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A recessive LRR-RLK gene causes hybrid breakdown in cotton

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Abstract

Key message An LRR-RLK gene causing interspecific hybrid breakdown between Gossypium. anomalum and G. hirsutum was identified by deploying a map-based cloning strategy. The self-destructing symptoms of hybrid incompatibility in most cases are attributed to autoimmunity. The cloning of genes responsible for hybrid incompatibility in cotton is helpful to clarify the mechanisms underlying hybrid incompatibility and can break the barriers in distant hybridization. In this study, a temperature-dependent lethality was identified in CSSL11-9 (chromosome segment substitution line) with Gossypium anomalum chromosome segment on chromosome A11. Transcriptome analysis showed the differentially expressed genes related to autoimmune responses were highly enriched, suggesting that expression of CSSL11-9 plant lethal gene activated autoimmunity in the absence of any pathogen or external stimulus, inducing programmed cell death (PCD) and causing a lethal phenotype. The lethal phenotype was controlled by a pair of recessive genes, and then fine mapped between JAAS3191-JAAS3050 interval, which covered 63.87 kb in G. hirsutum genome and 98.66 kb in G. anomalum. We demonstrated that an LRR-RLK gene designated as hybrid breakdown 1 (GoanoHBD1) was the causal gene underlying this locus for interspecific hybrid incompatibility between G. anomalum and G. hirsutum. Silencing this LRR-RLK gene could restore CSSL11-9 plants from a lethal to a normal phenotype. Our findings provide new insights into reproductive isolation and may benefit cotton breeding.

Key Message

An LRR-RLK gene causing interspecific hybrid breakdown between *Gossypium. anomalum* and *G. hirsutum* was identified by deploying a map-based cloning strategy.

Introduction

In the process of cotton breeding and germplasm innovation, the improvement of yield and quality is accompanied by the narrowing of genetic basis of variety resources. The narrow genetic basis makes crops vulnerable to diseases and pests and challenges the sustainable development of agriculture through continuous genetic improvement. Therefore, more attention has been paid to the introduction of elite genes from *Gossypium* wild species into cotton cultivars to enrich their genetic basis and enhance their resistance. The wild species contain abundant genetic variations and considerable desirable traits which are lacking in the cultivated species. The potential elite fiber quality genes, disease resistance genes, adaptability and fertility genes are the material basis for the variety to be improved in the aspects of yield, quality and stress resistance.

As in other crops, the domestication of cotton has led to a dramatic loss of allelic diversity. The genus *Gossypium* is composed of four cultivated species (*G. hirsutum*, *G. barbadense*, *G. herbaceum* and *G. arboreum*) and 46 wild species (Fryxell 1992). *Gossypium* wild species present an impressive range of variation in many characters. Transferring desired characters from wild species into cultivated cotton is therefore important for the continued improvement of cotton varieties, but interspecific reproductive

barriers limits the use of wild resources in breeding. There are mainly three types of reproductive isolation (Stebbins 1950). The first type thereof is geographic isolation, which indicates that species of the same kind are divided into different populations due to geographic barriers, such that gene flow fails to occur among the populations. The second type thereof is reproductive isolation before fertilization, which indicates that a hybrid zygote cannot be formed because species have different flowering times, or a pollen tube cannot germinate or cannot completely pass through a style and reach an ovary after a plant is pollinated, or male and female gametes are incompatible. The third type thereof is reproductive isolation after fertilization called hybrid incompatibility, which includes hybrid lethality (also referred to as hybrid weakness) and hybrid breakdown. Hybrid lethality or hybrid weakness occurs in the F_1 generation, showing plant lethality, sterility, shortness, and reduced viability. Some interspecific hybridization can produce normal hybrid F_1, but F_2 generation plants thereof often show weakness or lethality, which is referred to as hybrid breakdown (Yamamoto et al. 2007). Hybrid lethality or hybrid breakdown has become a serious barrier, preventing the combination of desirable traits from different *Gossypium* genotypes and limiting the utilization of heterosis from specific parental lines in cotton.

Hybrid lethality and hybrid breakdown cases have been reported in different plants, which have simple inheritance modes. The hybrid lethality phenotype occurs in F_1 generation, and is mostly controlled by one or two pairs of dominant genes. The hybrid breakdown phenotype occurs in the F_2 generation, and is mostly controlled by one or two pairs of recessive genes. The Bateson-Dobzhansky-Muller (BDM) model (Dobzhansky 1937; Muller 1942) is proposed to explain the genetic basis of hybrid lethality and hybrid breakdown. The classic BDM model includes the epistatic interaction of two loci. Each of branches derived from the common ancestor can form different new loci during an evolution process, and these loci are harmless in their respective genomes. However, in hybrids, these evolved genes interact, where when a new mutant gene is dominant, the F_1 generation shows hybrid lethality; and when a new mutant gene is recessive, the F_2 generation shows hybrid breakdown. Most reports of hybrid lethality and hybrid breakdown (Bomblies et al. 2007a; Yamamoto et al. 2007; Chen et al. 2013) are results of action of the two-locus model. The BDM interaction pattern can also occur between single loci (Smith et al. 2011).

The cloning of genes responsible for hybrid incompatibility is a molecular basis for the analysis of hybrid incompatibility mechanism and is also a key to fundamentally interpreting the mechanism of hybrid incompatibility in plants, which helps to understand the importance of hybrid incompatibility gene evolution in reproductive isolation and speciation. In existing studies, genes related to hybrid lethality (Zuellig et al. 2018; Jia et al. 2021; Si et al. 2021b; Zhang et al. 2022) and hybrid breakdown (Yamamoto et al. 2007; Jiang et al. 2008; Plötner et al. 2017; Yoneya et al. 2021) have been subjected to genetic mapping in different species, and some of which have been cloned (Bomblies et al. 2007a; Chae et al. 2014; Chen et al. 2014; Barragan et al. 2020; Si et al. 2021a). At present, the research on cotton hybrid incompatibility remains relatively rudimentary. Many scholars have discovered the phenomenon of cotton hybrid incompatibility. However, there was few more in-depth research of cotton hybrid lethality. Deng et al. (2019) confirmed that the CC-NLR gene is the encoding gene of the *Le4* locus, which determines the lethality of interspecific hybridization between *G. barbadense* and *G. hirsutum*.

Clarifying the mechanism of reproductive isolation can help to break reproductive barriers for traditional crop breeding and promote the interspecific genome exchange, which will facilitate the application of excellent traits of wild species in modern breeding. Herein, an LRR-RLK gene causing interspecific hybrid breakdown was identified in *Gossypium* by deploying a map-based cloning strategy. Our findings provide new insights into reproductive isolation and may benefit cotton breeding.

Materials And Methods

Plant materials

In a previous study, a synthesized hexaploid derived from an interspecific hybrid between *G. hirsutum* and *G. anomalum* was produced by colchicine treatment, and its hybridity and doubled status were demonstrated using morphological, cytological, and molecular marker methods (Zhang et al. 2014). With the hexaploid F_1 as the female parent and Su8289 as the recurrent parent, continuous backcrossing was conducted four times. Through marker assisted selection, a set of CSSLs (chromosome segment substitution lines) were obtained, among which CSSL11-9 with a single chromosome segment in the marker region JAAS3310-NAU5192 on chromosome A11 was identified to confer lethality in the absence of any pathogen or external stimulus. The development of the CSSL population was described in Xu et al. 2022. Individuals heterozygous (equivalent to F_1) in this introgression segment on chromosome A11 were selected and self-crossed to generate a F_2 population containing 2337 individuals at the Lishui Plant Experiment Station, Nanjing, China in 2017. For the temperature shift experiment, plants were grown under a 16-h light/8-h light dark cycle in a growth chamber. Plants were maintained at 23 °C from germination until two leaves had formed and then shifted to 30 °C.

Transcriptome analysis

Plants (CSSL11-9 and Su8289) were cultivated at a constant 23 °C from germination until two leaves had formed and then shifted to constant 30 °C. Leaves were harvested immediately before the temperature shift (set as the 0 point) and at three and five days after. Three biological replicates were conducted for each time point. Total RNA was isolated from frozen tissues using an improved CTAB method. Individual RNA samples were quantified using a Qubit 2.0 Fluorometer and an Agilent 2100 Bioanalyzer prior to library construction. The cDNA library was then constructed using Illumina chemistry following the manufacturer's instructions and sequenced using the Illumina HiSeq™ 2000 system. Adaptors and low-quality reads were excluded from further analysis. Reads were aligned to the reference genome (Zhang et al. 2015) using Bowtie2. Expression levels in two or more samples and statistically significant differences in expression were calculated with DESeq (Anders et al. 2010). A statistical analysis of the frequency of each read in different cDNA libraries was performed to compare changes in gene expression between stages. Multiple testing was corrected for using the false discovery rate, with FDR≤0.001 and the absolute value of $|\log_2{}^{\text{Ratio}}| \geq 1$ used as the thresholds to judge the significance of differences in gene expression.

Measurement of free salicylic acid (SA) and jasmonic acid (JA)

Free salicylic acid (SA) and jasmonic acid (JA) were extracted from leaves (300 mg) of CSSL11-9 and Su8289. The tissues were frozen immediately in liquid nitrogen and ground to a fine powder. Homogenates were extracted in acetonitrile solution at 4 °C overnight. The next day, the samples were centrifuged at 12,000 rpm for 5 min and residues re-extracted twice with acetonitrile solution; supernatants from these extractions were combined. A Sep-Pak C18 cartridge (Waters, Milford, MA, USA) was used for aqueous phase purification, after which the supernatants were blow-dried with nitrogen, redissolved in 200 μL methanol, and filtered through a 0.22 μm organic phase filtration membrane in preparation for the HPLC-MS/MS assay.

To perform the assay, a 2 μL aliquot of the sample was injected onto a Poroshell 120 SB-C18 reversed-phase column (2.1×150, 2.7 μm) at 30 °C. The run was performed in gradient mode, with the mobile phase comprising methanol/0.1% formic acid as solvent A and water / 0.1% formic acid as solvent B. The eluant flow rate was 0.3 ml min^{-1}. The mass spectrometer was set to collect data in the selected ion recording mode using electrospray ionization (ESI) in both positive and negative ion modes. The optimized conditions were as follows: spray voltage -4000 V, atomizing gas pressure 65 psi, auxiliary gas pressure 70 psi, and atomization temperature 400°C.

Diaminoaniline (DAB) staining

Leaves of CSSL11-9 and Su8289 were completely immersed in 1 mg/ml DAB staining solution (pH=3.8) at 25 ℃ for 8 h. Subsequently, the leaves were boiled in 96% ethanol for 10 min to remove the chlorophyll. The final polymerization products were photographed or observed under a microscope.

Fusarium and verticillium wilt resistance evaluation

Fusarium wilt (FW) and Verticillium wilt (VW) resistance evaluation was conducted in a growth chamber under a constant 24 °C temperature. Three independently repeated experiments were performed in three different chambers at the same time. For detailed methods of the FW and VW resistance evaluations, refer to the protocols described by Liu et al. (2021) and Zhao et al. (2018), respectively.

Fine mapping and map-based cloning of the lethal gene

A total of nine polymorphic SSR markers previously mapped on chromosome A11 were selected and used to analyze the target region. Primer sequences for the markers were obtained from Hu et al. (2019); Zhai et al. (2015) and are listed in Supplementary Table 1. DNA was extracted using the CTAB method. PCR amplifications were performed as described in Zhang et al. (2002). The resulting products were electrophoresed on a 10% non-denatured polyacrylamide gel and visualized by staining with silver nitrate, also following the procedure of Zhang et al. (2002). For the collection of genotypic data, banding patterns were categorized as follows: the same band type as *G. hirsutum* was labelled “1”; the same band type as

G. anomalum was labelled “2”; the same band type as the F_1 was labelled “3”; and missing and unclear band types were labelled “0”.

Substitution mapping was conducted according to Xu et al. (2017). Gene positions were determined by examining the graphical genotypes of different recombination classes in conjunction with their phenotypes. BlastN (Altschul et al. 1990) was applied to anchor markers to the cotton reference genome sequence, considering only alignments of E-value < 1e-10. The physical region between JAAS3191 and JAAS3050 was identified in the *Gossypium* genome and scanned for annotated genes. Candidate genes were explored based on gene annotations in the *G. anomalum*(Xu et al. 2022) and *G. hirsutum* acc. TM-1 genomes (Hu et al. 2019), and were amplified from Su8289 and *G. anomalum* using the primers listed in Supplementary Table 1.

Virus-induced gene silencing (VIGS) assays

Fragments of about 300-500 bp were isolated from eight candidate genes in CSSL11-9. The specific primers used for this amplification are listed in Supplementary Table 1. The obtained fragments were sequenced and cloned into double-digested digested pTRV2; the resulting new vectors are denoted by suffixing the gene name (pTRV2::gene). The helper plasmid pTRV1, pTRV2::gene, and the positive control pTRV2::CLA1 and negative control pTRV2::00 were introduced into *Agrobacterium tumefaciens* strain GV3101 using the heat shock method. Subsequently, the virus-induced gene silencing (VIGS) assay was performed according to the protocol described by Gao et al. (2011). A ratio of 1:1 mixed pTRV1 and pTRV2::gene were introduced into cotton seedlings with mature cotyledons but without a visible true leaf via syringe infiltration. The inoculated seedlings were grown in an incubator at 23 °C under a 16-h light and 8-h dark cycle, and then transferred to an incubator at 27 °C with the same photoperiod after the albino phenotype appeared on pTRV2::CLA1-inoculated plants. RNA was extracted from inoculated plants and the VIGS effect confirmed by qRT-PCR. More than ten individuals were infiltrated per gene for the purpose of phenotypic identification.VIGS assays were carried out independently with three replicates.

Expression analysis of high-temperature-induced genes

Total RNA at each stage was extracted using a Plant RNA Kit (Omega, Norcross, GA, USA). PrimeScript RT Master Mix (Perfect Real Time) from TaKaRa (Dalian, China) was used to synthesize first-strand cDNA. qRT-PCR was performed on an ABI QuantStudio 5 RealTime PCR System with reference to the method described by Xu et al. (2017) using the TB Green Premix Ex Taq kit (TaKaRa). Three biological replicates were used. *Actin* was employed as the reference gene. Gene-specific primers were designed with the Beacon Designer software and are listed in Supplementary Table 1.

Results

A lethal syndrome of a chromosome segment substitution line of *G. anomalum*

In previous studies, a set of CSSLs were obtained through marker assisted selection using the elite cotton variety Su8289 as the recurrent parent and a *Gossypium* wild species (*G. anomalum*) as the donor parent, among which the CSSL11-9 line with a single chromosome segment derived from *G. anomalum* on chromosome A11 (Fig. 1a, 1b) exhibited a lethal phenotype. Phenotypic identification of the lethal phenotype was conducted in the field on individual plants of the F_2 population every two weeks from the seedling stage to the mature stage. The phenotypic traits were divided into a lethal phenotype (Fig. 1c, 1e, 1g) and a normal phenotype (Fig. 1d, 1f, 1h). The lethal phenotype was specifically as follows: when a plant grew to have about seven to eight fruit-bearing shoots, the top leaves first turned red, then other leaves throughout the plant gradually turned red (Fig. 1c), and finally all leaves withered and fell off, during which the top of the plant went necrotic; at the end, the plant was bare (Fig. 1e).

CSSL11-9 and Su8289 were cultivated under various temperatures to test whether expression of the lethal gene was temperature-dependent. Interestingly, we found that the lethal syndrome did not appear at temperatures continuously lower than 26 ℃. When the ambient temperature was continuously higher than 26 ℃, CSSL11-9 plants would show a lethal symptom in withered apical leaves (Fig. 1g), and the symptoms become more severe at higher temperatures.

Autoimmune activation of lethal plants

Transcriptome analysis was conducted to unravel the global changes in gene expression in 23 ℃-grown seedlings after being shifted to the 30 ℃ condition at day 0, 1, 3 and 5. A total of 26,858 genes showed twofold or more differential expression between CSSL11-9 and Su8289 during at least one of the three time points. *PR1*, *PR4*, and other resistance genes did not exhibit differential expression at 23 ℃, but were dramatically induced in CSSL11-9 after the 30 ℃ treatment. Activation of some pathogenesis-related genes was consistently associated with the occurrence of lethal syndrome. In the later stage of the 30 ℃ treatment, more disease resistance genes were significantly differentially expressed between CSSL11-9 and Su8289 (Fig. 2a). Gene Ontology analysis indicated that genes involved in “response to biotic stimulus” were highly enriched.

Salicylic acid (SA) and jasmonic acid (JA) are two well-known phytohormones involved in defense responses. SA accumulation was greater in normal Su8289 plants. The trend of SA content variation in the CSSL11-9 lethal plants was consistent with that in the normal plants (Fig. 2b). Meanwhile, JA was highly accumulated in the CSSL11-9 lethal plants, especially after five days of 30 ℃ treatment (Fig. 2c).

Reactive oxygen species (ROS) are important signaling molecules in programmed cell death (PCD) regulation in plants. We measured ROS content in the apex leaves of lethal plants (CSSL11-9) and normal plants (Su8289) after 30 °C treatment for one, three, and five days. In lethal plants, we found that ROS accumulated from the bases of the leaves, and as the duration of the 30 °C treatment increased, the ROS content gradually extended along the veins and ultimately to the entire leaves. However, the ROS content in Su8289 leaves was not significantly changed, and only a small amount accumulated during the treatment period (Supplementary Fig. 1).

PCD and apoptosis have key functions in development and disease resistance. Accordingly, we evaluated the VW and FW resistance of CSSL11-9 and Su8289. The VW disease index of CSSL11-9 was significantly lower than that of Su8289, indicating CSSL11-9 to have enhanced resistance to VW. Likewise, the FW disease index of CSSL11-9 was significantly lower than that of Su8289, indicating CSSL11-9 to have enhanced resistance to FW (Fig. 2d).

Taken together, these results suggest that expression of the gene responsible for lethality in CSSL11-9 activated the downstream defense response in the absence of any pathogen or external stimulus, triggering the immune system, inducing PCD, and ultimately causing the lethal phenotype.

Genetic analysis of the lethal gene derived from *G. anomalum*

Individuals heterozygous in the marker region JAAS3310-NAU5192 on chromosome 11 were selected and self-crossed to generate a F_2 population containing 2337 individuals. Phenotypic identification was conducted on these individual plants in the field once every two weeks from the seedling stage to the mature stage. The phenotypic traits were divided into a lethal type and a normal type. Individual plants with leaves red on both upper and lower sides were classified as the lethal type, while individual plants with normal green leaves throughout were classified as the normal type. Among the 2337 F_2 individual plants, 545 showed the lethal phenotype and 1,792 the normal phenotype. Accordingly, $\chi_c^2=3.516 < \chi_c^2{}_{0.05,1}=3.84$, indicating that the normal phenotype and the lethal phenotype segregate in a 3:1 ratio (Table 1). Therefore, it was concluded that the lethal phenotype derived from *G. anomalum* was controlled by a pair of recessive genes in this population. With the appearance of the lethal phenotype in the F_2 generation, this is a representative example of hybrid breakdown.

Molecular mapping and map-based cloning of the lethal genederived from *G. anomalum*

In order to fine-map this lethal gene, the target region (JAAS3310-NAU5192) was refined with a total of nine SSR markers in the 2337 individuals of the F_2 population. This revealed nine types of recombinants with distinct recombination breakpoints. The individual plant numbered 1605, which exhibited the lethal phenotype, contained a homologous *G. anomalum* chromosome segment from JAAS3191 to JAAS3310, indicating the lethal gene to be located between those markers. The individual plant numbered 12, which exhibited a normal phenotype, contained a homologous *G. anomalum* chromosome segment from JAAS3050 to JAAS3310, indicating that the lethal gene was not located between those markers. The individual plant numbered 1474, which exhibited a normal phenotype, contained a homologous *G. anomalum* chromosome segment from NAU5192 to JAAS3191, indicating that the lethal gene was not located between those markers. Taken together, these results suggested the lethal gene to lie within a 60.91 kb interval from JAAS3191 to JAAS3050 based on the available *G. hirsutum* genome sequence (Hu et al. 2019), and a 98.66 kb interval based on the available *G. anomalum* genome sequence (Xu et al. 2022) (Fig. 3). This region encompassed eight candidate causal genes. The functional annotations of the candidate genes are detailed in Table 2.

Silencing of GoanoORF3 led to a normal phenotype in lethal plants

To identify the causal gene of the lethal symptom, virus-induced gene silencing (VIGS) was employed to repress each of the eight candidate genes in CSSL11-9 plants. Briefly, fragments of the eight candidate genes were cloned and inserted into pTRV2, then introduced into *Agrobacterium tumefasciens*. Seedlings inoculated with the transformed bacteria were first grown in incubators at 23 °C under a 16-h light and 8-h dark cycle, then transferred to incubators at 27 °C under a 16-h light and 8-h dark cycle after the albino phenotype appeared on pTRV2::CLA1-inoculated plants. Three biological replicates were performed. Expression of each targeted gene was significantly reduced compared to pTRV2::00-inoculated plants, indicating effective silencing. Differences in phenotype were observed at 5~7 days after transferal of inoculated plants to 27 °C. The lethal phenotype was observed in plants inoculated with pTRV2::CLA1, pTRV2::00, pTRV2::GoanoORF1, pTRV2::GoanoORF2, pTRV2::GoanoORF4, pTRV2::GoanoORF5, pTRV2::GoanoORF6, pTRV2::GoanoORF7, and pTRV2::Goano_ORF8, as well as in untreated control CSSL11-9 plants. Distinct from those cases, plants inoculated with pTRV2::GoanoORF3 consistently exhibited a normal phenotype (Fig. 4), indicating that GoanoORF3 was the causal gene derived from *G. anomalum*, henceforth designated hybrid breakdown 1 (*GoanoHBD1*). *GoanoHBD1* is a member of the LRR-RLK gene family and contains both an N-terminal LRR domain and a C-terminal kinase domain.

Expression analysis and structure variations of *GoanoHBD1*

CSSL11-9 and Heterozygous plants in this introgression segment (equivalent to F_1 ofSu8289 × CSSL11-9) were incubated at 23 °C until two genuine leaves had formed, after which all seedlings were shifted to conditions of 30 ℃, 26 ℃, and 23 ℃ for one, three, and five days, respectively. The expression of *GoanoHBD1* was then evaluated. In CSSL11-9 plants, *GoanoHBD1* was dramatically induced by higher temperature (30 ℃ and 26 ℃), increased gradually at the later stage, and reached its highest level when the lethal phenotype began to appear in apical leaves. However, in the heterozygous (Su8289 × CSSL11-9) F_1 plants, *GoanoHBD1* was not induced by high temperature; moreover, its basal expression level was significantly lower than in the CSSL11-9 lethal plants. For CSSL11-9 seedlings grown at 23 ℃, *GoanoHBD1* expression was independent of temperature (Fig. 5).

Guided by the sequences of *G. anomalum* (Xu et al. 2022) and TM-1 (Hu et al. 2019), we next cloned the coding regions of *GoanoHBD1* and *GhHBD1* from *G. anomalum* and Su8289, respectively. This revealed a large structural variation between *G. anomalum* and Su8289 (Supplementary Fig. 2). We hypothesized that mutation of *GoanoHBD1* led to amino acid changes that affected the activity and structural stability of the expressed products and ultimately resulted in the different phenotypes. It can be reasonably speculated that this structural variation of *GoanoHBD1* is likely to cause the lethal syndrome observed in CSSL11-9 plants.

Discussion

Several studies of hybrid incompatibility have been reported in cotton. Hutchinson (1932) observed lethal F_1 hybrids in crosses between certain strains of *G. arboretum* var. *soudanensis* and a range of other *arboretum* and *herbaceum* strains. Silow (1941) reported hybrid lethality between *G. herbaceum* and other species including *G. arboretum* and *G. gossypioides*. Stephens (1946) reported lethal hybrids between certain strains of *G. hirsutum* var. *marie-galante* and *G. barbadense*. In addition, lethal syndrome was also observed in hybrids of *G. hirsutum* and *G. gossypioides* (Phillips and Merritt 1972), *G. klotzchianum* (Phillips and Reid 1975), *G. davidsonii* (Lee 1981), or *G. barbadense* (Song et al. 2009). All of the above phenomena were reported in F_1 hybrids and referred to as hybrid lethality or hybrid weakness. In this study, the lethal phenotype occurred in F_2 generation and was controlled by a pair of recessive genes from *G. anomalum*, indicating a representative example of hybrid breakdown. This is the first case of hybrid breakdown reported in cotton.

Studies have shown that hybrid lethality is sensitive to temperature, with most examples reported to date being induced by low temperature (Bomblies et al. 2007b; Jeuken et al. 2009; Ueno et al. 2016; Jia et al. 2021). The underlying causal genes of low-temperature-dependent hybrid lethality are usually members of the immune receptor family encoding nucleotide-binding domain and leucine-rich repeat (NLR). Ambient temperature is a key environmental factor affecting the strength of plant immune responses along with pathogen virulence; generally, higher temperatures reduce immunity, especially NLR-dependent responses, whereas lower temperatures enhance immunity (Cheng et al. 2019). However, some cases of hybrid lethality activated by high temperature have also been observed; for example, rice Hwi1 and Hwi2 function in high-temperature-triggered resistance and thereby induce the hybrid weakness event (Chen et al. 2013). It is well known that high temperature may decrease plant resistance to pathogens; however, the proteins encoded by the bacterial blight resistance gene *Xa7* in rice (Webb et al. 2010) and the wheat strip rust resistance gene *Yr36* (Fu et al. 2009) are more effective at higher temperatures. In high-temperature-dependent hybrid weakness, underlying causal genes are usually members of the immune receptor family and encode either a receptor kinase or a receptor protein. In this study, we found that elevated expression of *GoanoHBD1* improved CSSL11-9 resistance to VW and FW, indicating associated induction of plant defense responses. This discovery implies that whether hybrid lethality depends on low or high temperature may be determined by the function of the causal gene.

Several genes that cause hybrid incompatibility have been cloned. Bomblies et al. (2007a) and Chae et al. (2014) found simultaneous presence of the two incompatible genomic regions Dangerous mix1 (DM1) and DM2d to lead to F_1 hybrid lethality in Arabidopsis; both regions encode NLR proteins. Barragan et al. (2020) reported another two new Arabidopsis incompatibility loci, where an epistatic interaction between DM10, which encodes a truncated NLR gene, and DM11 results in F_1 hybrid lethality. Additionally, one Arabidopsis LRR–RLK gene, Strubbelig receptor family 3, has been shown to interact with another NBS–LRR gene at the DM2 locus to trigger hybrid weakness(Alcázar et al. 2010). Chen et al. (2014) likewise confirmed that two LRR-RLK genes at locus Hwi1 and a secreted subtilisin-like protease at locus Hwi2 determine interspecific hybrid weakness in rice. Si et al. (2021a) found that the wheat gene *Ne2*, which encodes a CC-NLR protein, causes whole-seedling necrosis. Collectively, these studies suggest that in

almost all hybrid lethality events, at least one of the loci is likely to encode a member of the RK/RP immune-related protein or NLR immune receptor families.

In this study, we identified an LRR-RLK transmembrane receptor-like kinase gene as the causal gene underlying a locus for interspecific hybrid incompatibility between *G. anomalum* and *G. hirsutum*. It is generally believed that hybrid lethality or hybrid breakdown is associated with autoimmunity, which suggests that *GoanoHBD1* may function in cotton defense responses. LRR-RLKs are usually employed as receptors to monitor signaling molecules released by pathogens, and cue endogenous signal peptides to induce immune reactions. Receptor kinases respond to a variety of pathogen molecules in the extracellular space to achieve fundamental resistance to pathogens, which contributes to pattern-triggered immunity (PTI). Hitherto, only a few LRR receptor-like genes have been documented to encode PRRs and function as immune receptors. Some family members, such as BAK1 also function as co-receptors, forming complexes with multiple LRR-type proteins and thereby activating immune responses (Chinchilla et al. 2009). The rice LRR-RLK *Xa21* is known to be involved in rice immunity (Pruitt et al. 2015).

Declarations

Author contribution statement

Experiments were designed by XS. Experiments were performed by PX, JX, QG, ZX, JC, WJ, HY, LZ, JZ, JL and XC. PX drafted the manuscript, XS revised the manuscript. All authors read and approved the final manuscript.

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Conflict of interest The authors declare that there are no conflicts of interest in the reported research.

Ethical standards The authors note that this research is performed and reported in accordance with ethical standards of the scientific conduct.

Data availability

All data used in the current study are included in this published article or are available from the corresponding author on reasonable request.

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Tables

Table 1 Genetic analysis of the lethal gene derived from *G. anomalum*

	Normal Nor	Lethality Le	Total
Observe (O)	1792	545	2337
Expect (E)	1752.75	584.25	2337
O-E	39.25	-39.25	
$(O-E)^2$	1540.563	1540.563	
$(O-E)^2/E$	0.879	2.637	
$\chi^2=\sum(O-E)^2/E$	3.516		$\chi^2=3.516<\chi^2_{0.05,1}=3.84$

Table 2 Eight candidate genes and their annotations

Gene	Annotation
GoanoORF1	TUDOR-SN protein 1
GoanoORF2	Disease resistance protein (CC-NBS-LRR class) family
GoanoORF3	Leucine-rich repeat transmembrane protein kinase
GoanoORF4	Ethylene responsive element binding factor 1
GoanoORF5	Integrase-type DNA-binding superfamily protein
GoanoORF6	ARABIDILLO-1
GoanoORF7	2-oxoglutarate (2OG) and Fe (II)-dependent oxygenase superfamily protein
GoanoORF8	Uncharacterized conserved protein

Figures

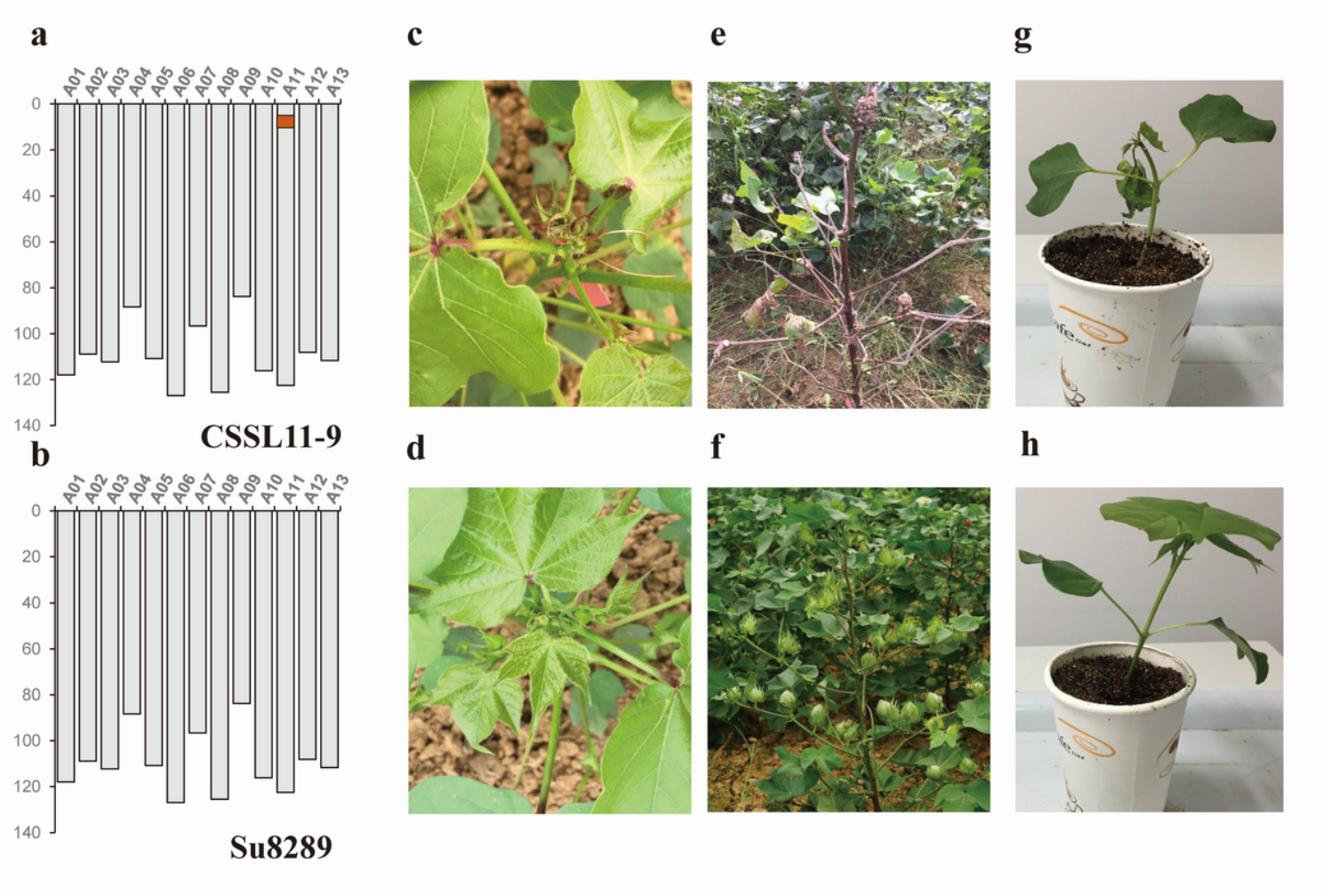

Figure 1

Graphical genotypes and phenotypic identification of the lethal phenotype derived from *G. anomolum*. The gray column represents the genotype of recurrent parent Su8289 and the orange column represents the genotype of *G. anomolum*.

(a) (b) Graphical genotype of CSSL11-9 in Su8289 genetic background. (c) (e) (g) The lethal phenotype at different stages. (d) (f) (h) The normal phenotype at different stages.

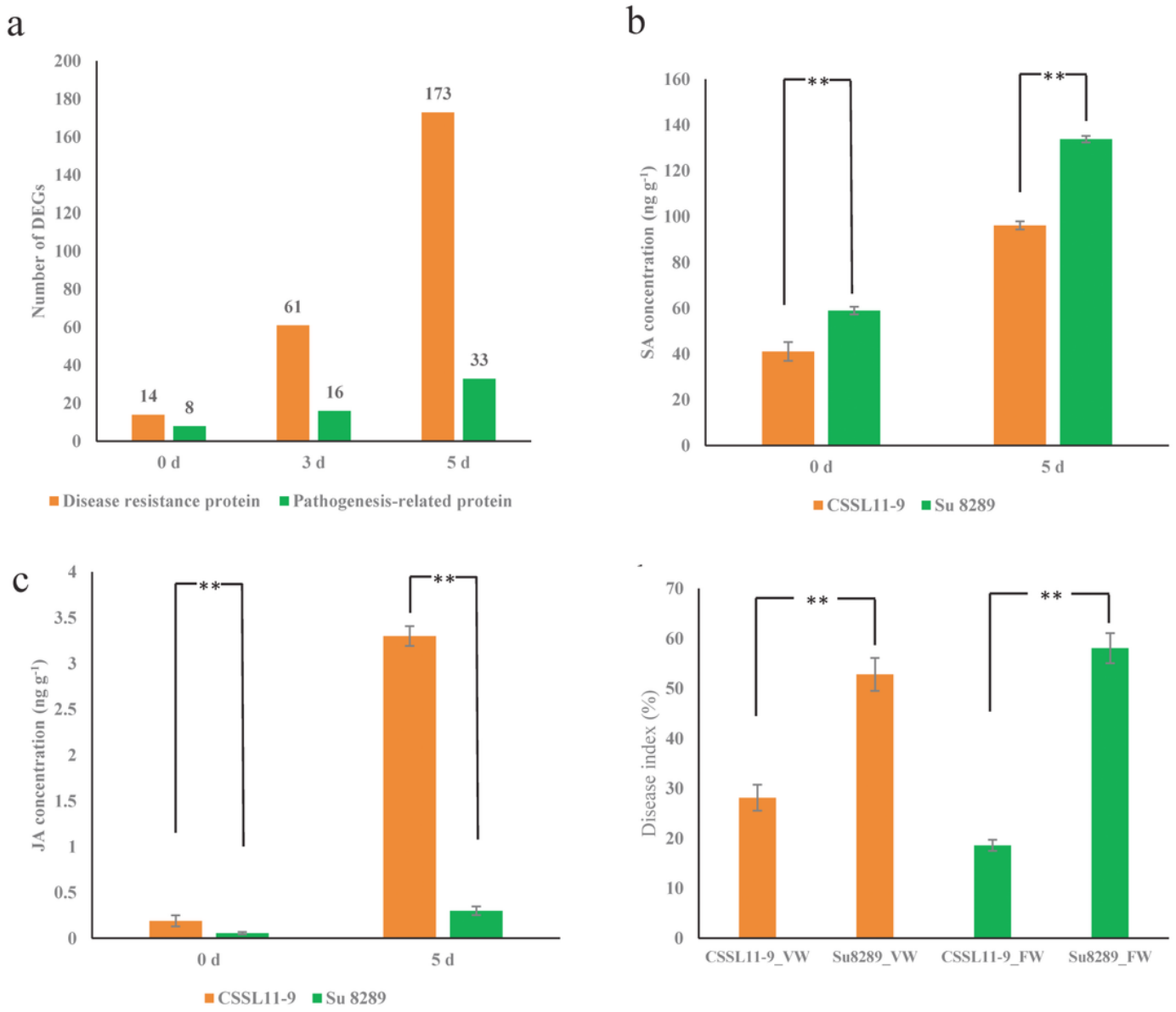

Figure 2

Autoimmune characterization of the lethal plant CSSL11-9

(a) Analysis of differentially expressed pathogenesis-related genes and disease resistance genes between CSSL11-9 and Su8289. (b) The free salicylic acid content in leaves of CSSL11-9 and Su8289; (c) the free jasmonic acid content in leaves of CSSL11-9 and Su8289; ** indicate statistical significance at the 0.01

probability level. (d) The *Verticillium wilt* and *Fusarium wilt* resistance of CSSL11-9 and Su8289, CSSL11-9_VW: the *Verticillium wilt* disease index of CSSL11-9; Su8289_VW: the *Verticillium wilt* disease index of Su8289; CSSL11-9_FW: the *Fusarium wilt* disease index of CSSL11-9; Su8289_FW: the *Fusarium wilt* disease index of Su8289.

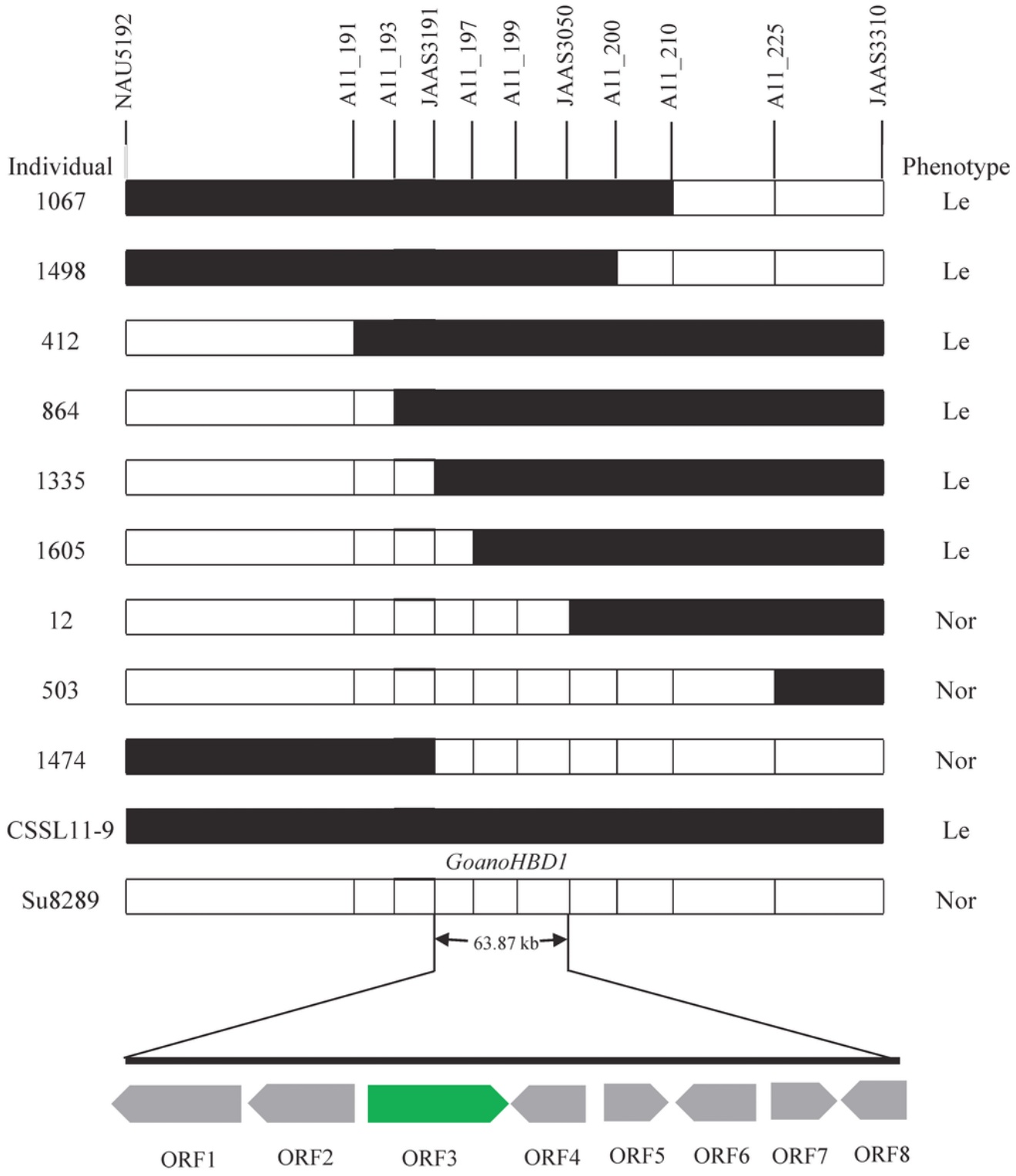

Figure 3

Fine mapping and map-based cloning of the lethal gene derived from *G. anomolum*

White portions of the graph indicate homozygous Su8289 chromosome segments, black regions indicate *G. anomolum* chromosomes.

Le: Lethal phenotype; Nor: Normal phenotype

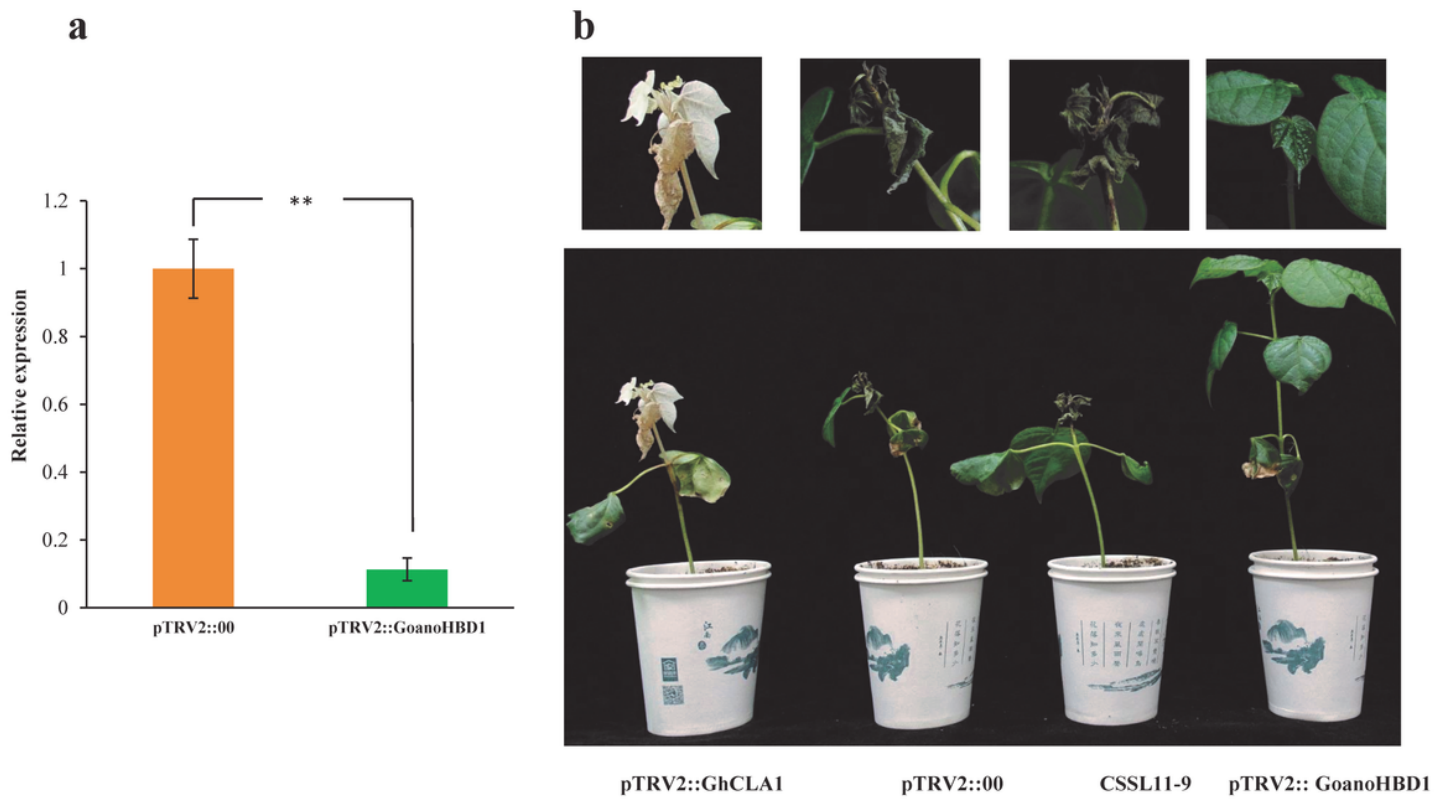

Figure 4

Phenotypes of VIGS plants and suppression of endogenous transcripts in cotton.

(a) Transcript levels of *GoanoHBD1* in pTRV2 :: 00 and pTRV2 :: GoanoHBD1 inoculated plants.

The cotton *Actin*gene was used as an internal reference. The data are mean ± SE of three biological replicates. ** indicate statistical significance at the 0.01 probability level.

(b) Phenotype of the VIGS lines and its control

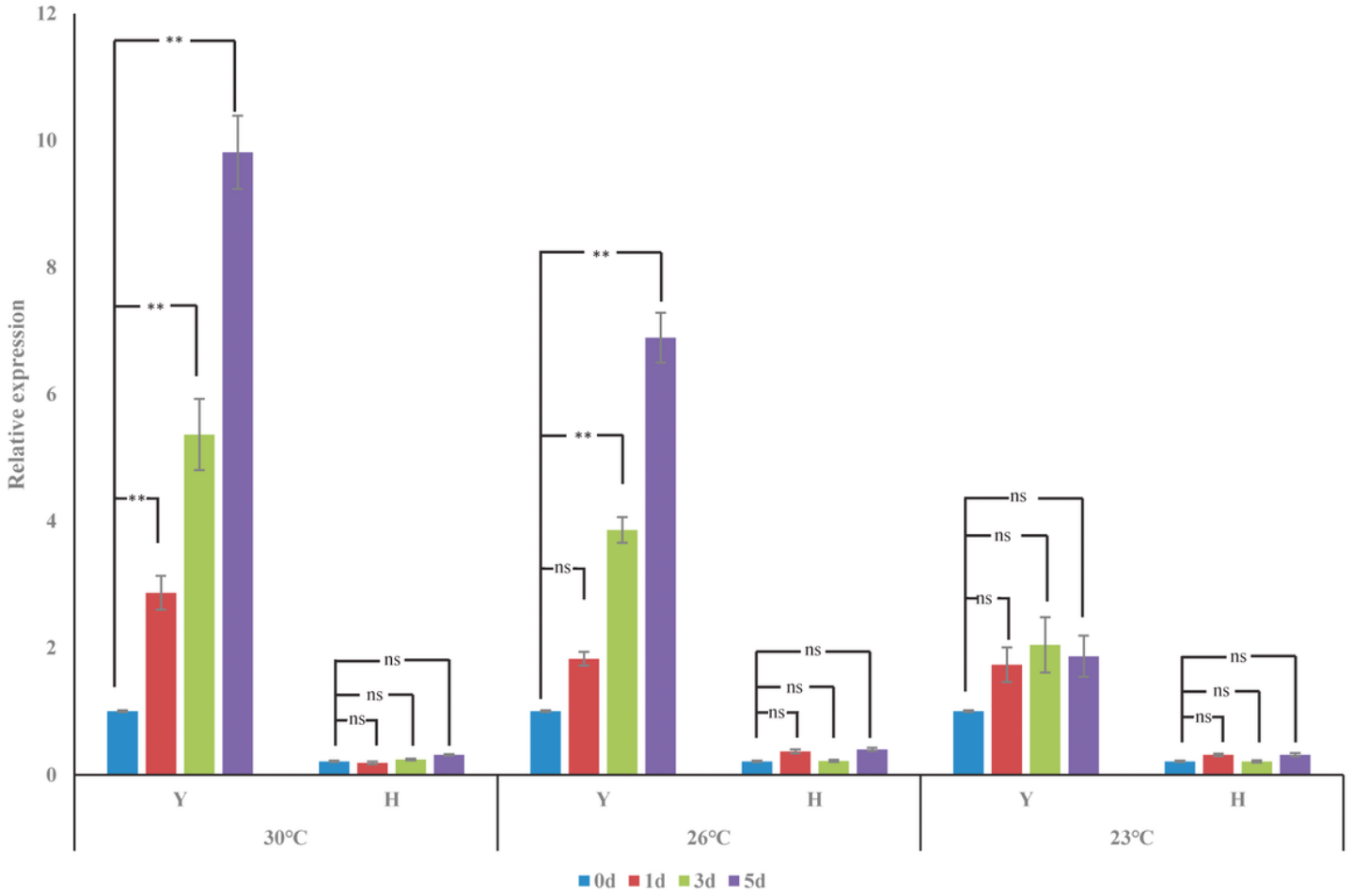

Figure 5

Expression profiles of *GoanoHBD1* and *GhHBD1* at different temperature in CSSL11-9 and heterozygous F_1 plants of Su8289 × CSSL11-9

Y: CSSL11-9; H: Heterozygous F_1 plants of Su8289 × CSSL11-9

Supplementary Files

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