the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2024) doi: 10.1111/tpj.16750

SR45a plays a key role in enhancing cotton resistance to *Verticillium dahliae* by alternative splicing of immunity genes

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Received 8 January 2024; revised 22 February 2024; accepted 20 March 2024.
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SUMMARY

Alternative splicing (AS) of pre-mRNAs increases the diversity of transcriptome and proteome and plays fundamental roles in plant development and stress responses. However, the prevalent changes in AS events and the regulating mechanisms of plants in response to pathogens remain largely unknown. Here, we show that AS changes are an important mechanism conferring cotton immunity to *Verticillium dahliae* (*Vd*). *GauSR45a*, encoding a serine/arginine-rich RNA binding protein, was upregulated expression and underwent AS in response to *Vd* infection in *Gossypium australe*, a wild diploid cotton species highly resistant to *Vd*. Silencing *GauSR45a* substantially reduced the splicing ratio of *Vd*-induced immune-associated genes, including *GauBAK1* (*BRI1-associated kinase 1*) and *GauCERK1* (chitin elicitor receptor kinase 1). GauSR45a binds to the *GAAGA* motif that is commonly found in the pre-mRNA of genes essential for PTI, ETI, and defense. The binding between GauSR45a and the *GAAGA* motif in the pre-mRNA of *BAK1* was enhanced by two splicing factors of GauU2AF35B and GauU1-70 K, thereby facilitating exon splicing; silencing either *AtU2AF35B* or *AtU1-70 K* decreased the resistance to *Vd* in transgenic *GauSR45a* Arabidopsis. Overexpressing the short splicing variant of *BAK1GauBAK1.1* resulted in enhanced Verticillium wilt resistance rather than the long one *GauBAK1.2*. *Vd*-induced far more AS events were in *G. barbadense* (resistant tetraploid cotton) than those in *G. hirsutum* (susceptible tetraploid cotton) during *Vd* infection, indicating resistance divergence in immune responses at a genome-wide scale. We provided evidence showing a fundamental mechanism by which GauSR45a enhances cotton resistance to *Vd* through global regulation of AS of immunity genes.

Keywords: alternative splicing, plant immunity, SR protein, splicing regulating factor, *Verticillium dahliae*, cotton.

INTRODUCTION

A complex innate immune system plays key roles in protecting plants from pathogen invasion (Wang et al., 2022). During plant–pathogen interactions, the robust innate immune response in plants is mainly activated by surface receptors and intracellular receptors (Jones & Dangl, 2006; Zhou & Zhang, 2020). To subvert plant immunity, pathogenic microbes secrete numerous virulence effectors targeting and regulating these receptors to create a permissive niche for infection (Zhou & Chai, 2008). In response, plants increase the diversity of immune receptors to confer new effector recognition and initiate the immune defense response (Cui et al., 2015). *Verticillium dahliae* Kleb. (*Vd*), the predominant causal agent of Verticillium wilt (VW), is a notorious pathogen leading to the most devastating soil-borne fungal diseases of crops worldwide (Klosterman et al., 2009). Great progress has been made in the identification of disease-resistance genes in crops, but how posttranscriptional regulation is involved in plant defense against *Vd* remains unclear.

Alternative splicing (AS) of precursor-mRNAs (pre-mRNAs) is a ubiquitous form of post-transcriptional regulation in eukaryotes, leading to the generation of more than one mature mRNA from an individual gene (Chaudhary et al., 2019) and thus greatly enrich the transcriptome complexity and diversity of functional proteins (Jia et al., 2020). In general, there are five types of AS, i.e. exon skipping (SE), intron retention (RI), mutually exclusive exon inclusion (MXE), and alternative 5′ (A5SS) or 3′ (A3SS) splice site selection in eukaryotes. AS not only contributes to the coevolution of intracellular recognition receptors and effectors but also acts as a key regulator in plant immunity by improving the plasticity of the transcripts encoding proteins of the genes needed for pattern recognition receptor-triggered immunity (PTI) (Sanabria & Dubery, 2016) and effector-triggered immunity (ETI) (Yang et al., 2014) as well. For instance, the receptor-like kinase Nt-Sd-RLK undergoes AS in pathogen recognition (Sanabria & Dubery, 2016); Resistant-to-*P. syringae*4 (*RPS4*), a nucleotide binding/leucine-rich repeat (NLR) gene of Arabidopsis, is alternatively spliced in disease resistance (Zhang & Gassmann, 2003). Multiple transcripts of the *N* resistance gene are necessary for complete resistance to tobacco mosaic virus (Dinesh-Kumar & Baker, 2000). Both plants and animals may employ AS to diversify the signaling repertoire toward diseases or pathogens by adapting and fine-tuning hormonal signaling (Jiang et al., 2015).

AS is regulated by hundreds of splicing factors (SFs), including the core snRNP and the auxiliary RNA-binding proteins (RBPs). RBPs harbor various RNA-binding domains such as RNA recognition motif (RRM), K homology domain (KH), zinc finger domain, DEAD-box domain, and Pumilio/FBF domain (Gehring et al., 2017). RBPs could participate in response to pathogens in direct or indirect ways (Woloshen et al., 2011). For example, the RNA-binding protein-defense related 1 (AtRBP-DR1) positively regulates AS of SA signaling-related genes (Qi et al., 2010); GRP7, a heterogeneous nuclear ribonucleoprotein (hnRNP)-like RBP, directly binds to the target mRNAs to regulate AS in response to environmental stimuli (Streitner et al., 2012). However, how RBPs regulate AS to tune plant immunity remains largely unknown, especially in crops.

Serine- and arginine-rich (SR) protein is one of the most relevant protein superfamilies of general SFs and contributes to the generation of unique spatiotemporal regulated AS patterns by establishing complex synergistic and antagonistic interactions (Pandit et al., 2013). SR proteins bind pre-mRNAs and function as activators or repressors of AS in animals (Graveley & Maniatis, 1998), whereas SRs can interact with the spliceosome to regulate splicing site selection in plants (Reddy, 2007). Exonic splicing enhancers (ESEs) generally bind to SR proteins to promote exon splicing, while exonic splicing silencers (ESSs) typically bind with hnRNPs to antagonize the positive effect of SRs on splice site usage (Wahl et al., 2009). Besides regulating AS of target RNAs, *SR* genes themselves can produce more than 90 transcripts resulted from AS (Palusa et al., 2007). In particular, the AS of *SR* is inducible by stress or hormone stimulation (Albaqami et al., 2019; Reddy & Shad Ali, 2011). Several SR proteins are associated with plant immunity (Kufel et al., 2022; Xu et al., 2011; Zhang et al., 2023); however, the mechanisms of how SR proteins regulate AS in plant immunity are poorly understood.

To reveal whether and how AS is involved in cotton VW resistance, we first analyzed *Vd*-induced genome-wide AS by joint analyses of *Gossypium australe* transcriptomic data by Illumina sequencing and single-molecule real-time sequencing (SMRT). We found that a large set of genes involved PTI and/or ETI underwent AS in the immune response to *Vd* in cotton, SR45a collaborated with splicing factors U2AF35B and U1-70K to regulate AS of immune-associated genes. Our results provide new evidence showing that SR45a may play a central role in the regulation of AS of plant immunity genes in response to *Vd* invasion, and thus deepen our insights into the mechanisms of VW resistance in cotton.

RESULTS

Alternative splicing constitutes a distinct regulatory layer controlling gene expression in cotton response to *V. dahliae*

Global transcriptional reprogramming has been recognized as a fundamental immune regulatory mechanism in plants, while alternative splicing events continue to be discovered in an ever-increasing variety of plants in the context of physiologically normal and stress states. The expression of some PAMP receptors is modulated at both the transcriptional and posttranscriptional levels (Xu et al., 2017). To investigate the gene expression reprogramming events essential for VW-resistance, we performed paired-end Illumina RNA-seq analyses to profile the transcriptomes of the roots of a highly VW resistant wild cotton species *G. australe* inoculated with the *Vd* V991 isolate for 1, 2, and 3 days, respectively. The full-length mRNA sequences of *G. australe* previously generated by using SMRT sequencing technology were used as the reference to produce an accurate catalog of transcript isoforms (Feng et al., 2019) and improve annotation of the *G. australe* genome (Figure 1). The comprehensive AS landscape was further investigated using these sequencing data (Figure S1). The AS events, including RI, SE, A3SS, A5SS, and MXE, were identified by junction-mapped reads (Figure 1b), and gene expression reprogramming in response to *Vd* was classified into differentially expressed genes (DEGs) and differentially AS genes (DASGs) (Figure S2). A total of 537, 494, and 1986 DEGs and 3243, 3218, and 3333 DASGs were

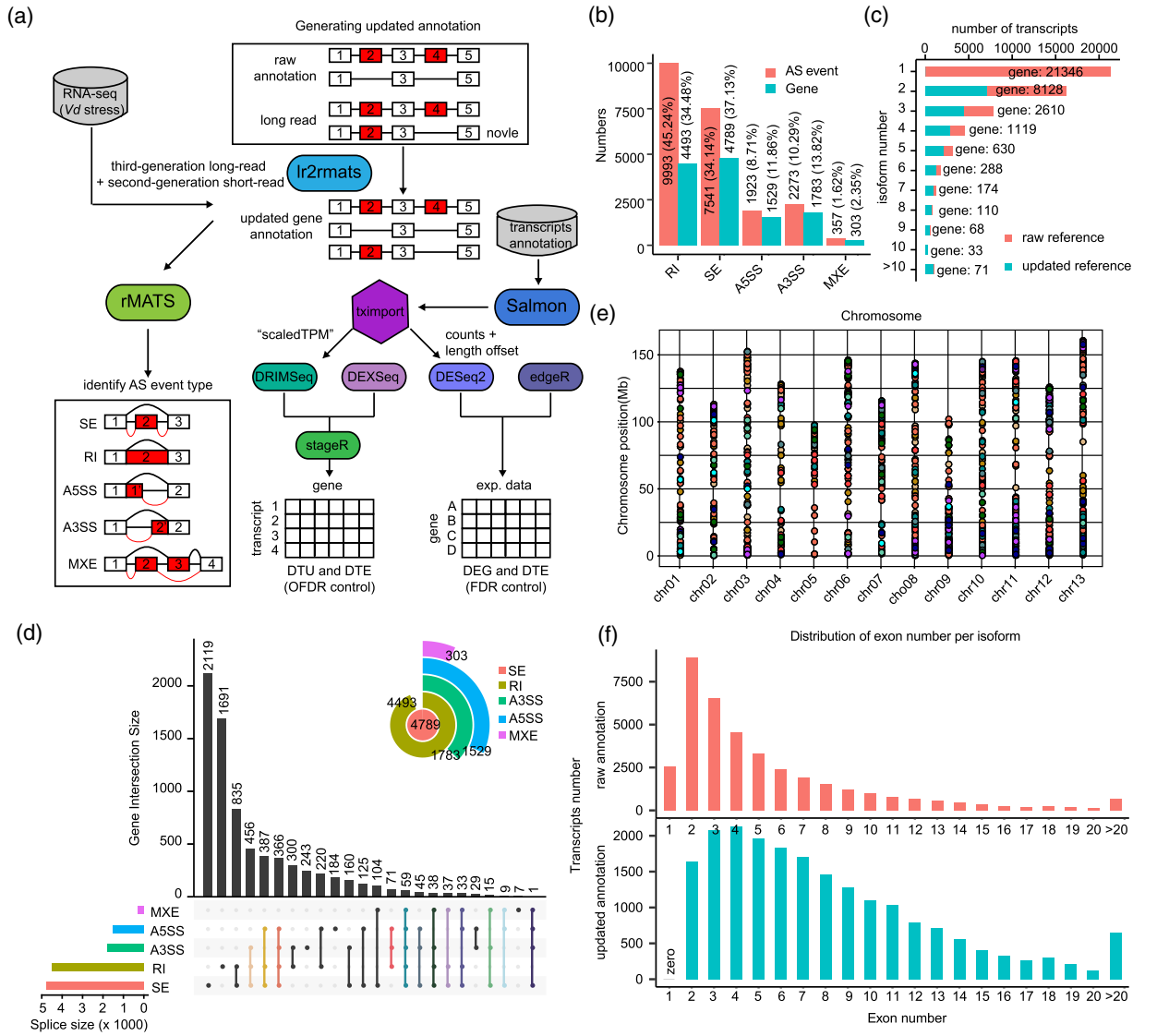

Figure 1. Generation of updated *G. australe* transcriptome annotation and identification of alternative splicing (AS) events during *V. dahliae* (*Vd*) infection.
(a) Flowchart for updated *G. australe* gene annotation and identifying AS event types and systematic profiling of differentially expressed genes and differentially used transcripts during *Vd* infection.
(b, c) Summary of AS events in updated transcriptome annotation. Common types of AS events include intron retention (RI), exon skipping (SE), alternative 5′ splicing site (A5SS), alternative 3′ splicing site (A3SS), and mutually exclusive exon (MXE) splicing.
(d) Comparison of the types of AS events within the *G. australe* genome.
(e) Chromosome localization of multiple types of AS-containing genes.
(f) The distribution of exon number per isoform in raw and updated gene annotations.

identified after *Vd* infection for 1, 2, and 3 days, respectively (Table S4–S6). Notably, very few of the DASGs were differentially expressed during *Vd* infection (Figure S3); except for 1517 genes that underwent multiple types of AS, most genes underwent only one type of AS (Figure 1d, e). Moreover, AS of genes in cotton were prevalent in response to *Vd* infection (Figure S5 and Table S6). AS events detected by RNA-seq analysis can be verified by using RT-PCR analysis. For example, when we arbitrarily selected *GauOTU9* to determine the AS events by RT-PCR, the results were consistent with those based on RNA-seq analysis (Figure S4). Subsequently, we performed GO enrichment analysis for 4281 DASGs and 2186 DEGs after *Vd* induction (Figure S6a). The results showed that *Vd*-induced specific DASGs and DEGs were significantly enriched in immune-related biological processes (Figure S6b,c). This result indicated that transcriptional regulation (rGE) and AS regulation (rAS) were two different but perhaps complementary strategies taken by *G. australe* to defend against *Vd*.

We further analyzed the immune-related genes that underwent DAS in *Vd* invasion from RNA-seq data (Table S7). RNA-seq analysis revealed that the key PTI and ETI-related genes, such as *GauBAK1* (BRI1-associated kinase 1) (Zhou & Zhang, 2020), *GauCERK1* (chitin elicitor receptor kinase 1) (Gong et al., 2020), *GauRLK4* (Couto & Zipfel, 2016), *GauLRR3* (Zhou & Zhang, 2020), *GauWAK14* (wall-associated kinase 14) (Wang et al., 2020), *GauPRL* (pathogenesis-related protein) (Breen et al., 2017), *GauLRR4* (Zhou & Zhang, 2020), *GauBIR2* (BAK1-interacting RLK2) (Halter et al., 2014), and *GauNPR3* (Ding et al., 2018) produced multiple transcript variants characterized by different extents of exon splicing during the infection of *Vd* (Figure 2a–l; Figure S7a–f). The increased splicing ratios of these genes were verified with qRT-PCR assays (Figure 2; Figure S7).

GauSR45a is alternatively spliced, and only two variants are involved in response to *Vd*

SRs regulate gene expression at both transcriptional and posttranscriptional levels in eukaryotes (Day et al., 2012). This prompted us to evaluate the roles of SR family genes

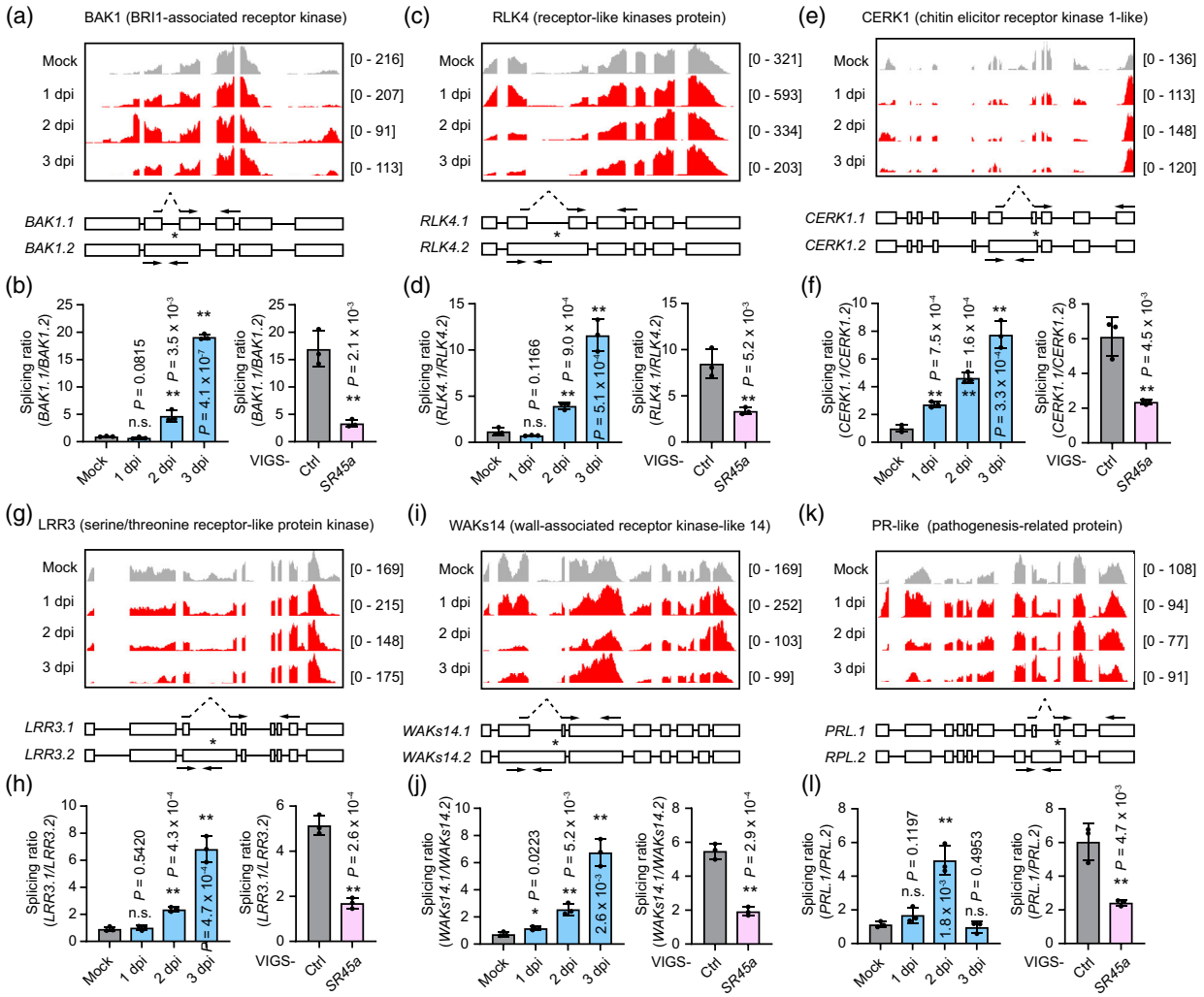

Figure 2. Validation of altered splicing ratios of cotton immune-associated genes by *Vd* infection.
(a) Validation of altered splicing ratios of *the BAK1* gene by *Vd* infection. The top panel shows wiggle plots of RNA-seq data for the *BAK1* gene. The schematic gene models of different splicing forms are shown in the bottom panel. The asterisks indicate a premature termination codon.
(b) The splicing ratio of *BAK1* increased during *Vd* infection. The *Vd*-induced enhancement splicing ratios of *BAK1* were dependent on SR45a. The result was confirmed using qRT-PCR in VIGS-*SR45a* and control plants. Isoform-specific primers were designed to cross the exon–exon junction, and primers detecting unspliced products were designed to span the intron-exon junction. Total RNA was extracted from roots infected with *Vd* 1, 2, and 3 dpi or roots from VIGS-*SR45a* plants infected with *Vd* 2 dpi or 3 dpi. Sterile water treatment was used as a mock control. The results were normalized to the internal control gene *histone3* ($n = 3$).
(c–l) Validation of increased splicing ratios of *the RLK4* (c, d), *CERK1* (e, f), *LRR3* (g, h), *WAKs14* (i, j), *PRL* (k, l) gene by *Vd* infection. The data are shown as the mean ± s.e. from three independent repeats. The *P* values indicate the results from pairwise comparisons of one-way ANOVA tests. * $P < 0.05$, ** $P < 0.01$, n.s., not significant. The above experiments were performed at least three times with similar results.

of cotton in response to *Vd*. The expression of *GauSR45a-like* (*SR45a-L*) was upregulated at 2-, and 3- dpi of *Vd*, while the expression of the other SR genes showed no remarkable fluctuation (Figure S8a). We further identified six *GauSR45a-L* AS variants generated by differential exon inclusions from exon 3–8 (Figure S9a): *GauSR45a-L2* contains 1189 nucleotides encoding a full-length GauSR45a protein characterized by an RNA recognition motif (RRM) domain flanked by Arg/Ser-rich (RS) domains at each end, *GauSR45a-L1* contains a premature termination codon (PTC) in exon 9 and thus generates a truncated RS domain at the C-terminus (Figure S9b), and four transcript variants encode shorter truncated isoforms without the C-terminal RS domain or with a truncated RRM domain (Figure S9b). Among them, only *GauSR45a-L2* (*PB.26297.1*) was highly induced during *Vd* infection (Figure S8b). To investigate the correlation between *SR45a-L* genes and cotton resistance to VW, we further analyzed the expression and AS patterns of the orthologous *SR45a-Ls* of *G. barbadense* and *G. hirsutum*, which exhibit resistance and susceptibility to *Vd*, respectively. The results showed that *GbSR45a-L* and *GhSR45a-L* were ubiquitously expressed in various tissues (Figure S8e,f), but were downregulated at 2–6 h post-inoculation (hpi) compared to at 0 hpi and then upregulated at 12 to 96 hpi compared to at 6 hpi (Figure S8c,d). GUS (*β-glucuronidase*) reporter gene driven by the promoter of *GauSR45a-L* or *GbSR45a-L* can be activated by *Vd* infection to express in *N. benthamiana* leaves (Figure S8g), the promoter activity indicated by GUS staining is consistent with the abundance of GbSR45a-L transcripts by RNA-seq analysis. RT-PCR analysis showed that six transcript variants were produced by AS (Figure S9c–e), and only *GbSR45a-L1* and *GbSR45a-L2* were differentially accumulated during *Vd* infection. The splicing patterns of the *SR45a-L* were similar in the two resistant cotton species. In contrast, the AS patterns of the *GhSR45a-L* in susceptible *G. hirsutum* were significantly different (Figure S9f). These results suggest that SR45a-L may perform a key role in cotton VW resistance.

Each of the six transcript variants was tagged with GFP and transiently expressed in *N. benthamiana* epidermal cells to investigate the cellular localization of their proteins. The results showed that all the isoforms were colocalized with AtSR34-RFP, the nuclear speckle marker (Huang et al., 2020) of nuclear speckles (Figure S10), the storage sites of splicing machinery. In addition, GauSR45a-L1 and GauSR45a-L2 were also observed in the cytoplasm and PM, implying that they may have functions different from those of other isoforms.

SR45a positively regulates cotton resistance against *Vd* via AS regulation of immune-related genes

To further examine whether SR45a contributes to cotton resistance to VW, we employed virus-induced gene silencing (VIGS) to silence the *SR45a* gene in *G. australe* and *G. barbadense* cv. Hai7124 (Figure 3a). As the target genes were efficiently silenced in cotton roots (Figure 3b), VIGS plants were inoculated with spores of the *Vd* V991 isolate by using a root-dipping method. Silencing *SR45a* genes gave rise to typical symptoms including leaf chlorosis, curling and necrosis, and vascular discoloration (Figure 3a,d). The fungal recovery rates from stems (Figure 3c), relative fungal biomass (Figure 3e), and the disease index (DI) were significantly increased in the silenced plants (Figure 3f). Notably, silencing *SR45a* resulted in more severe disease symptoms, such as wilting, in *G. australe* than in *G. barbadense* after inoculation with *Vd* (Figure 3a).

We further ectopically expressed each of the six *GauSR45a* splicing variants from *G. australe* in Arabidopsis to examine their potential roles in defense against *Vd* infection. The results showed that overexpression of *GauSR45a-L1* or *GauSR45a-L2* significantly boosted the VW resistance of Arabidopsis (Figure 3g,h), characterized by significantly lowered fungal biomass (Figure 3i) and DI (Figure 3j) at 20 dpi, but overexpression of *GauSR45a-L2* resulted in relatively higher resistance to *Vd* than overexpression of *GauSR45a-L1* (Figure 3g). Taken together, *GauSR45a-L1* and *GauSR45a-L2* alternative splicing variants of *SR45a-L* positively regulate plant resistance to *Vd*.

To further explore whether SR45a is involved in the AS regulation of immune-related genes, we used reverse transcription PCR to detect the splicing events of immune-related genes in the roots of VIGS-*GauSR45a* cotton after a challenge with *Vd*. The results showed that the AS ratios of *GauBAK1*, *GauCERK1*, *GauRLK4*, *GauLRR3*, *GauWAK14*, and *GauPRL* were significantly attenuated in *GauSR45a*-silenced cotton (Figure 2). Indeed, some immunity-related genes showed more complex alternative splicing patterns, for example, the splicing ratio of *GauLRR4* was increased in response to *Vd* in WT but decreased at 3 dpi in *GauSR45a*-silenced cotton plants; while splicing ratio of *GauBIR2* or *GauNPR3* was significantly reduced after *Vd* infection, but was increased in *GauSR45a*-silenced cotton (Figure S7c–f). These results suggest that *GauSR45a* confers cotton resistance to *Vd* largely by regulating the alternative splicing of the key genes involved in PTI, ETI, and defense in response to *Vd*.

GauSR45a promotes exon skipping of immune-related genes

We performed LUC (firefly luciferase)-splicing reporter system assays in *N. benthamiana* leaves to determine how GauSR45a regulates the splicing of immune-related genes. In this assay system, exon skipping of reporter constructs permits expression of the LUC protein, while intron retention generates a premature termination codon and is thus unable to express the LUC protein. The alternatively

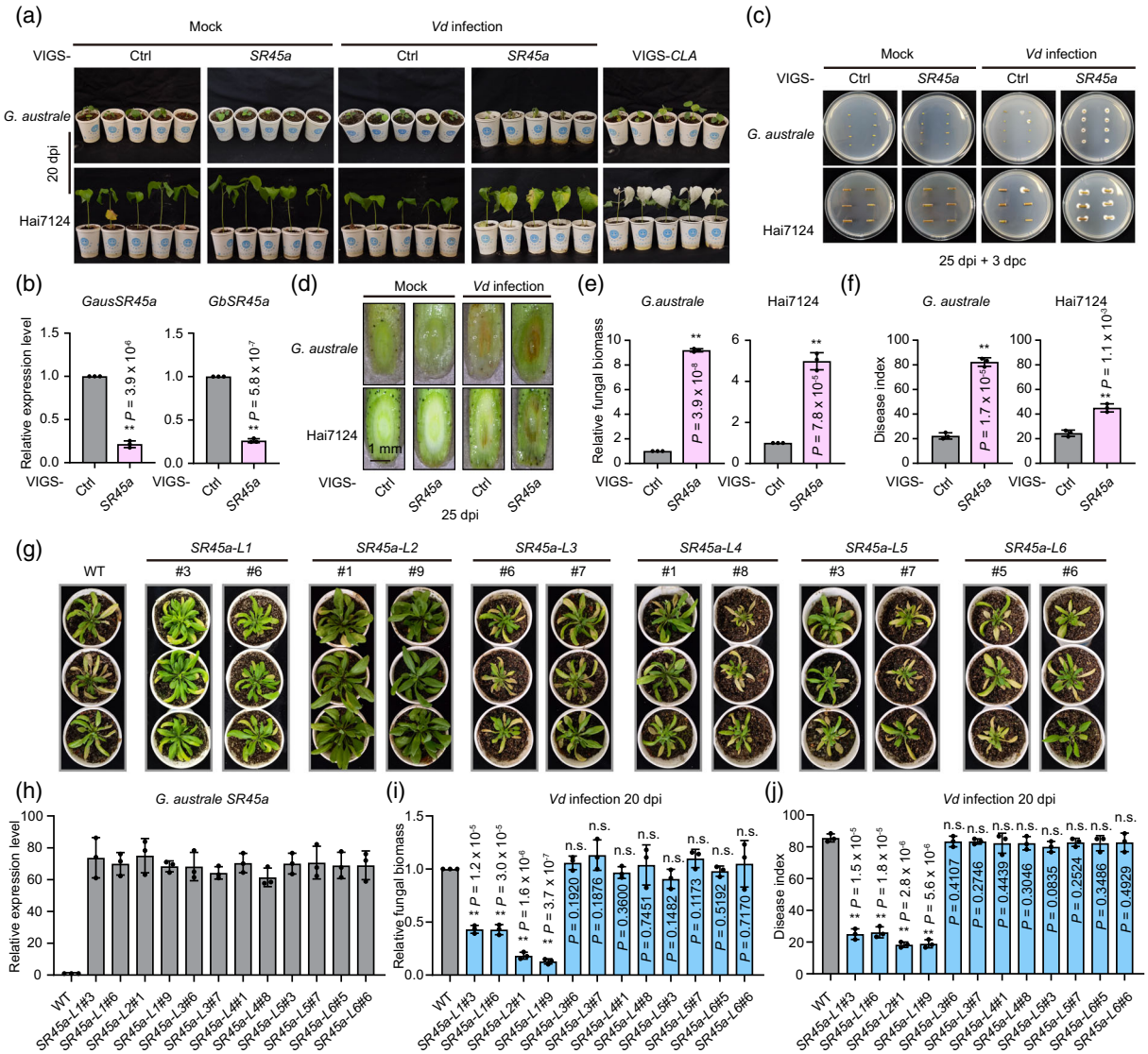

Figure 3. *SR45a-Like* is involved in cotton defense responses against Verticillium wilt.

(a) The disease symptoms of VIGS-*GauSR45a*, *-GbSR45a* and control plants inoculated with the *Vd* V991 isolate for 20 days. Sterile water treatment was used as a mock treatment. The plants were inoculated with the V991 isolate at an inoculum of 1×10^7 spores ml^{-1} using the root-dipping method.

(b) Expression of the *GauSR45a* or *GbSR45a* gene in VIGS-silenced and control plants. Cotyledons from 10-day-old cotton were used for the VIGS assay to silence *GauSR45a*, *GbSR45a*, or empty vector control (Ctrl). The results were normalized to the internal control gene *histone3* ($n = 3$).

(c) Fungal recovery experiments of stem sections in VIGS-*GauSR45a*, *-GbSR45a* gene and control plants. Stem sections were plated on potato dextrose agar (PDA) medium and incubated at 25°C. Images were taken at 3 dpc (days post culture).

(d) Vascular discoloration of VIGS-*GbSR45a* and control plants after inoculation with V991. Images were taken at 20 dpi. Scale bar, 1 mm.

(e) Quantification of *V. dahliae* biomass on roots of VIGS-*GauSR45a*, *-GbSR45a* gene and control plants at 20 dpi with V991 ($n = 3$). qRT–PCR was performed to quantify *V. dahliae* DNA using a *V. dahliae*-specific *primer pair (ITS1-F and STVe1-R).*

(f) The disease index of VIGS plants after V991 infection at 20 dpi.

(g) *GauSR45a-L2*-OE Arabidopsis enhanced resistance to *V. dahliae* infections. Four-week-old Arabidopsis seedlings were inoculated with the V991 isolate at an inoculum of 1×10^6 spores ml^{-1} using the root-dipping method. The images were taken 14 days after V991 infection.

(h) Relative expression levels of *GauSR45a* in transgenic Arabidopsis and wild-type (WT). The results were normalized to the internal control gene *Actin* ($n = 3$).

(i) Quantification of *V. dahliae* biomass on roots of transgenic Arabidopsis and WT plants at 20 dpi inoculation with V991 ($n = 3$).

(j) The disease index of transgenic Arabidopsis and WT plants after V991 infection at 20 dpi. The data are shown as the mean ± s.e. from three independent repeats. The *P* values indicate the results from pairwise comparisons of one-way ANOVA tests. ** $P < 0.01$, n.s., not significant. The above experiments were performed at least three times with similar results.

spliced regions in the pre-mRNAs of the *GauBAK1*, *GauRLK4*, *GauCERK1*, *GauWAKs14*, *GauLRR3*, and *GauPRL* genes were fused with the *LUC* gene and co-expressed with *GauSR45a* in *N. benthamiana* leaf cells. Only in the presence of *GauSR45a* could strong luminescence signals and luciferase activity be detected, indicating that *GauSR45a* enhanced the splicing of the six immune-related gene introns (Figure 4a–i). Among these genes, *GauBAK1* and *GauRLK4* exhibited much stronger splicing efficiency than other splicing constructs (Figure 4a–c). These results indicate that *GauSR45a* directly regulates the splicing of these genes and that *GauBAK1* and *GauRLK4* are preferentially targeted for splicing. To test whether GauSR45a-driven exon skipping of *BAK1* contributes to VW resistance, we overexpressed the two splicing variants of *BAK1* (*BAK1.1* and *BAK1.2*) in Arabidopsis and found that overexpression of *BAK1.1* conferred stronger VW resistance than *BAK1.2* in Arabidopsis (Figure S11a–c). Taken together, these results suggest that *GauSR45a*, as a splicing regulator, may enhance resistance to *Vd* by promoting the production of functional transcript variants of immune-related genes with high performance.

Immune-related genes containing the *GAAGA* motif were differentially regulated by AS between *G. barbadense* and *G. hirsutum* during *Vd* invasion

To explore the repertoire of AS regulation in cotton species with different resistance in response to *Vd*, we further investigated AS events in response to *Vd* invasion in the genomes of *G. barbadense* acc Hai7124 and *G. hirsutum* acc TM-1, which exhibits moderate resistance and is susceptible to *Vd*, respectively. The results showed that no differences in the number and proportion of AS events were observed between the At and Dt genomes of either TM-1 or Hai7124 during *Vd* infection (Figure 5a, b). However, the rate of SE events induced in the At genome of Hai7124 (23.65%) was higher than that of TM-1 (7.47%), while the rates of A3SS, A5SS, and MXE were

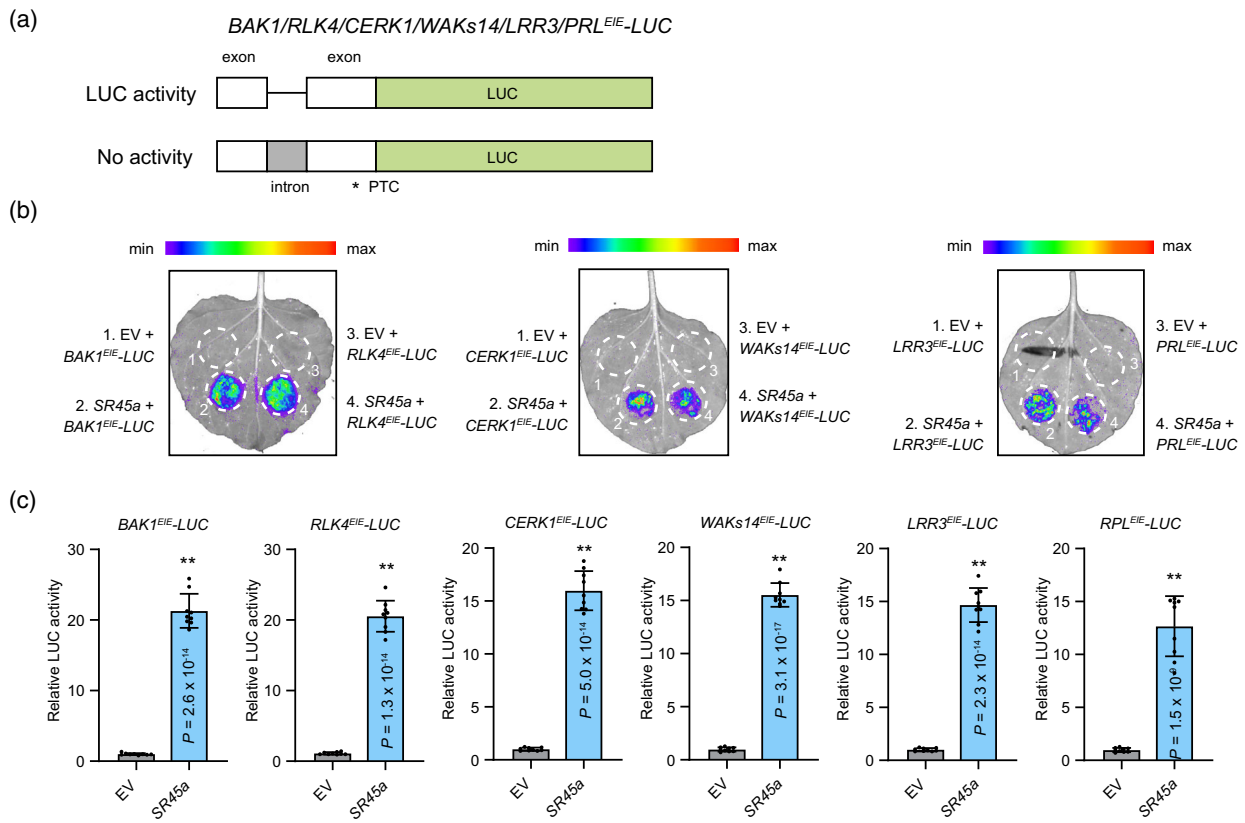

Figure 4. SR45a as a splicing regulator promotes exon skipping of cotton defense-related genes.
(a) Schematic representation of the genes (*BAK1*/*RLK4*/*CERK1*/*WAKs14*/*LRR3*/*PRL*) splicing reporter system. The alternatively spliced regions of genes were fused with the *luciferase* (LUC) gene to yield *BAK1*/*RLK4*/*CERK1*/*WAKs14*/*LRR3*/*PRL*^{EIE}-*LUC* reporter constructs. The exon skipping of reporter constructs produced a functional LUC protein, while the retained intron had a premature termination codon and did not produce LUC activity.
(b) SR45a was involved in promoting exon skipping of *BAK1*, *RLK4*, *CERK1*, *WAKs14*, *LRR3* and *PRL*. SR45a was co-infiltrated with *BAK1*/*RLK4*/*CERK1*/*WAKs14*/*LRR3*/*PRL*^{EIE}-*LUC* in four-week-old tobacco leaves for 3 days, and images were collected by a plant *in vivo* molecular imaging system.
(c) Leaf discs were collected from infiltration sites at 48 h after transfection, and LUC activity was detected (n = 9). The data are shown as the mean ± s.e. from three independent repeats. The P values indicate the results from pairwise comparisons of one-way ANOVA tests. ** $P < 0.01$. The above experiments were performed at least three times with similar results.

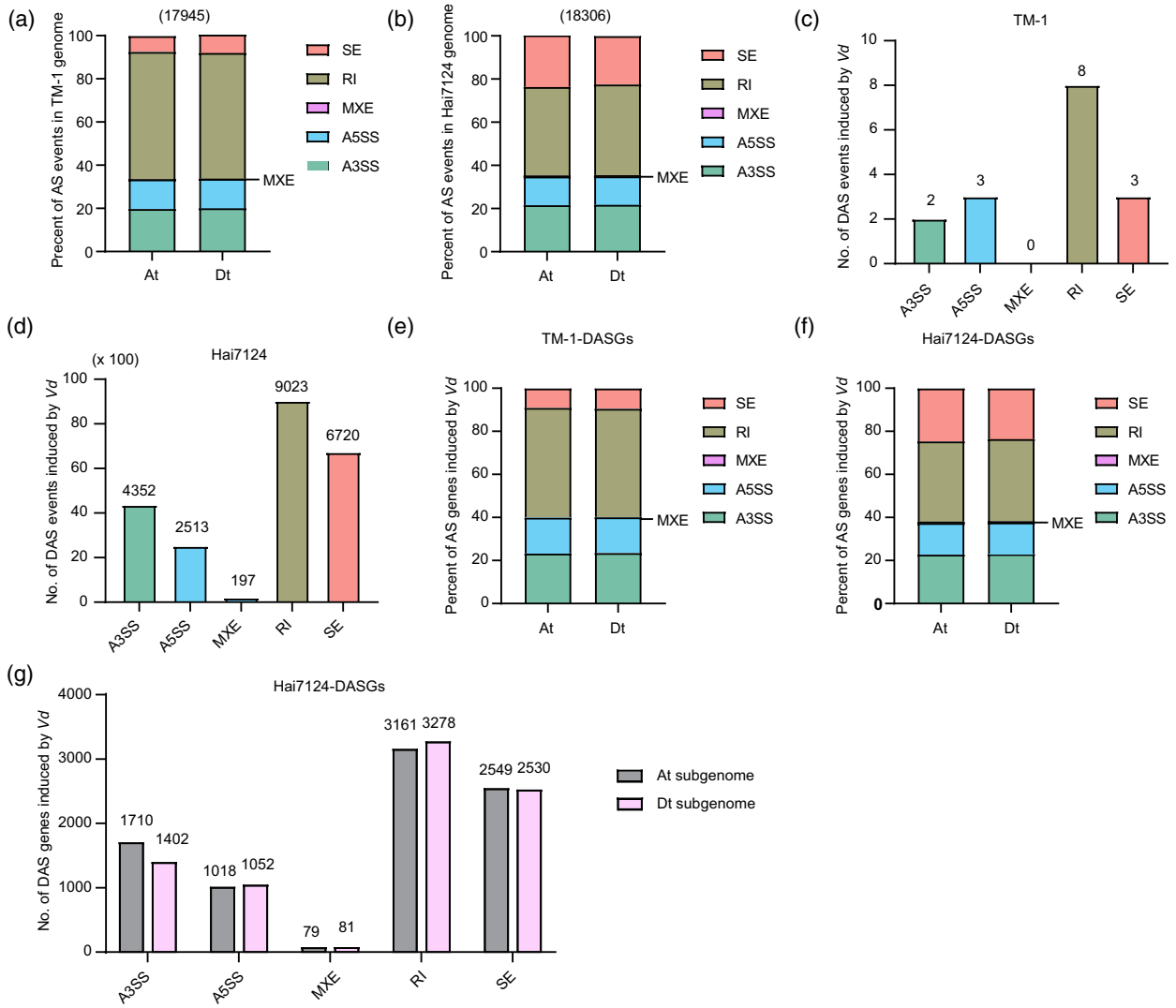

Figure 5. Differences in genomic AS in the immune response in *G. hirsutum* and *G. barbadense* cotton.
(a) AS events in the A and D subgenomes of *G. hirsutum* cv. TM-1 by *Vd* induction.
(b) AS events in the A and D subgenomes of *G. barbadense* cv. Hai7124 by *Vd* induction.
(c) The number of differentially expressed AS events in TM-1 after *Vd* induction.
(d) The number of differentially expressed AS events in Hai7124 after *Vd* induction.
(e) The proportion of differentially expressed AS genes in the A and D subgenomes of TM-1.
(f) The proportion of differentially expressed AS genes in the A and D subgenomes of Hai7124.
(g) The number of differential AS events in the A and D subgenomes of Hai7124 after *Vd* induction.

comparable (Figure 5a,b), so then, much more genes were alternatively spliced in Hai7124 than in TM-1 (Figure 5c,d), although no difference in the proportion of AS types between the At and Dt subgenomes in either TM-1 or Hai7124 (Figure 5e–g). These results indicated that AS regulation in response to *Vd* invasion was different between cotton species with different tolerance to WV without subgenome preference.

We further used public transcriptome data to compare genome-wide AS events in *G. barbadense* and *G. hirsutum*. In consistence with those from our sequencing data, the results showed that *G. barbadense* genome mobilized more extensive genome-wide AS during *Vd* invasion (Figure 6a), and concurrently, many more DASGs occurred in *G. barbadense* than in *G. hirsutum* (Figure 6a). This phenomenon could be attributed to interspecific differentiation after the formation of tetraploid species.

We carried out an unbiased search for consensus motifs in DASGs of *G. australe* during *Vd* infection by using the DREME algorithm of MEME and found that the motif 5′-*GAAGA*-3′ was the most significantly enriched (*P*-value = $4.9e^{-246}$, *e*-value = $2.4e^{-241}$) (Figure 6b), resembling the binding site preference of an SR protein. Subsequently, the *GAAGA* motif was used to search

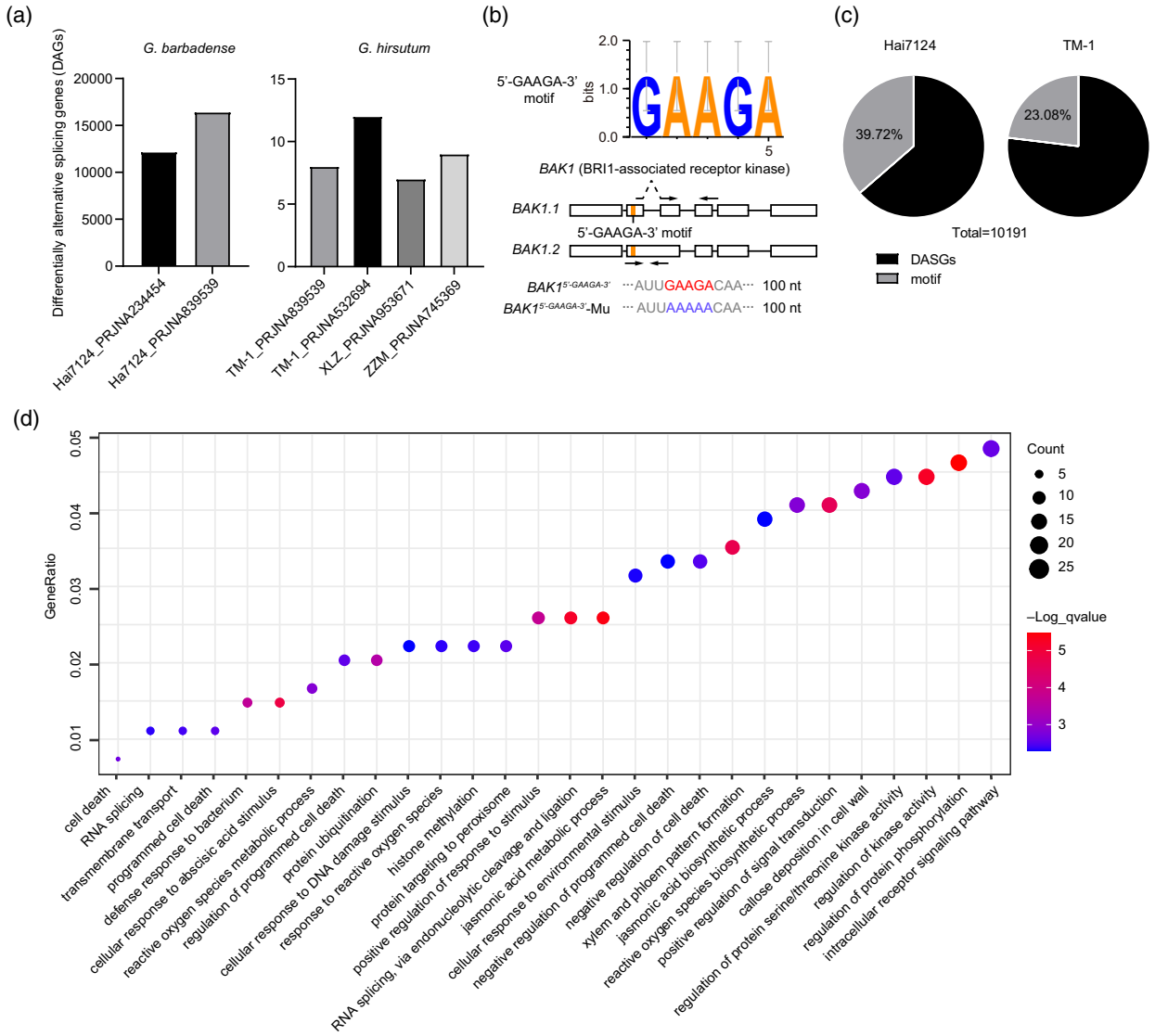

Figure 6. There is no GAAGA motif in the homologous genes of immune-associated DASGs in upland cotton.
(a) The number of *Vd*-induced DASGs between Island cotton and upland cotton in publicly available transcriptome data. For Sea Island cotton, *Vd*-inoculated transcriptome data from Hai7124 in PRJNA234454 and PRJNA839539 were analyzed. For upland cotton, *Vd*-inoculated transcriptome data from TM-1 in PRJNA839539 and PRJNA532694 were analyzed. The transcriptome data of the upland cotton cultivar Xinluzao-36 were derived from PRJNA953671, and the transcriptome data of Zhongzhimian-2 were derived from PRJNA745369.
(b) Sequence logo of the enriched motif among the differentially alternative splicing genes during *Vd* infection. Schematic diagram of *BAK1*$^{5'\text{-}GAAGA\text{-}3'}$ and *BAK1*$^{5'\text{-}GAAGA\text{-}3'}$-Mu RNA probe sequences. The arrows indicate the primer locations used to measure the splicing ratio. The consensus of the motif was analyzed by WebLogo3.
(c) Proportion of *Vd*-induced DASGs containing *GAAGA* motifs in Hai7124 and proportion of homologous genes containing *GAAGA* motifs in TM-1.
(d) GO enrichment analysis of motif-containing genes specific to Hai7124 (Hai7124 contains *GAAGA*, while the corresponding homologous gene does not contain *GAAGA* in TM-1).

against the DASGs of *G. barbadense* and *G. hirsutum* by BLASTN-short program, the results showed that 39.72% and 23.08% of DASGs in *G. barbadense* and *G. hirsutum* contained the GAAGA motif, respectively (Figure 6c). We extracted 1473 unique *GAAGA*-containing DASGs from *G. barbadense* for GO enrichment analysis and found that they were significantly enriched in plant immune-related biological processes (Figure 6d). We further investigated the sequence variation of the *GAAGA* containing genes in 130 re-sequenced *G. hirsutum* genomes and 70 re-sequenced *G. barbadense* genomes, the results showed that the *GAAGA* sequence of these genes was conserved in natural populations of both *G. barbadense* and *G. hirsutum* (Figures S12 and S13). Taken together, these results

suggest that *GAAGA*-containing genes were ubiquitous and differentially regulated by AS during *Vd* invasion both in *G. barbadense* and *G. hirsutum*.

GauSR45a associates with either GauU2AF35B or GauU1-70 K to regulate *BAK1* pre-mRNA splicing by binding to the *GAAGA* motif

To dissect the molecular mechanism of GauSR45a, we identified GauU1-70 K and GauU2AF35B as the proteins potentially interacting with GauSR45a-L1 and GauSR45a-L2 by a yeast two-hybrid (Y2H) assay (Figure S14a,b). GauSR45a-L2 showed stronger interaction with GauU1-70 K or GauU2AF35B than GauSR45a-L1, while GauSR45a-L1 displayed stronger interaction with GauU1-70 K than GauU2AF45B in yeast (Figure S14a,b). The bimolecular fluorescence complementation (BiFC) assays indicated that both GauSR45a-L1 and GauSR45a-L2 interacted with GauU1-70 K or GauU2AF35B in the nucleus of tobacco leaves (Figure S14c). Pull-down assays further confirmed these interactions *in vitro* (Figure S14d). Coimmunoprecipitation (Co-IP) assays further revealed that GauU1-70 K or GauU2AF35B protein could be precipitated by GFP-GauSR45a-L1 and GFP-GauSR45a-L2 (Figure S14e). Split-luciferase (LUC) assays also supported the interaction between GauSR45a-L2/L1 and GauU1-70 K or GauU2AF35B (Figures S14f–h and S15a). Moreover, U1-70K strongly interacted with SR45a-L1 compared with U2AF35B in Co-IP and split-LUC assays (Figures S14e,h and S15b). These results indicate that the RS2 domain is more needed for GauU2AF35B to interact with GauSR45a than for GauU1-70 K. To characterize the interactions between GauSR45a and GauU1-70 K or GauU2AF35B, we generated five GauSR45a variants by deleting one or two domains for protein interaction assays (GauSR45a$^{\Delta RS2}$: C-terminal RS2 domain deletion; GauSR45a$^{\Delta RS1}$: N-terminal RS1 domain deletion; GauSR45a^{RS1}: only RS1 domain; GauSR45a^{RRM}: only RRM domain; GauSR45a^{RS2}: only RS2 domain) (Figure S16a). Y2H analyses showed that either BD-GauU1-70 K or GauU2AF35B could only interact with GauSR45a$^{\Delta RS1}$ and GauSR45a^{RS2}, indicating that GauU1-70 K physically interacted with the RS2 domain of GauSR45a in yeast cells (Figure S16b). Pull-down and Co-IP assays showed that both GauSR45a$^{\Delta RS1}$ and GauSR45a^{RS2} interacted with GauU1-70 K and GauU2AF35B (Figure S16c–f). These results indicate that the RS2 domain is indispensable for GauSR45a to interact with GauU1-70 K and GauU2AF35B.

GauSR45a is able to interact with GauU2AF35B and GauU1-70 K to regulate the splicing of immune-related genes, which inspired us to determine whether GauSR45a directly binds to target pre-mRNAs to regulate splicing. Sequence analysis showed that the 5′-*GAAGA*-3′ motif appears in the exon immediately upstream of the alternative splicing site of *GauBAK1* pre-mRNA (Figure 6b). RNA electrophoretic mobility shift assay (RNA-EMSA) analysis showed retardation of mRNA migration for the *GauBAK1*$^{5'\text{-}GAAGA\text{-}3'}$ probe, and this binding could be inhibited by the competitive probe (Figure 7a,b). RNA pull-down assays further confirmed this binding (Figure 7c,d). Next, we tested whether GauSR45a can bind to GauBAK1 mRNA *in vivo* by using RNA chromatin immunoprecipitation in cotton protoplasts expressing *35S: GauSR45a-GFP* followed by qPCR (RIP-qPCR) assays with three pairs of primers to amplify different regions of *GauBAK1* (Figure 7e). The results showed that GauSR45a strongly bound to the P2 region containing the 5′-*GAAGA*-3′ motif in *GauBAK1* mRNA but not to the P1 or P3 region (Figure 7e). The biotin-labeled *GauBAK1* mRNA fragment containing the splicing region was immobilized on streptavidin magnetic beads and then incubated with GST-GauSR45a and GST-GauSR45a$^{\Delta RS2}$ proteins. An RNA affinity assay revealed that the *GauBAK1* mRNA-GauSR45a interaction was independent of the RS2 domain (Figure 7c, d).

Given that GauSR45a could interact with splicing factors GauU2AF35B and GauU1-70 K (Figure S14), we next investigated how GauU2AF35B and GauU1-70 K are involved in regulating the splicing of GauSR45a-bound mRNA. RNA-EMSAs revealed that GauSR45a binding with the *GauBAK1*$^{5'\text{-}GAAGA\text{-}3'}$ probe was significantly increased in the presence of either GauU2AF35B (Figure 7a) or GauU1-70 K (Figure 7b), but neither of them directly bound to the mRNA probe. RNA affinity assays confirmed that GauU2AF35B or GauU1-70 K enhanced the binding of GauSR45a to *GauBAK1* mRNA (Figure 7c,d), but they did not affect GauSR45a$^{\Delta RS2}$ binding to *GauBAK1* mRNA (Figure 7c, d), suggesting that the elevated RNA-binding affinity may depend on the protein interaction mediated by the RS2 domain. Moreover, GauU2AF35B and GauU1-70 K themselves could bind to the mRNA bait containing the splicing region. These results revealed that GauU2AF35B and GauU1-70 K were involved in GauSR45a-mediated splicing of *GauBAK1* mRNA by enhancing the binding affinity of GauSR45a to *GauBAK1* mRNA. Furthermore, we found that silencing either the *GauU2AF35B* or *GauU1-70 K* gene significantly reduced the splicing rate of *GauBAK1* (Figure 7f).

As GauSR45a promotes exon skipping of a subset of immune-related genes (Figure 4), we further investigated whether GauSR45a binds to the RNA of *GauRLK4*, *GauCERK1*, *GauWAKs14*, *GauLRR3*, and *GauPRL* using RIP-qPCR. The results showed that GauSR45a also bound to the regions containing the 5′-*GAAGA*-3′ motif in these genes (Figure S17), and consistently, the splicing ratios of these genes were significantly decreased in *GauU2AF35B*- or *GauU1-70 K*-silenced cotton during *Vd* infection (Figure S17). We further found that silencing *AtU2AF35B* or *AtU1-70 K* in transgenic Arabidopsis ectopically overexpressing *GauSR45a* led to severe VW disease symptoms with significantly increased fungal biomass and DI

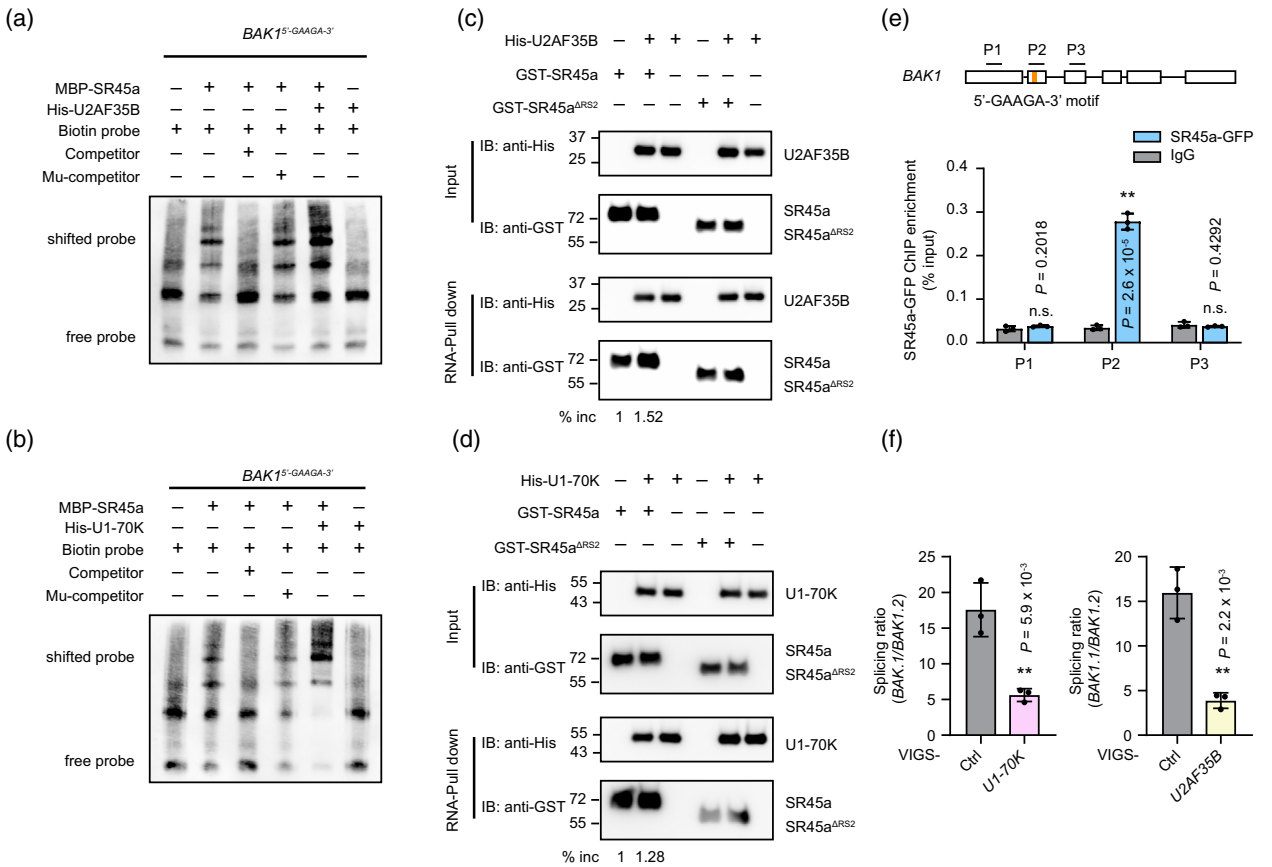

Figure 7. SR45a association with U2AF35B or U1-70K regulates *BAK1* pre-mRNA splicing by binding to its *GAAGA* motif.
(a) SR45a directly binds to the 5′-*GAAGA*-3′ motif of *BAK1* mRNA in the RNA-EMSA. MBP-SR45a and His-U2AF35B were expressed in *E. coli* and purified using amylose resin or Ni-NTA MagBeads. Biotin-labeled RNA probes were incubated alone or with recombinant purified MBP-SR45a or His-U1-70K. The 125-fold excess of the corresponding unlabeled probes was used as a competitor.
(b) RNA-EMSA confirmation of the direct binding of SR45a to the 5′-*GAAGA*-3′ motif of *BAK1* mRNA. RNA-EMSAs were performed as described in (a).
(c) RNA-SR45a-U2AF35B affinity assay. The biotin-labeled *BAK1* mRNA containing the splicing region was immobilized on streptavidin beads and incubated alone or with His-U2AF35B, GST-SR45a or GST-SR45a$^{\Delta RS2}$. The proteins were analyzed by immunoblotting with anti-GST or anti-His antibodies. The binding intensity is indicated below the band.
(d) RNA-SR45a-U1-70K affinity assay. The assay was performed as described in (c).
(e) Binding of SR45a-GFP to the 5′-*GAAGA*-3′ motif of *BAK1* mRNA was confirmed by RIP-qPCR. IgG was used as the negative control. The RNA-ChIP signals were normalized to input.
(f) The altered splicing ratio of *BAK1* was partially dependent on U1-70K or U2AF35B, which was corroborated by qRT–PCR assays in VIGS-U1-70K or -U2AF35B and control plants. The results were normalized to the internal control gene *histone3* ($n = 3$). The data are shown as the mean ± s.e. from three independent repeats. The *P* values indicate the results from pairwise comparisons of one-way ANOVA tests. ** $P < 0.01$, n.s., not significant. The above experiments were performed at least three times with similar results.

(Figure S18a–c). Moreover, *U2AF35B*-silenced plants displayed more susceptibility to *Vd* infection than *U1-70K*-silenced plants (Figure S18a).

Taken together, these data demonstrate that GauSR45a binds to the *GAAGA* motif and interacts with GauU2AF35B or GauU1-70 K to coordinately regulate pre-mRNA splicing of immune-related genes in defense against *Vd* infection.

DISCUSSION

In eukaryotic RNA processing, pre-mRNA splicing is a key step in gene expression regulation, which enhances transcriptome plasticity and proteome complexity (Chaudhary et al., 2019; Dinesh-Kumar & Baker, 2000; Reddy & Shad Ali, 2011). The precise excision of introns from the pre-mRNA is executed by the spliceosome (Wahl et al., 2009). AS regulation has emerged as an important immunity regulation layer to respond to pathogenic invasion (Huang et al., 2020). A previous study revealed that SR proteins were involved in both constitutive and alternative pre-mRNA splicing in higher eukaryotes (Zhong et al., 2009). Here, we performed a genome-wide AS analysis and revealed that AS and transcriptional regulation are synergistically involved in the plant immune defense response to *Vd*. GauSR45a is an important splicing regulator whose loss of function increases plant susceptibility to *Vd* infection. Furthermore, we found that GauSR45a

interaction with GauU2AF35B or GauU1-70 K regulates pre-mRNA splicing of immune-associated genes in the defense response to *Vd* infection by binding to the *GAAGA* motif. The GauSR45a interaction with GauU2AF35B or GauU1-70 K enhances the binding of GauSR45a to pre-mRNA targets, thereby promoting exon skipping to produce full-length functional proteins and resulting in disease resistance. In contrast, the intron retention of the pre-mRNA targets produced truncated proteins, ultimately leading to disease susceptibility (Figure 8). Thus, our study sheds light on the function of GauSR45a in AS regulation and elucidates the potential molecular mechanism of AS regulation in plant immunity.

Up to now, many VW resistance genes have been reported through transcriptome data analysis. For example, eleven possible resistance genes were identified in cotton by comparative RNA-seq analysis using infected Xinluzao-36 (susceptible) and Zhongzhimian-2 (resistant) by *Vd* (Zhang et al., 2021). In another study, adenosine triphosphate (ATP)-binding cassette (ABC) protein was identified to enhance *Vd* resistance using RNA-seq analysis (Dong et al., 2019). Whether these genes exist AS and their AS is regulated by GauSR45a remains to be further studied.

In the past decade, emerging evidence has revealed that AS reprogramming is a key regulator of plant immunity (Chaudhary et al., 2019). For example, genome-wide analysis of AS landscapes in *Brachypodium distachyon* and *Zea mays* revealed that the stress-responsive genes underwent specific alternative splicing in response to virus infection (Mandadi & Scholthof, 2015). AS reprogramming was also exhibited in response to bacterial infection and herbivorous insect infestation in plants (Howard et al., 2013; Ling et al., 2015). In this study, we integrated Illumina and SMRT sequencing data and analyzed genome-wide AS landscapes. Our results revealed that few of the DAS genes were differentially expressed during *Vd* infection, indicating that transcriptional regulation and AS were two different but complementary strategies in cotton to defend against *Vd*. Moreover, by the enhanced annotation of genes in *G. australe*, further exploration of cotton splicing events indicated that the AS of cotton pre-mRNA was prevalent in response to *Vd* infection. During *Vd* invasion, AS also occurred widely in the two cultivated tetraploid cotton species *G. hirsutum* and *G. barbadense*. Furthermore, a wide range of AS is found in *G. barbadense* compared to *G. hirsutum*.

In plant–microbe interactions, the plant defense machinery is very sophisticated and tightly linked with immune regulation (Kong et al., 2021; Wang et al., 2019; Yuan et al., 2017). Recent evidence indicates that pathogenic microbes secrete numerous effectors into plant cells to interfere with host AS regulation for successful colonization (Fu et al., 2007). For instance, several RXLR effectors of *Phytophthora infestans* were found to suppress host splicing by binding to splicing factors (Huang et al., 2020). Pst_A23, as a new *Puccinia striiformis* arginine-rich effector, reprograms the AS of *TaXa21-H* and *TaWRKY53* to suppress wheat immunity (Tang et al., 2022). We showed here that SR45a underwent a dramatic AS response to *Vd* infection in cotton. Intriguingly, the subcellular localization of the protein encoded by multiple splicing variants of

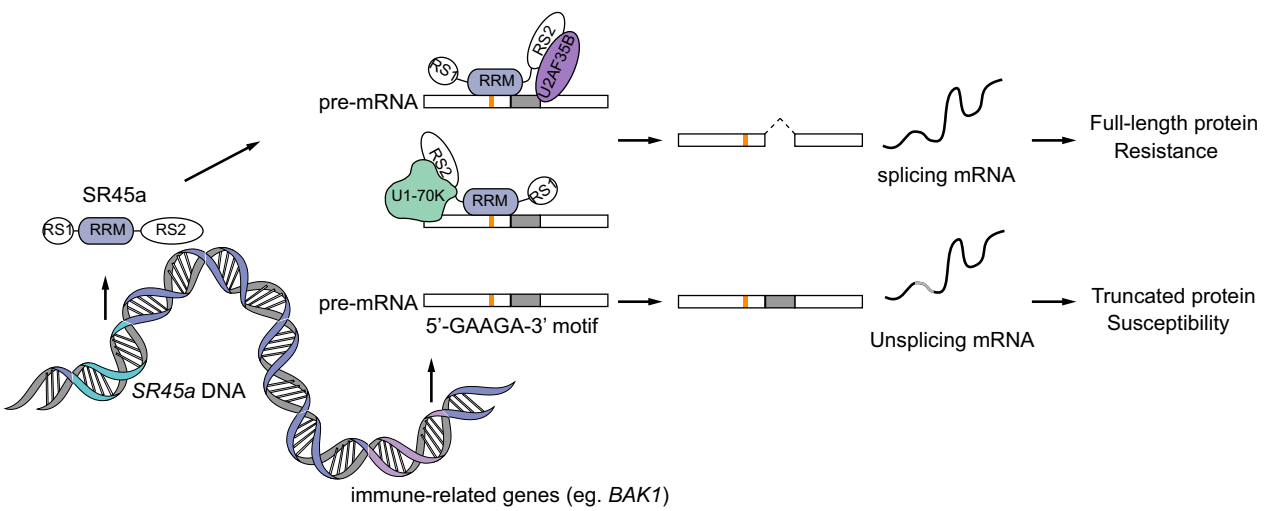

Figure 8. A proposed model for SR45a association with U2AF35B or U1-70K to regulate immune-related pre-mRNA splicing by binding to its *GAAGA* motif. To respond to *Vd* invasion, SR45a acts as a splicing regulator that binds to the 5′-*GAAGA*-3′ motif to promote immune-related gene splicing. The interaction between SR45a and U2AF35B or U1-70K enhances the binding of SR45a to pre-mRNAs of immune-related genes, thereby promoting exon skipping to produce full-length functional proteins and resulting in disease resistance. In contrast, the intron retention of immune-related genes' pre-mRNAs produces truncated proteins, ultimately leading to disease susceptibility.

GauSR45a was different, implying potential functional divergence among *GauSR45a* splicing isoforms. However, the expression analysis revealed that *GbSR45a* in *G. barbadense* and *GhSR45a* in *G. hirsutum* were transiently downregulated at 2–6 hpi, which implies that the transcript and AS regulation of *SR45a* is complex in response to *Vd* infection. We speculate that SR45a was once an attack target of pathogen effectors and that pathogen effectors might be overcome later by plants through AS regulation during evolution.

Both plants and animals deploy AS for the regulation of gene expression to increase the diversification of the transcriptome and proteome toward diseases or pathogens (Jia et al., 2020). In this study, the AS of *GauSR45a* produced two relatively long functional transcripts, *GauSR45a-L1* and *GauSR45a-L2*. Subcellular localization analysis indicated that these two transcripts were localized not only in nuclear speckles but also in the cytoplasm and PM. Subsequently, protein interaction analysis revealed that GauSR45a-L1 (with a truncated RS2 domain) had a weak association with splicing factors. Compared with *GauSR45a-L2* overexpression, *GauSR45L1* overexpression showed slight resistance in inoculation assays. In addition, the GauU1-70 K interaction with GauSR45a-L1 was stronger than that with GauU2AF35B. Together, these results indicate that the RS2 domain is more important for GauU2AF35B interaction with GauSR45a than for GauU1-70 K.

Several previous reports revealed that plant SR proteins were involved in the regulation of splicing site choice by associating with the spliceosome (Isshiki et al., 2006; Reddy, 2007). To respond to splicing signals, SR proteins generally bind to ESEs to promote exon splicing (Shen & Green, 2006; Wahl et al., 2009). Previous studies in Arabidopsis have shown that SR protein SR45 could suppress the expression of defense genes and negatively regulate innate immunity in plants (Zhang et al., 2017). However, in our study, the expression of *SR45* did not significantly change during pathogen infection. *GauSR45a* expression was upregulated by *Vd* induction. In *G. australe*, GauSR45 and GauSR45a were two completely different proteins with different functions. Moreover, we demonstrated that GauSR45a interacted with splicing regulators and contributed to the splicing regulation of a subset of immune-associated genes. A previous report indicated that U2AF35B and U1-70K were essential components of U1 snRNP and U2AF, respectively, and were involved in 5′SS or 3′SS recognition in the early steps of spliceosome assembly (Wahl et al., 2009). The splicing ratio analysis and LUC-splicing reporter system *in vivo* revealed that the splicing of *GauBAK1* and *GauRLK4* exhibited the largest changes in GauSR45a-mediated splicing regulation during *Vd* infection, implying that *GauBAK1* and *GauRLK4* are very important genes in AS regulation in response to *Vd*. Moreover, the splicing ratio of GauSR45a-mediated defense genes was lower in *GauU2AF35B*-silenced plants than in *GauU1-70 K*-silenced plants, indicating that GauU2AF35B played a crucial role in GauSR45a-mediated splicing.

In animal systems, SR proteins, as activators or repressors bind to pre-mRNAs regulating splicing (Graveley & Maniatis, 1998). SR proteins are generally characterized by conserved RRMs and C-terminal RS domains (Reddy & Shad Ali, 2011). Its RRM is responsible for RNA binding and the RS domain for protein–protein interactions, and sometimes their functions are interchanged. The deletion of the RS domain in the Arabidopsis SR protein RRC1 causes splicing defects in key genes and abnormal phytochrome B signaling, implying that the RS domain of the SR protein is crucial for splicing regulation in plants (Shikata et al., 2012). Furthermore, the phosphorylation status of the RS domain is highly regulated, which may contribute to SR activity in animals and plants (Tripathi et al., 2010). In the current study, we identified a 5′-*GAAGA*-3′ motif and confirmed that GauSR45a is directly bound to this motif in pre-mRNA targets. The binding of GauSR45a to pre-mRNA targets was independent of the RS2 domain. Both GauU2AF35B and GauU1-70 K enhanced the binding activity of GauSR45a to pre-mRNA in the RNA-EMSA and RNA affinity assays. The enhanced RNA-binding affinity of GauSR45a was dependent on the protein's interaction between splicing factors and the RS2 domain of SR45a. Furthermore, GauU2AF35B and GauU1-70 K did not bind directly to the 5′-*GAAGA*-3′ motif but to the pre-mRNA targets. Therefore, we summarize that GauU2AF35B and GauU1-70 K are functionally involved in GauSR45a-mediated splicing regulation and that the RS2 domain plays important roles in GauSR45a splicing regulation.

Understanding the molecular functions of AS regulation in plant immunity will contribute to elucidating the complete framework of plant–pathogen interactions. Our data suggest that GauSR45a association with GauU2AF35B or GauU1-70 K regulates the splicing of the key immunity gene *GauBAK1*, which directly binds the 5′-*GAAGA*-3′ motif that is deposited in pre-mRNA targets, thereby regulating the defense response against *Vd* infection.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Gossypium australe (highly resistant, G genome), *Gossypium barbadense* "Hai7124" (resistant, AD genome), and *Gossypium hirsutum* "Junmian1" (susceptible, AD genome) were grown in a growth chamber under a photoperiod of 16 h light:8 h dark with cool-white fluorescent light 120 μmol photons m^{-2} sec^{-1} at 30 ± 2°C/25 ± 2°C (day/night) conditions. Two-week-old cotton seedlings were used for VIGS assays and protoplast isolation. Plants were treated by VIGS for 2 weeks and then inoculated with

Verticillium dahliae (*Vd*) "V991" isolate. Arabidopsis ecotype Columbia-0 (Col-0) was grown in soil in a growth chamber at 21 ± 2°C with a photoperiod of 10 h light:14 h dark, and wild-type tobacco (*N. benthamiana*) was grown in a growth chamber at 23 ± 2°C with a photoperiod of 16 h light:8 h dark. *Pro35S: GauSR45a-GFP* and its splicing isoform constructs were introduced into Col-0 by the floral-dip method (Zhang et al., 2006) to generate transgenic overexpression lines.

Methods for pathogenicity assays, RNA isolation and qRT-PCR, virus-induced gene silencing, RNA-seq analysis and AS isoform identification, transient expression and LUC-splicing receptor assays, Y2H, BiFC, pull-down, Co-IP, split-luciferase, and GUS activity assays are presented in Methods S1.

RNA electrophoretic mobility shift assays (RNA-EMSAs)

RNA from the *BAK1* (BRI1-associated kinase 1) gene containing the 5′-*GAAGA*-3′ motif (~100 nt) was obtained by *in vitro* transcription using T7 RNA polymerase following the manufacturer's instructions (Takara, Dalian, China). The mutant RNA probe was synthesized (Tsingke Biotech, Nanjing, China). The biotin-labeled RNA probes (2 nM) were incubated with different recombinant purified proteins. For competition assays, 125-fold unlabeled or mutant competitor RNA probes were also added to the reactions. The sample was separated on a native polyacrylamide gel, and then RNA was transferred onto a nylon membrane (Beyotime Biotechnology, Shanghai, China, Cat No. FFN13). After UV cross-linking, the labeled RNA was detected using a chemiluminescent method.

RIP-qPCR assays

Cotton protoplasts were transfected with the indicated constructs and used for RIP-qPCR assays as previously described (Wang et al., 2015). For RIP-qPCR, the fold enrichment of tested genes was calculated using the equation $100 \times 2^{(\text{Ct of input} - \text{Ct of IP})} \times 0.1$. The primers used for RIP-qPCR are listed in Table S1.

Confocal microscopy

Confocal laser microscopy was used to observe the fluorescence of GFP and RFP (Leica TCS SP8, Germany). The GFP and RFP fluorescence was excited at 488 and 587 nm, respectively, and emissions were detected at 490–530 and 590–630 nm, respectively. The Leica Application Suite X software was used for image capture and analysis.

Statistical analysis

GraphPad Prism 8.0.1 (GraphPad Software) or Excel (Microsoft, version 16) was used for all statistical analyses. Data are given in means ± s.e., and *n* means numbers of sample sizes or biological replicates described in detail in figure legends. *P* values >0.05 were considered nonsignificant. The image was used for average optical density quantification with ImageJ. All source data for figures are listed in Table S2. The ANOVA and *t*-test results are shown in Table S3.

ACCESSION NUMBERS

The RNA-seq data from this study are openly available in the NCBI Sequence Read Archive with the BioProject IDs PRJNA850158, PRJNA532694, and PRJNA234454. Gene sequences used in this study were obtained from the Cotton Functional Genomics Database (CottonFGD) (https://cottonfgd.net/) and the Cottongen (https://cottonfgd.org/) database. The accession numbers of genes are as follows: *GauSR45a* (EPI10_029788), *GbSR45a* (GB_A13G2680), *GhSR45a* (GH_A13G2531), *GauU1-70 K* (EPI10_020129), *GauU2AF35B* (EPI10_014750), *GauBAK1* (EPI10_032265), *GauRLK4* (EPI10_019974), *GauCERK1* (EPI10_024966), *GauLRR3* (EPI10_018650), *GauWAKs14* (EPI10_024611), *GauPR-like* (EPI10_033685), *GauLRR4* (EPI10_022362), *GauBIR2* (EPI10_029109), and *GauNPR3* (EPI10_019046) and *GauOUT9* (EPI10_003414). Source data are provided within this paper. The unprocessed western blots are provided in Figures S19-S24.

ACKNOWLEDGMENTS

This study was funded by the National Natural Science Foundation of China (31771845, 31271771, and 32172083), the Key Scientific and Technological Projects of the Eighth Division of Xinjiang Production and Construction Corps (XPCC) (2019NY01), the Key Scientific and Technological Project of XPCC (2021AB010), and the Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX21_0601). This study was also supported by the high-performance computing platform of Bioinformatics Center, Nanjing Agricultural University. We also appreciate Prof. Lin Ling (Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, Nanjing, China) for providing *V. dahliae* strain V991.

AUTHOR CONTRIBUTIONS

BLZ and FJL conceived and designed the project. FJL and SC performed the experiments and analyzed the data. SC, LJD, NJA, GLF, and NSW assisted in completing part of the experiment. BLZ, KL, and WLZ supervised and revised the manuscript.

CONFLICT OF INTEREST

The authors declare no competing interests.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Sequences of primers used in this study.

Table S2. Source data.

Table S3. Summary of statistical analyses.

Table S4. Differentially expressed genes and differentially expressed transcripts during *Vd* infection.

Table S5. Expression of SE-, RI- and MXE-containing genes.

Table S6. Differentially alternatively spliced genes during *Vd* infection.

Table S7. Differentially alternatively spliced defense-related genes during *Vd* infection.

Methods S1. Supplementary details about the Materials and Methods section.

Figure S1. Chromosomal landscape of multiple splicing event-containing genes in the updated transcriptome annotation.

Figure S2. Comparison analysis of differentially expressed genes and differential transcript usage (DTU) during *Vd* infection.

Figure S3. DTU and DTE were independent layers of gene expression regulation in response to *Vd*.

Figure S4. Validation of AS events for the *GauOTU9* gene.

Figure S5. Extensive changes in cotton mRNA alternative splicing occurred in response to *Vd* infection.

Figure S6. The biological functions of *Vd*-induced DASGs and DEGs were different but complementary.

Figure S7. Validation of altered splicing ratios of cotton defense genes or susceptibility genes by *Vd* infection.

Figure S8. The response of serine/arginine-rich-related genes to *Vd* infection and the expression characteristics of SR45a.

Figure S9. Cotton *SR45a-like* genes are involved in the response to *Vd* infection.

Figure S10. Subcellular localization of SR45a and its variants.

Figure S11. Full-length BAK1 enhanced resistance to *Vd* infection.

Figure S12. Variation in the unique motif gene of Hai7124 in the natural *G. barbadense* population.

Figure S13. Variation in the unique motif homologous genes of TM-1 in the *G. hirsutum* natural population.

Figure S14. SR45a interaction with splicing factors U1-70K and U2AF35B.

Figure S15. Luciferase activities in split-luciferase and VIGS efficiencies.

Figure S16. The RS2 domain is needed for SR45a interaction with U1-70K and U2AF35B.

Figure S17. SR45a binding to the *GAAGA* motif of immune-related genes and the altered splicing ratios of these genes by *Vd* infection were partially dependent on U1-70K or U2AF35B.

Figure S18. U1-70K and U2AF35B were important for SR45a-mediated resistance to *Vd*.

Figure S19. Unprocessed gels in Figure S7.

Figure S20. Unprocessed gels in Figure S4.

Figure S21. Unprocessed western blots in Figure S9.

Figure S22. Unprocessed western blots in Figure S9.

Figure S23. Unprocessed western blots in Figure S11.

Figure S24. Unprocessed western blots in Figure 5.

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