where the expression level of *BhDDL4.1* in GX-7 was significantly higher than that in my-1 at each leaf tip (#1–4) in leaf tissues 5, 6, and 7. The expression of GX-7 was higher than that of my-1 at all seven positions. A highly significant difference was observed between the expression levels of *BhDDL4.1* in the most evident part (#7) of the deeply lobed leaves in parents. These data indicated that the differential expression of *BhDDL4.1* may play a key role in the formation of lobed leaves in wax gourds.

3.6 Molecular marker validation

An InDel marker (InDel623) was developed via insertion into *BhDDL4.1*. Genotypic and phenotypic concordance was examined using 60 pure wax gourd samples with extreme traits, as well as their parents and plants in the F₁ generation. Among 60 samples, ten had deeply lobed leaves and 50 had lobed leaves. The results showed that 38 genotypic bands in lobed samples were consistent with GX-7 phenotypic bands and seven genotypic bands for deeply lobed samples were consistent with my-1 phenotypic bands (Fig. 8). A total of 12 genotypic bands inconsistent with the GX-7 phenotypic band and three genotypic bands inconsistent with the my-1 phenotypic band were observed. F1 was the parent, and the genotype–phenotype coincidence rate was 74.03%.

3.7 Phylogenetic analysis

To analyze the relationship between BhDDL4.1 and homologous proteins, we obtained protein sequences via NCBI BLAST and generated a phylogenetic tree using the NJM and 1,000 bootstrap replicates based on the sequences from wax gourd and other cucurbits, as well as from yam and turnip. The phylogenetic tree showed that BhDDL4.1 was closely related to XP_031743737.1 in melon and KAA0031691 in cucumber (Fig. 9) and more distantly related to homologs in pumpkin and silver-seeded cucumber.

Discussion

4.1 Growth and development of lobed leaves in wax gourd

The results of this study indicated that the early period of leaf growth and development is crucial for the rapid formation of lobed leaves. Leaf shape morphogenesis is a multifaceted process and has a polygenic basis (Sluis and Hake 2015).

In the present study, histological observations of transverse sections of mature parental leaves revealed differences in cell size and number between GX-7 and my-1. A significant difference was observed in the number of thin-walled cells at the visible sites of lobed leaves (nos. 6 and 7) compared with that in parental leaves (Fig. 5e). With respect to cytology, leaf growth and development are mainly regulated by cell proliferation and expansion (Zhang et al. 2021b), and these processes are regulated by many functionally interrelated genes. In *Arabidopsis*, the following six gene modules control leaf growth and development and participate in cell proliferation: DA1-DA1 enhancer (EOD1); growth regulatory factor (GRF)-GRF interaction factor (GIF); SWITCH/sucrose non-fermentation (SWI/SNF); gibberellin (GA)-DELLA; KLU; and PEAPOD (Vercruysse et al. 2019). In poplar, GRFs finely control the leaf size by regulating cell proliferation and expansion. Further studies are needed to determine whether *BhDDL4.1*, which regulates the lobed leaves in wax gourd, regulates the cell size and proliferation.

4.2 Molecular mechanism underlying the regulation of lobed leaves in wax gourd by BhDDL4.1

Bch04G012650 (*BhDDL4.1*) may play the leading role in regulation of lobed leaves in wax gourd. The results of qPCR analysis of seven different parts of the leaf were consistent with those of previously

reported qPCR results of candidate genes in lobed leaves in zucchini (Bo et al. 2022a). In this study, the functional annotation of candidate genes showed that BhDDL4.1 may promote the interaction between calcium and calcium or calmodulin-dependent threonine protein kinases, which regulate kinase activity and participate in several key intracellular signal transduction pathways.

In plant cells, Ca^{2+} engages in many signal transduction processes, including those involving environmental stimuli. As a second messenger in eukaryotic cells (Li et al., 2024), Ca^{2+} regulates cell division and apoptosis and plays an important role in regulating various cellular processes (Islam 2020); (Rosendo-Pineda et al. 2020). Calmodulin, an important mediator of calcium in the cell cycle, regulates numerous kinases, such as cyclin dependent kinase (CDK1) (Berridge et al. 2000), as well as the phosphatases involved in these processes. It also interacts with many other cellular proteins and plays a signaling role in various cells (Moser et al. 1995); (Park et al. 2019). During mitosis, CDK1 regulates and reduces the activity of Orai1 by phosphorylating STIM1. During mitosis, proteases, protein kinases, and protein phosphatases are highly regulated during transitions between stages, thereby ensuring precise cell division and smooth cell cycle progression (Tyson et al., 2008; (Nurse 2000). The intracellular Ca^{2+} concentration is strictly and precisely regulated via a complex calcium homeostasis system consisting of various calcium channels, calcium pumps, and calcium antiporters (Cyert and Philpott 2013). This finding indicates the existence of a close interaction between calcium ions and threonine protein kinase. Calcium ions regulate the activity of threonine protein kinase, thereby affecting its downstream signaling process; alternatively, threonine acid protein kinase regulates the activity of calcium ion channels via phosphorylation, further affecting concentration of intracellular Ca^{2+} . Such interactions enable plants to finely regulate physiological functions coping with complex external environments.

Declarations

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Competing Interests

The authors declare no conflicts of interest.

Author Contributions

ZL, PW and XY conceived and designed the study. WB conducted all experiments. YD, ZC, WY, TL, LN, LS, and ZC participated in some experiments. WB wrote the manuscript. ZL, PW and XY revised the

manuscript. All authors reviewed the manuscript.

Data Availability

The data presented in this study are available in this article and as supplementary materials.

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Figures



Figure 1

Ratio of the split depth to lateral vein length (L-S/L), $\frac{S}{L}$ split length, $\frac{L}{L}$ lateral vein length

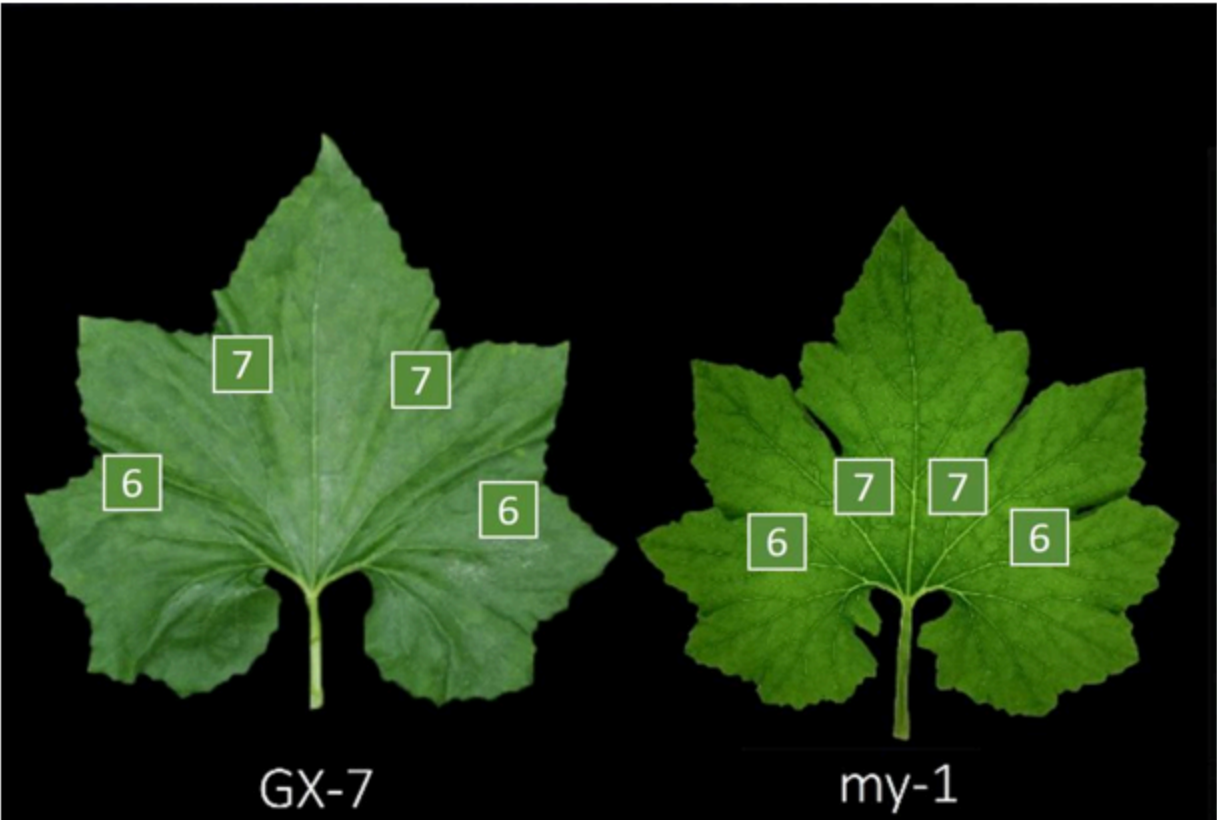


Figure 2

Schematic of lobed leaf sampling for GX-7 and my-1

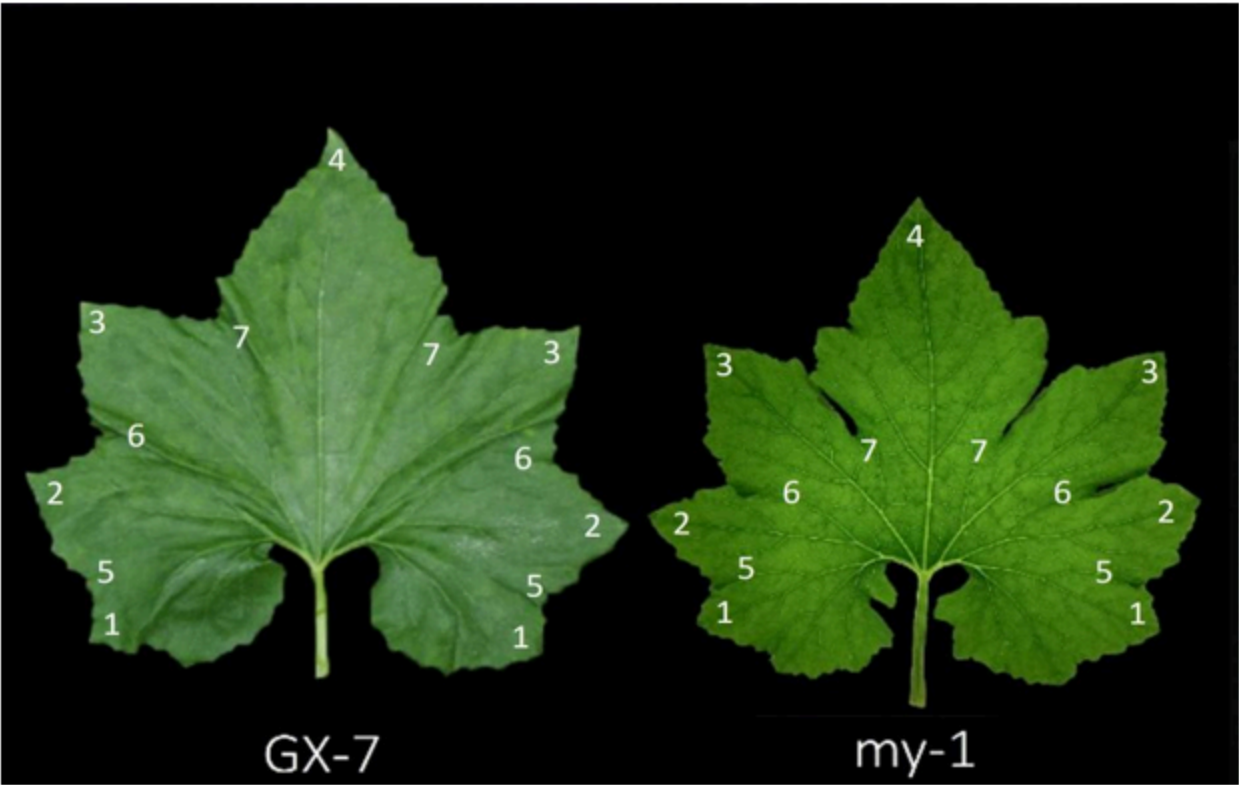


Figure 3

Sampling sites used for qPCR

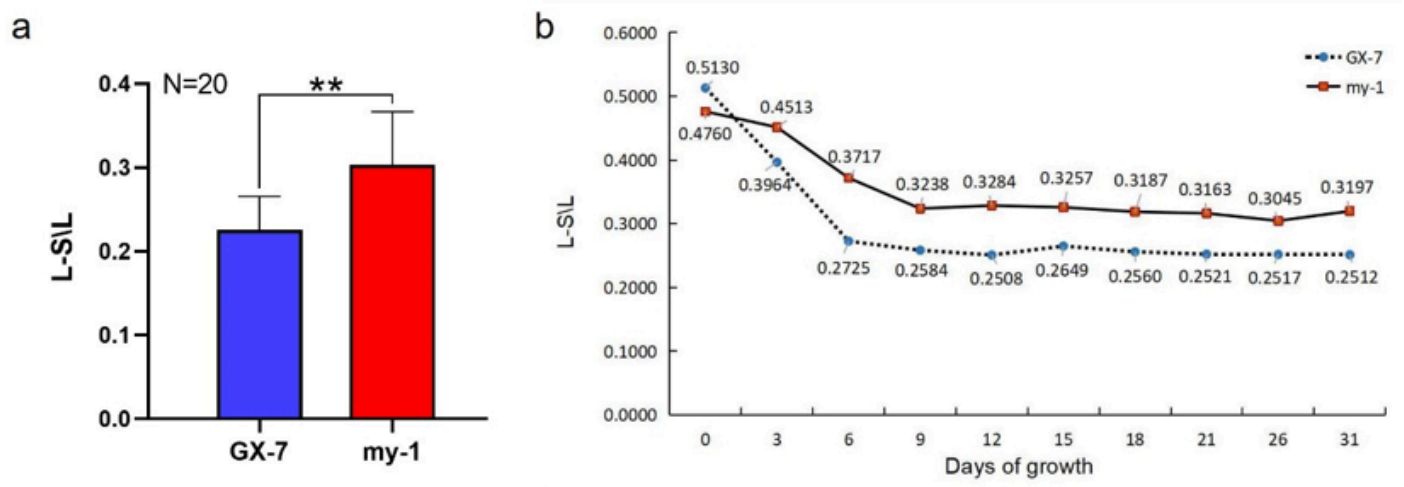


Figure 4

Analysis of phenotypic differences of lobed leaf in wax gourd. (a) difference of the ratio of the split depth to lateral vein length (L-S/L) between GX-7 and my-1 (N: number of leaves measured); **P < 0.01 (t-test); values are expressed as means $\pm$ SE; (b) growth curves of split depth/lateral vein length in GX-7 and my-1

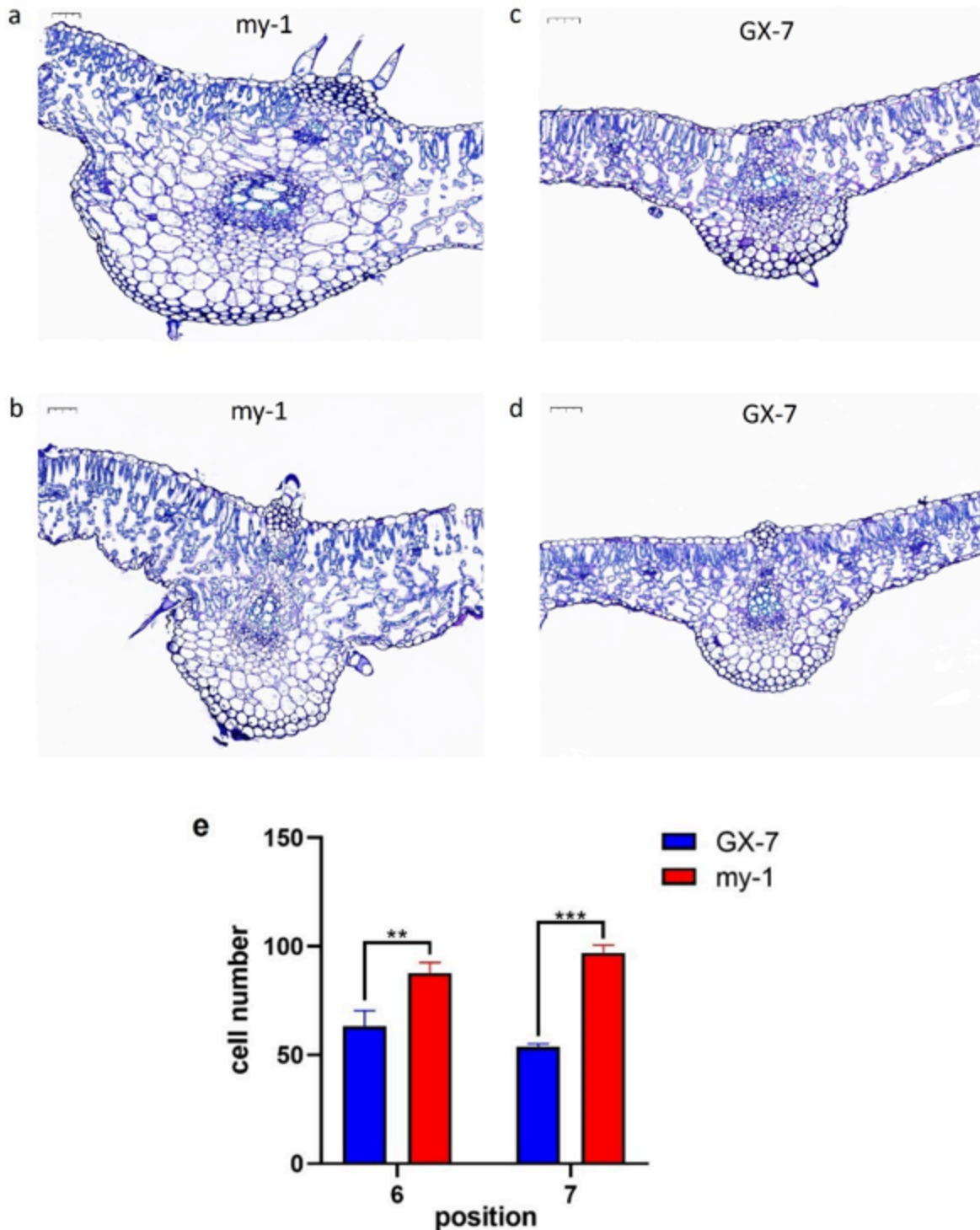


Figure 5

Cell size and cell count analyses of paraffin sections of 30-day leaves of wax gourd in areas of visible foliation: (a) Paraffin section of my-1 leaf at No. 7; (b) Paraffin section of my-1 leaf at No. 6 : (c) Paraffin section of GX-7 leaf at No. 7; (d) Paraffin section of GX-7 leaf at No. 6; scale bar = 0.1 mm; (e) Differential analysis of cell number of GX-7 and my-1 at the two sites (t-test; ** $P < 0.01$ and *** $P < 0.001$, respectively); values are expressed as means $\pm$ SE

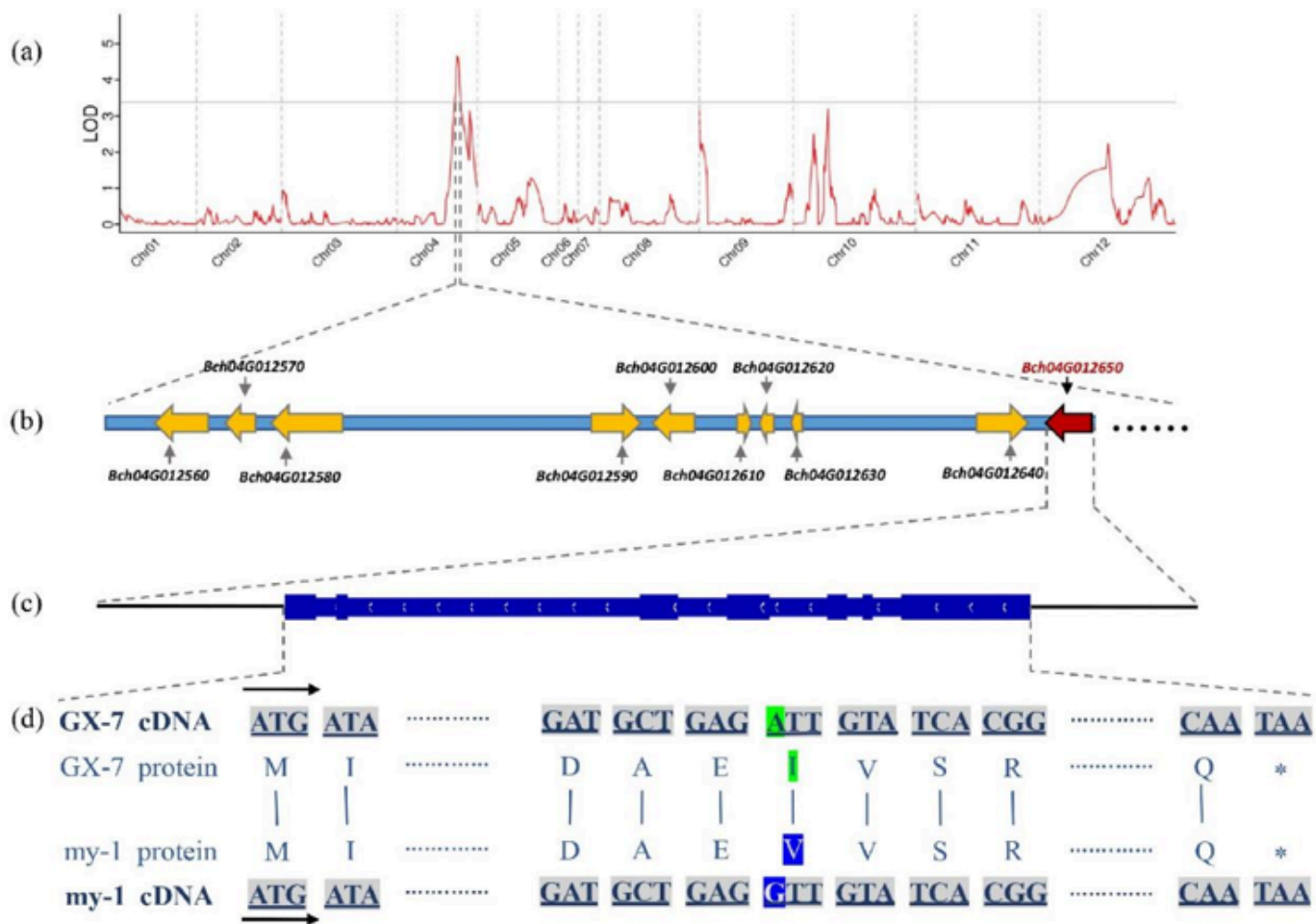


Figure 6

Quantitative trait loci (QTL) mapping: (a) High-density genetic map of Chr04; (b) QTL analysis of Chr04 in lobed leaves (c) Candidate genes within the QTL interval; (d) CDS and protein sequences of BhDDL4.1 in the two parents; * indicates translation termination

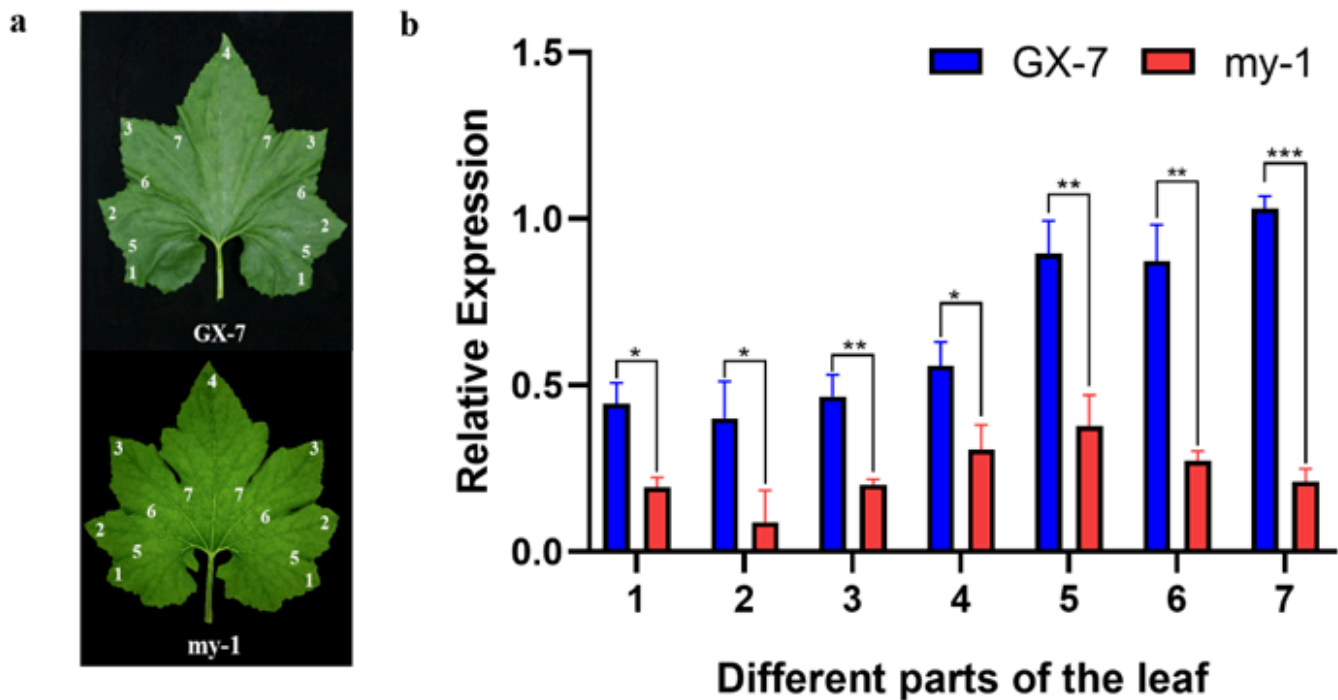


Figure 7

Expression levels of *BhDDL4.1* in GX-7 and my-1: (a) Seven leaf segments (1–7) from GX-7 and my-1 were sampled for qPCR; (b) Relative expression levels of *BhDDL4.1* in different parts of GX-7 and my-1. (*t*-test **P* < 0.05, ***P* < 0.01, and ****P* < 0.001)

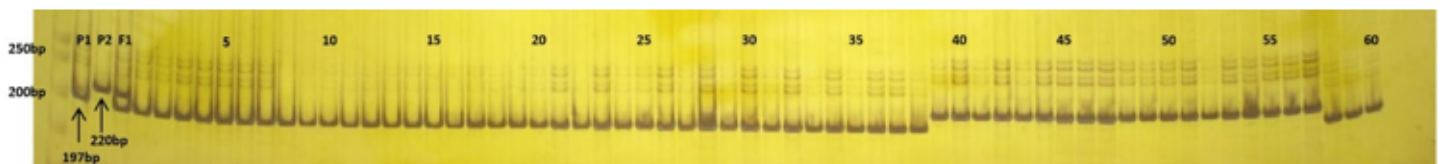


Figure 8

Genotyping of 60 germplasm resources of wax gourd using InDel markers at the *BhDDL4.1* locus. P1 and P2, respectively, represent GX-7 and my-1; 1–50 are shallowly lobed leaves and 51–60 are deeply lobed leaves

