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The island cotton NBS-LRR gene *GbaNA1* confers resistance to the non-race 1 *Verticillium dahliae* isolate Vd991

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SUMMARY

Wilt caused by *Verticillium dahliae* significantly reduces cotton yields, as host resistance in commercially cultivated *Gossypium* species is lacking. Understanding the molecular basis of disease resistance in non-commercial *Gossypium* species could galvanize the development of Verticillium wilt resistance in cultivated species. Nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins play a central role in plant defence against pathogens. In this study, we focused on the relationship between a locus enriched with eight NBS-LRR genes and Verticillium wilt resistance in *G. barbadense*. Independent virus-induced gene silencing of each of the eight NBS-LRR genes in *G. barbadense* cultivar Hai 7124 revealed that silencing of *GbaNA1* alone compromised the resistance of *G. barbadense* to *V. dahliae* isolate Vd991. In cultivar Hai 7124, *GbaNA1* could be induced by *V. dahliae* isolate Vd991 and by ethylene, jasmonic acid and salicylic acid. Nuclear protein localization of GbaNA1 was demonstrated by transient expression. Sequencing of the *GbaNA1* orthologue in nine *G. hirsutum* accessions revealed that all carried a non-functional allele, caused by a premature peptide truncation. In addition, all 10 *G. barbadense* and nine *G. hirsutum* accessions tested carried a full-length (~1140 amino acids) homologue of the *V. dahliae* race 1 resistance gene *Gbve1*, although some sequence polymorphisms were observed. *Verticillium dahliae* Vd991 is a non-race 1 isolate that lacks the *Ave1* gene. Thus, the resistance imparted by *GbaNA1* appears to be mediated by a mechanism distinct from recognition of the fungal effector Ave1.

Keywords: defence response, *Gossypium barbadense*, NBS-LRR, Verticillium wilt resistance.

INTRODUCTION

To counteract pathogen invasion, plants have evolved different types of resistance (R) proteins which activate immune responses and restrict pathogen proliferation (Chisholm *et al.*, 2006; Dodds and Rathjen, 2010; Jones and Dangl, 2006). The first layer of defence involves the recognition of conserved pathogen-associated molecular patterns (PAMPs) by membrane-resident pattern recognition receptors. The second line of defence is mediated by intracellular host R proteins that recognize specific secreted effectors, which are employed by pathogens as modifiers of host metabolism or defence mechanisms (Chisholm *et al.*, 2006; Jones and Dangl, 2006; Zipfel, 2008). Effector-triggered activation of R proteins leads to an array of protective responses, including reactive oxygen species (ROS) bursts, salicylic acid (SA) accumulation, pathogenesis-related (PR) gene induction and rapid programmed cell death (PCD), called the hypersensitive response (HR), at the infection site (Kandoth and Mitchum, 2013; Wu *et al.*, 2014), thereby preventing further ingress of the pathogen. Over the past few decades, more than 100 *R* genes that confer resistance to 122 different pathogens have been cloned and characterized from a diversity of plant species (Anderson *et al.*, 1997; Ellis *et al.*, 1999; Feuillet *et al.*, 2003; Hinsch and Staskawicz, 1996; Periyannan *et al.*, 2013; Sanseverino *et al.*, 2013; Shen *et al.*, 2007; Wang *et al.*, 2015; Whitham *et al.*, 1994; Zhu *et al.*, 2017).

R proteins can be classified into several super-families based primarily on the presence of specific conserved structural motifs. Important R protein motifs, which often occur in combination, include nucleotide-binding sites (NBSs), leucine-rich repeats (LRRs), Toll/interleukin-1 receptors (TIRs), coiled-coils (CCs) and transmembrane motifs (TMs) (Joshi and Nayak, 2011; Kruijt *et al.*, 2005; Martin *et al.*, 2003). The most common group of R proteins is NBS-LRRs (also called NB-LRRs or NB-ARC-LRRs) (Tameling and Takken, 2008). NBS-LRR proteins generally have a tripartite domain architecture that roughly corresponds to an N-terminal response domain involved in downstream signalling (CC or TIR are examples), a central molecular switch domain (NB-ARC, a nucleotide-binding adaptor shared by the mammalian apoptosis regulator Apaf1 and the *Caenorhabditis elegans* apoptosis regulator CED4) and a C-terminal sensor domain containing LRRs (van der Biezen and Jones, 1998; Collier and Moffett, 2009; Maekawa *et al.*, 2011; Meyers *et al.*, 2003; Qi and Innes, 2013). The ARC domain can be further classified into two structurally and functionally distinct units, ARC1 and ARC2 (Albrecht and Takken, 2006;

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Rairdan and Moffett, 2006), which combine with the NB domain to form a functional nucleotide-binding pocket (Tameling *et al.*, 2002).

In the absence of a pathogen elicitor, NB-LRR proteins exist in an auto-inhibited state. The N-terminal, NB-ARC and LRR domains all seem to be critical for the control of the transition between auto-inhibited and activated forms in the presence of an elicitor. Specifically, the NB-ARC domain of NB-LRRs appears to function as a molecular switch, wherein the adenosine diphosphate (ADP) bound state represents the 'off' state and the adenosine triphosphate (ATP) bound state represents the 'on' state (Collier and Moffett, 2009; Lukasik and Takken, 2009; Moffett *et al.*, 2002; Slootweg *et al.*, 2013; Takken *et al.*, 2006). The conformational change coincides with the exchange of bound ADP for ATP in the NB-ARC, stabilizing the active conformation, and initiating the subsequent activation of immune signalling pathways (Collier and Moffett, 2009; Eitas and Dangl, 2010; Lukasik and Takken, 2009).

Verticillium wilt caused by *Verticillium dahliae* is the most destructive disease of cotton. It affects more than 50% of cotton acreage and significantly reduces yield and fibre quality (National Cotton Council of America-Disease Database, 2009 and 2010). Improvement of host resistance is considered to be the most optimal method to manage Verticillium wilt. At present, many Verticillium wilt resistance quantitative trait loci (QTLs) have been reported in cotton (Zhao *et al.*, 2014), and several genes have been characterized that contribute to defence responses against Verticillium wilt, including *GbCAD1* and *GbSSI2* (Gao W *et al.*, 2013), *GbRLK* (Zhao *et al.*, 2013), *GbSTK* (Zhang *et al.*, 2013), *GbTLP1* (Munis *et al.*, 2010), *GbSBT1* (Duan *et al.*, 2016), *GhPAO* (Mo *et al.*, 2015), *GbNRX1* (Li *et al.*, 2016), *GbRVd* (Yang *et al.*, 2016) and *GbVe/GbVe1/Gbvdr5* (Yang *et al.*, 2015; Zhang *et al.*, 2011, 2012). The receptor-like protein encoded by *GbVe1* is homologous to the well-characterized major resistance genes (Verticillium resistance gene 1) first described in tomato. By definition, *Ve* gene homologues confer resistance to *V. dahliae* race 1 isolates, which are defined by the presence of a gene encoding the secreted effector Ave1 (de Jonge *et al.*, 2012). *Ve* gene-mediated signalling in tomato requires both EDS1 (enhanced disease susceptibility 1) and NDR1 (non-race-specific disease resistance 1) (Fradin *et al.*, 2009). Homologues of *Ve* genes are known to occur widely in plants (Song *et al.*, 2017), and race 1 and non-race 1 isolates are distributed worldwide (Short *et al.*, 2014). Mounting evidence, however, points to the existence of more than two races in *V. dahliae* (Usami *et al.*, 2016). Currently, little is known about the molecular basis of resistance to non-race 1 *V. dahliae*.

Over 50 species of *Gossypium* exist (Li *et al.*, 2014) and, although *G. hirsutum* accounts for over 90% of cultivated cotton worldwide, it lacks genetic resistance to Verticillium wilt. *Gossypium arboreum* and *G. raimondii* are the putative diploid donors of chromosome groups to the tetraploid cotton species, including *G. hirsutum* and *G. barbadense*, which are highly resistant to Verticillium wilt. Comparative genomics between *G. arboreum* and *G. raimondii* has revealed the key role of a specific NBS gene family in conferring Verticillium wilt resistance (Li *et al.*, 2014). In the genome of *G. barbadense*, 26 *V. dahliae* resistance loci (VdRLs) have been shown to be involved in Verticillium wilt resistance and several encode NB-ARC domain-containing proteins (Chen *et al.*, 2015). One particular locus in the *G. raimondii* genome, VdRL08, is enriched with eight NBS-LRR protein-coding genes and is significantly associated with Verticillium wilt resistance (Chen *et al.*, 2015). As *G. raimondii* is a putative diploid donor to *G. barbadense*, this locus could be an important source of genetic resistance to Verticillium wilt.

The main objectives of the current study were: (i) to investigate which NB-LRR protein-coding genes in locus VdRL08 are involved in Verticillium wilt resistance in *G. barbadense*; (ii) to explore the relationship between plant hormones and defence responses mediated by a functional NB-LRR gene; (iii) to investigate the allelic divergence of an NB-LRR gene between resistant germplasm accessions of *G. barbadense* and susceptible accessions of *G. hirsutum*; and (iv) to investigate the presence and allelic divergence of the *V. dahliae* race 1 resistance gene *Gbve1* homologues in *G. barbadense* and *G. hirsutum* cultivars.

RESULTS

Identification of the Verticillium wilt resistance locus VdRL08 in *G. barbadense*

The potential Verticillium wilt resistance locus identified in *G. raimondii*, VdRL08, spans ~263 kb (chr7: 54093871–54356747) according to the *G. raimondii* genome sequence (Chen *et al.*, 2015; Paterson *et al.*, 2012). An analysis of the predicted gene content revealed that VdRL08 contained 21 resistance gene analogues putatively involved in disease resistance (Fig. 1). Eight genes (*GraNA1–GraNA8*) were predicted to encode NB-ARC domain-containing proteins, as well as two genes that encoded CC-NBS-LRR domain-containing proteins (NBS-LRR proteins that specifically contain a CC domain), two receptor-like proteins and one EF-Tu receptor (Fig. 1). To identify which of the genes in locus VdRL08 were associated with the Verticillium wilt resistance phenotype, gene expression patterns of VdRL08 genes were investigated in two cotton species 48 h post-inoculation (hpi) with the highly virulent non-race 1 *V. dahliae* strain Vd991. Gene expression data were filtered at $P < 0.001$, false discovery rate (FDR) < 0.001 and $|\log_2\text{Ratio}| \geq 2.0$. In the resistant *G. barbadense* cv. Hai 7124, nine genes were differentially expressed (seven up-regulated) post-inoculation with *V. dahliae* relative to uninoculated plants: five NB-ARC domain-containing genes (*GraNA1*, *GraNA4*, *GraNA5*, *GraNA6* and *GraNA7*), three auxin-responsive GH3 family genes (*GraAGH3.1–GraAGH3.3*) and the

Gene-ID	Alias	RGA	Domains (Interpro)	Gb-R	Gh-S
Gorai.007G323100.1	*GraNA1*		NB-ARC domain-containing	2.39	
Gorai.007G323200.1	*GraMFS1*		Major facilitator superfamily	-3.39	-1.44
Gorai.007G323300.1	*GraNA2*		NB-ARC domain-containing		
Gorai.007G323400.1	*GraAGH3.1*		Auxin-responsive GH3 family	2.04	1.47
Gorai.007G323500.1	*GraAGH3.2*		Auxin-responsive GH3 family	2.41	
Gorai.007G323600.1	*GraAGH3.3*		Auxin-responsive GH3 family	2.48	
Gorai.007G323700.1	*GraDRP1*		CC-NBS-LRR class		
Gorai.007G323800.1	*GraPETB*		Photosynthetic electron transfer B		
Gorai.007G323900.1	*GraNATR1*		NAC domain transcriptional regulator		
Gorai.007G324000.1	*GraHTP1*		Hypothetical protein		
Gorai.007G324100.1	*GraDRP2*		CC-NBS-LRR class		
Gorai.007G324200.1	*GraNA3*		NB-ARC domain-containing		
Gorai.007G324300.1	*GraNA4*		NB-ARC domain-containing	3.05	
Gorai.007G324400.1	*GraNA5*		NB-ARC domain-containing	1.13	
Gorai.007G324500.1	*GraRLP33.1*		Receptor like protein 33		
Gorai.007G324600.1	*GraNA6*		NB-ARC domain-containing	1.11	
Gorai.007G324700.1	*GraNA7*		NB-ARC domain-containing	-1.23	
Gorai.007G324800.1	*GraRLP33.2*		Receptor like protein 33		
Gorai.007G324900.1	*GraEF-Tu*		EF-Tu receptor		
Gorai.007G325000.1	*GraPase5.2*		Protein phosphatase 5.2		
Gorai.007G325100.1	*GraNA8*		NB-ARC domain-containing		

Fig. 1 Expression of 21 genes in the Verticillium wilt resistance locus VdRL08 in *Gossypium barbadense* and *G. hirsutum* after inoculation with *Verticillium dahliae* Vd991. The Verticillium wilt resistance locus VdRL08 has been identified previously (Chen *et al.*, 2015). 'Gene-ID' and 'Alias' refer to genes from the reference genome of *G. raimondii*. Red boxes indicate genes predicted to be resistance gene analogues (RGAs). Protein domains were annotated with the InterProScan database (Version 5.21). Values under Gb-R and Gh-S are transcript data from the resistant cultivar *G. barbadense* Hai 7124 and susceptible cultivar *G. hirsutum* Junmian No. 1, respectively, 48 h after inoculation with *V. dahliae* strain Vd991. Values represent averages from three biological replicates. The $\log_2$ fold change in gene expression relative to uninoculated controls is shown. Green shading indicates down-regulation and red shading indicates up-regulation. ARC, nucleotide-binding adaptor shared by the mammalian apoptosis regulator Apaf1 and the *Caenorhabditis elegans* apoptosis regulator CED4; CC, coiled-coil; LRR, leucine-rich repeat; NBS, nucleotide-binding site.

major facilitator superfamily gene *GraMFS1* (Fig. 1). However, in the susceptible *G. hirsutum* cv. Junmian No. 1, only two genes were differentially expressed post-inoculation: *GraMFS1* and *GraAGH3.1* displayed down-regulation ($\log_2$Ratio $= -1.14$) and up-regulation ($\log_2$Ratio $= 1.47$), respectively (Fig. 1). Thus, several genes in locus VdRL08 were identified that were differentially expressed exclusively in the resistant species when challenged with *V. dahliae* Vd991.

Silencing of *GbaNA1* impairs cotton resistance to *V. dahliae* Vd991

Polymerase chain reaction (PCR) primers based on the VdRL08 locus in the *G. raimondii* reference gene sequence were able to amplify fragments of 16 of 21 putative orthologues using genomic DNA of *G. barbadense* (Fig. S1, see Supporting Information). Sequenced PCR products shared a high identity to the gene sequences of *G. raimondii* (data not shown). To understand the function of the 16 candidate Verticillium wilt resistance genes in locus VdRL08, *Tobacco rattle virus* (TRV)-based virus-induced gene silencing (VIGS) was performed using the resistant *G. barbadense* cv. Hai 7124. For the VIGS assays, ~500-bp fragments of all 16 amplified genes were integrated separately into the vector pTRV2 to generate gene-deficient cotton lines. The gene-silenced efficiency was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) compared with the control after *CLA1* (cloroplastos alterados 1, essential for chloroplast development in plants)-deficient cotton lines presented a photobleaching phenotype on newly emerged leaves. qRT-PCR analyses indicated that the gene silencing efficiency of five selected NB-ARC domain-encoding genes was greater than 70% (Fig. S2, see Supporting Information). Among the seven NB-ARC-encoding genes in locus VdRL08 in *G. barbadense*, only the silencing of *GbaNA1* significantly impaired cotton Verticillium wilt

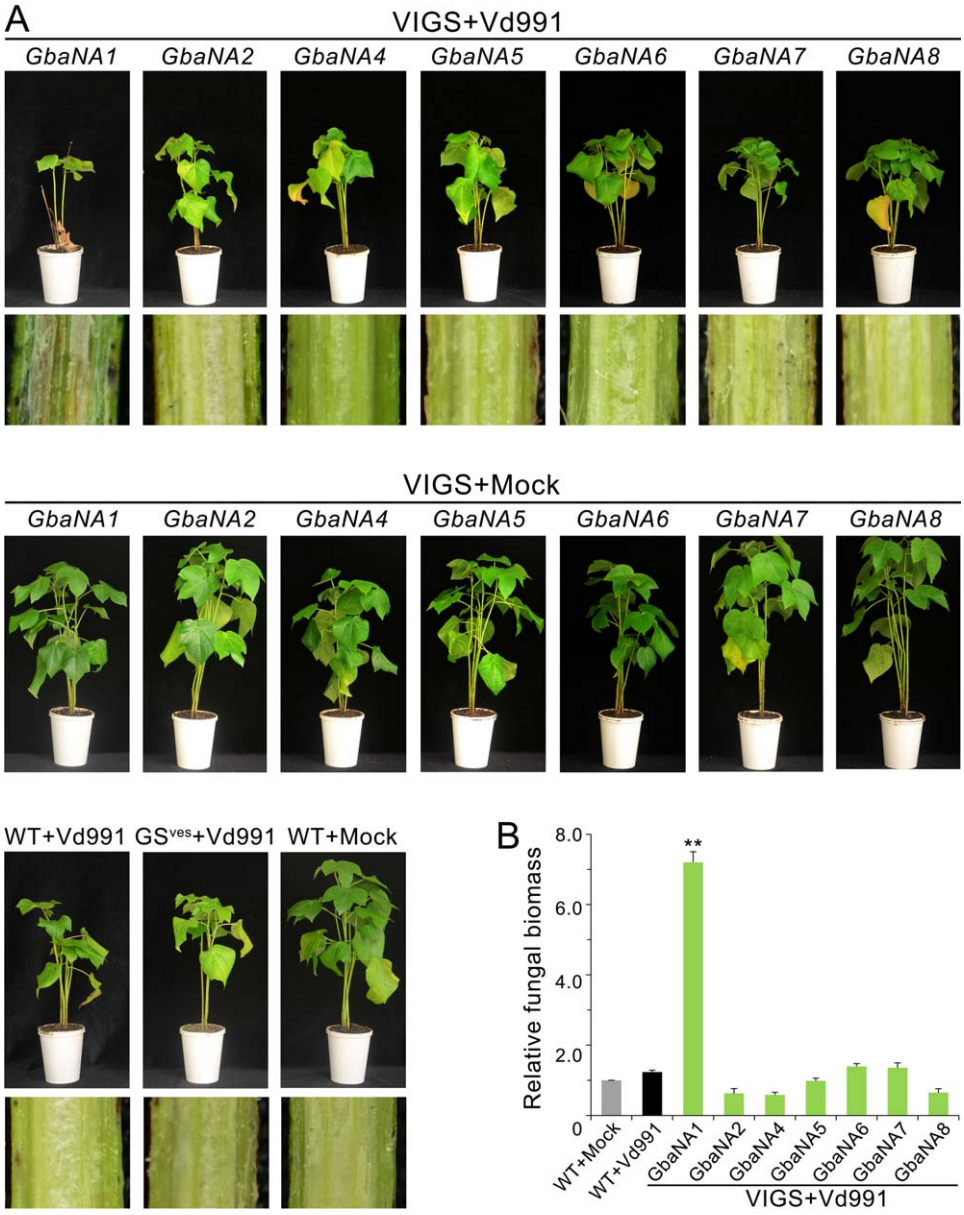


Fig. 2 Silencing of *GbaNA1* by virus-induced gene silencing (VIGS) compromises resistance to *Verticillium dahliae*. (A) Determination of Verticillium wilt resistance in NB-ARC gene-silenced plants. Approximately 14 days after the VIGS procedure in 3-week-old resistant *Gossypium barbadense* cv. Hai 7124, the gene-silenced and wild-type (WT) plants were inoculated with 5 mL of *V. dahliae* strain Vd991 conidial suspension (5×10^6 conidia/mL) or sterile water (mock) using a root-dip method. Experiments consisted of three replicates of 12 plants each arranged in a complete random block design. The Verticillium wilt phenotypes of wilting leaves and vascular discoloration were photographed 4 weeks after inoculation. Infiltration with the empty vector pTRV2 (GS^{vec}) served as a positive control. (B) Real-time polymerase chain reaction (PCR) quantification of fungal biomass in the gene-silenced cotton plants. Relative fungal biomass *in planta* was determined 4 weeks after inoculation from shoot RNA extractions using quantitative PCR. Bars represent *V. dahliae* elongation factor 1α (*EF-1α*) transcript levels relative to cotton *18S* gene transcript levels. Error bars represent standard errors. **Statistical significance ($P < 0.01$) according to unpaired Student's *t*-tests.

resistance in cv. Hai 7124; *GbaNA1*-silenced Hai 7124 plants displayed severe symptoms of wilting leaves and stunting (Fig. 2A). Nine additional genes in the *G. barbadense* VdRL08 locus were silenced independently in cv. Hai 7124, and plants were inoculated with Vd991. Resistance phenotypes were unchanged in eight of the nine gene-silenced lines, but *GbaAGH3.3*-deficient cotton lines displayed slightly more severe symptoms (Fig. S3A, see Supporting Information). Real-time quantitative polymerase chain reaction (qPCR) quantification of fungal biomass in cotton plants demonstrated that *GbaNA1*- and *GbaAGH3.1*-deficient cotton lines developed significantly more fungal biomass than the wild-type when inoculated with *V. dahliae* strain Vd991. However, the fungal biomass of the *GbaNA1*-deficient cotton line was more than twice as great as the fungal biomass in *GbaAGH3.1*-deficient cotton (Figs 2B, S3B). Therefore, *GbaNA1* was identified as a major functional gene in the VdRL08 locus involved in the Verticillium wilt resistance of *G. barbadense* cv. Hai 7124.

GbaNA1 encodes a typical NB-LRR protein

The genomic and full-length cDNA sequences of *GbaNA1* were cloned from the resistant *G. barbadense* cv. Hai 7124 (Fig. S4, see Supporting Information). A comparison of the genomic and cDNA sequences showed that *GbaNA1* contained a single open reading frame (ORF) of 2856 bp (GenBank Accession No.: MF078620), and encoded a protein of 951 amino acid residues with a

molecular weight of 109 kDa and a theoretical iso-electric point (pI) of 6.91. Analysis of the protein sequence structure by InterProScan showed that GbaNA1 was a typical NB-ARC domain-containing protein that contained an NB-ARC domain (155–473 amino acids) and LRR domain (496–889 amino acids) (Fig. 3A). Sequence alignment with known NB-ARC and LRR domain-containing proteins showed that several important motifs were present in the NB-ARC domain, including hhGRExE, P-loop, RNBS-A, Kinase 2, RNBS-B, RNBS-C, GLPL, RNBS-D and MHD, and that the LRR domain contained 13 imperfect LRRs (Fig. 3B).

Expression induction and subcellular localization of *GbaNA1*

To test whether *GbaNA1* expression changes in response to pathogen inoculation, we evaluated the expression of *GbaNA1* in the resistant *G. barbadense* cv. Hai 7124 after inoculation with the highly virulent *V. dahliae* strain Vd991 using qRT-PCR, relative to uninoculated controls. The relative expression of *GbaNA1* displayed two up-regulation peaks of 2.48 ± 0.22 and 3.08 ± 0.22 relative fold change at 12 and 48 hpi, respectively; but did not change earlier at 2 and 6 hpi (Fig. 4A). The expression pattern of *GbaNA1* was also affected after application of the hormones ethylene (ET) and methyl jasmonate (MeJA). On treatment with ethephon (ETH), *GbaNA1* expression was continuously significantly up-regulated until 72 hpi (Fig. 4B). In contrast, after treatment with MeJA, the expression of *GbaNA1* peaked at 2 hpi and then declined to a level similar to that of mock-inoculated plants (Fig. 4C). Compared with the treatments of ET and MeJA, the expression level of *GbaNA1* was less affected by treatment with SA (Fig. 4D).

GbaNA1 lacks an obvious signal peptide or any transmembrane structure, and typical subcellular localizations of GbaNA1 were not clearly predicted by WoLF PSORT (*kNN* value: nuclear, 3; cytosol, 3; plasma membrane, 3; vacuolar membrane, 2; endoplasmic reticulum, 2). To investigate the subcellular location of GbaNA1, localization of a GbaNA1-GFP (green fluorescent protein) fusion protein was assessed by *Cauliflower mosaic virus* (CaMV) 35S (35S) transient expression in onion epidermal cells. The fusion protein P35S:GbaNA1 was clearly localized in cell nuclei, but the control P35S:GFP protein encoded by the empty vector was observed throughout the onion epidermal cells (Fig. 4E), indicating that GbaNA1 was localized in the nucleus.

Sequence divergence and truncation of the GbaNA1 homologue in *G. hirsutum* results in a loss of resistance gene function

To study the features and evolution of *GbaNA1* in resistant and susceptible species, the *GbaNA1* homologues were sequenced in nine germplasm accessions each of *G. barbadense* and *G. hirsutum* (Table S2, see Supporting Information). Four weeks after inoculation with *V. dahliae* Vd991, relative fungal biomass *in planta* was compared; fungal biomass in the resistant *G. barbadense* cv. Hai 7124 was set as 1.0. As expected, accessions of *G. hirsutum* were susceptible to *V. dahliae* infection. In *G. barbadense*, fungal biomass ranged from 0.23 ± 0.09 to 1.69 ± 0.34; in *G. hirsutum*, fungal biomass ranged from 2.33 ± 0.45 to 19.66 ± 1.22 (unitless values) (Fig. 5A).

ORFs of *GbaNA1* homologues were PCR amplified with *GbaNA1*-specific primers from all 18 cotton accessions and sequenced. Sequenced amplicons were either 2856 or 2857 bp (GenBank Accession No.: MF078621) in all of the *G. barbadense* and *G. hirsutum* accessions, respectively (Table S2). Alignment of the ORF sequences showed that the *GbaNA1* homologues were highly conserved amongst the 18 cotton accessions. Four single nucleotide polymorphisms (SNPs) and a single 1-bp deletion–insertion (indel) were observed between *G. barbadense* and *G. hirsutum* accessions (Fig. S5, see Supporting Information). Interestingly, all of the ORFs of *GbaNA1* homologues from the *G. hirsutum* accessions contained a premature termination (stop codon at position 757–759 bp, TGA) as a result of the 1-bp insertion in position 713 bp (Fig. 5B). Thus, the premature termination of the protein encoded by *GbaNA1* homologues in the *G. hirsutum* accessions resulted in a truncated protein of 251 amino acids that lacked most conserved motifs in the NB-ARC domain (Fig. 5C). These results indicated extreme allelic divergence in *GbaNA1* homologues of the Verticillium wilt-susceptible *G. hirsutum* compared with the resistant *G. barbadense*.

Cloning of the *GbaNA1* homologue (*GhNA1*) from *G. hirsutum* accessions by RT-PCR confirmed that the coding sequence consisted of 756 bp and encoded 251 amino acids (Fig. 5D). Gene expression analysis showed that *GhNA1* was not responsive to *V. dahliae* Vd991 infection (Fig. 5E).

Gossypium barbadense and *G. hirsutum* accessions carry homologues of the *V. dahliae* race 1 resistance gene *GbVe1*

PCR primers designed from a gene sequence in the *G. arboretum* reference genome with >99% identity to *GbVe1* (Zhang *et al.*, 2012) successfully amplified genomic DNA from 10 *G. barbadense* and nine *G. hirsutum* germplasm accessions (Table S1, see Supporting Information). The sequenced amplicons were 3423 bp (GenBank Accession No.: MF078623) in length for all *G. barbadense* accessions and 3417 bp (GenBank Accession No.: MF078622) in length for all *G. hirsutum* accessions, and each species contained a single conserved allelic sequence. Several sequence polymorphisms were observed in both the nucleotide and peptide sequences between *G. barbadense* and *G. hirsutum*, and the reference sequence of *G. arboreum* (Fig. 6). Because the genome of *V. dahliae* strain Vd991 lacks the corresponding avirulence gene (*Ave1*) for *GbVe1* homologues (genome sequence Chen *et al.*, 2017; The Whole Genome Shotgun project has been deposited at

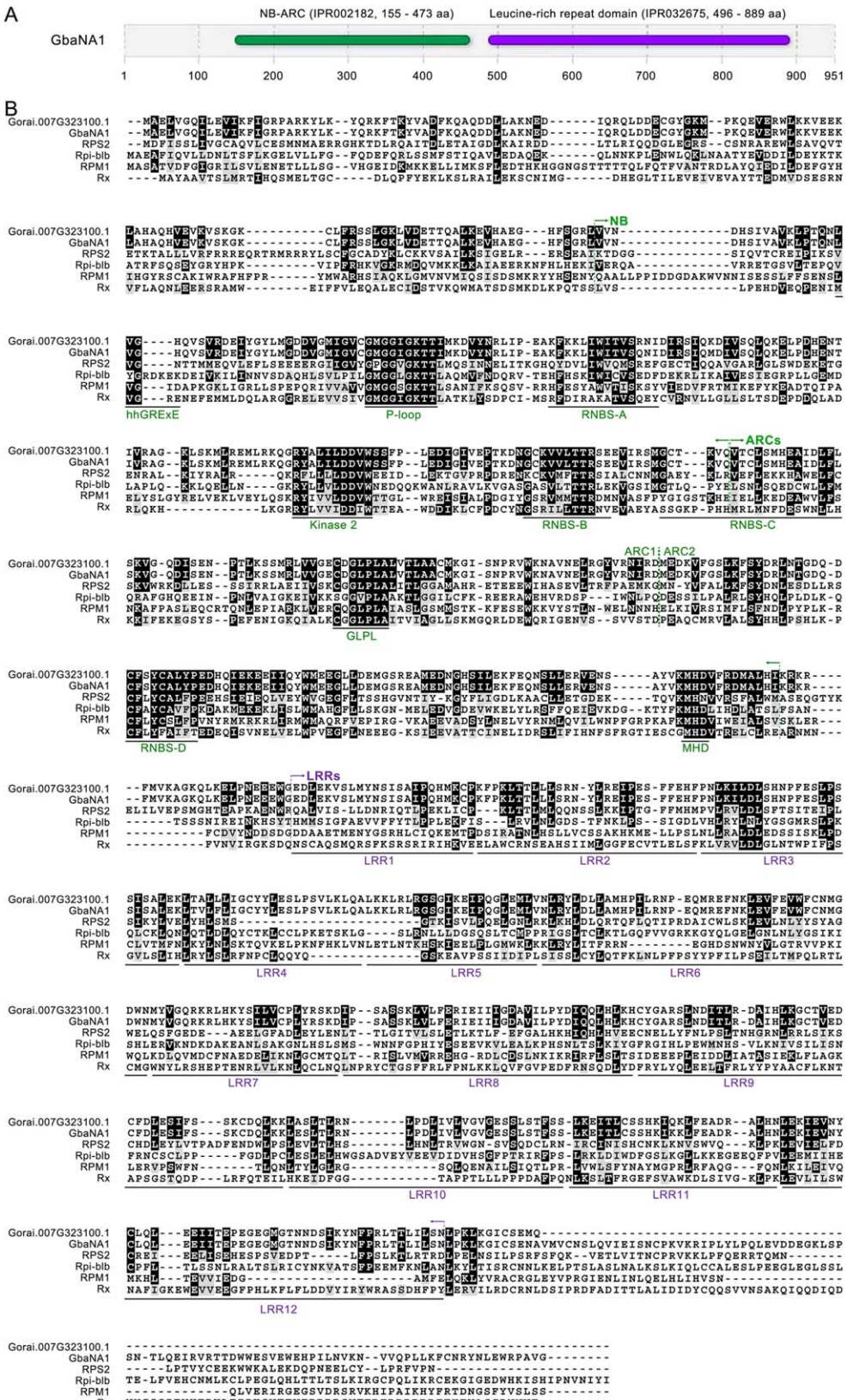


Fig. 3 Peptide domain prediction in GbaNA1.
(A) NB-ARC and LRR domains of GbaNA1 were predicted by InterProScan.
(B) Structure-based multiple sequence alignment of the NB, ARC1 and ARC2 subdomains of GbaNA1 to known NB-ARC proteins. The secondary structure assignments of the known NB-ARC proteins are depicted at the bottom of the alignment. The NB-ARC and LRR domain borders are indicated as vertical green and purple lines, respectively. The motifs in NB-ARC and LRRs are annotated as horizontal green and purple lines, respectively. Conserved residues are marked by asterisks. Gorai.007G323100.1, gene in the *Gossypium raimondii* genome (Paterson *et al.*, 2012); the known NB-ARC proteins include wild potato Rpi-blb (Q7XBQ9), potato Rx (Q9XGF5), mouse-ear cress RPM1 (Q39214) and RPS2 (Q42484).

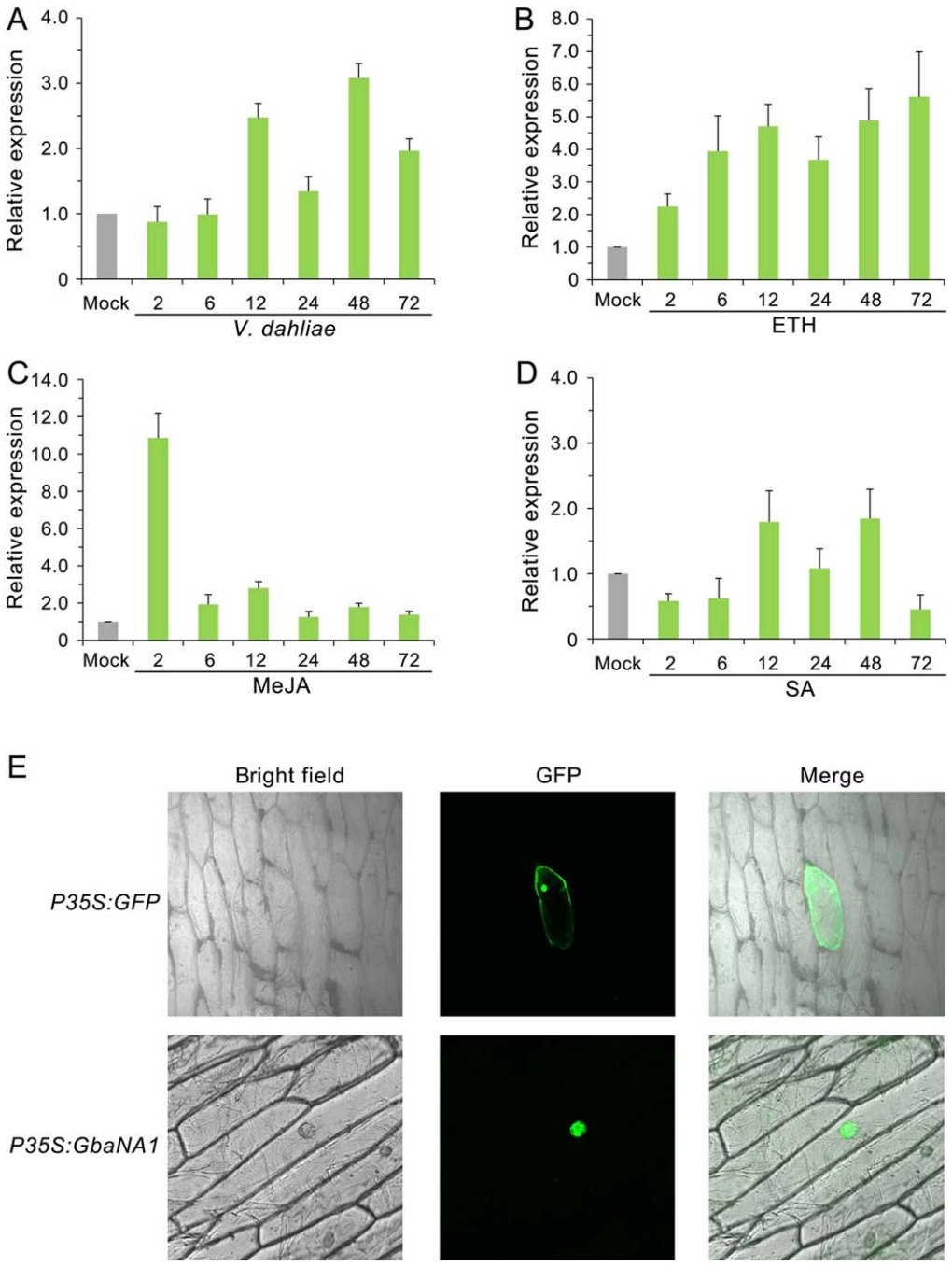
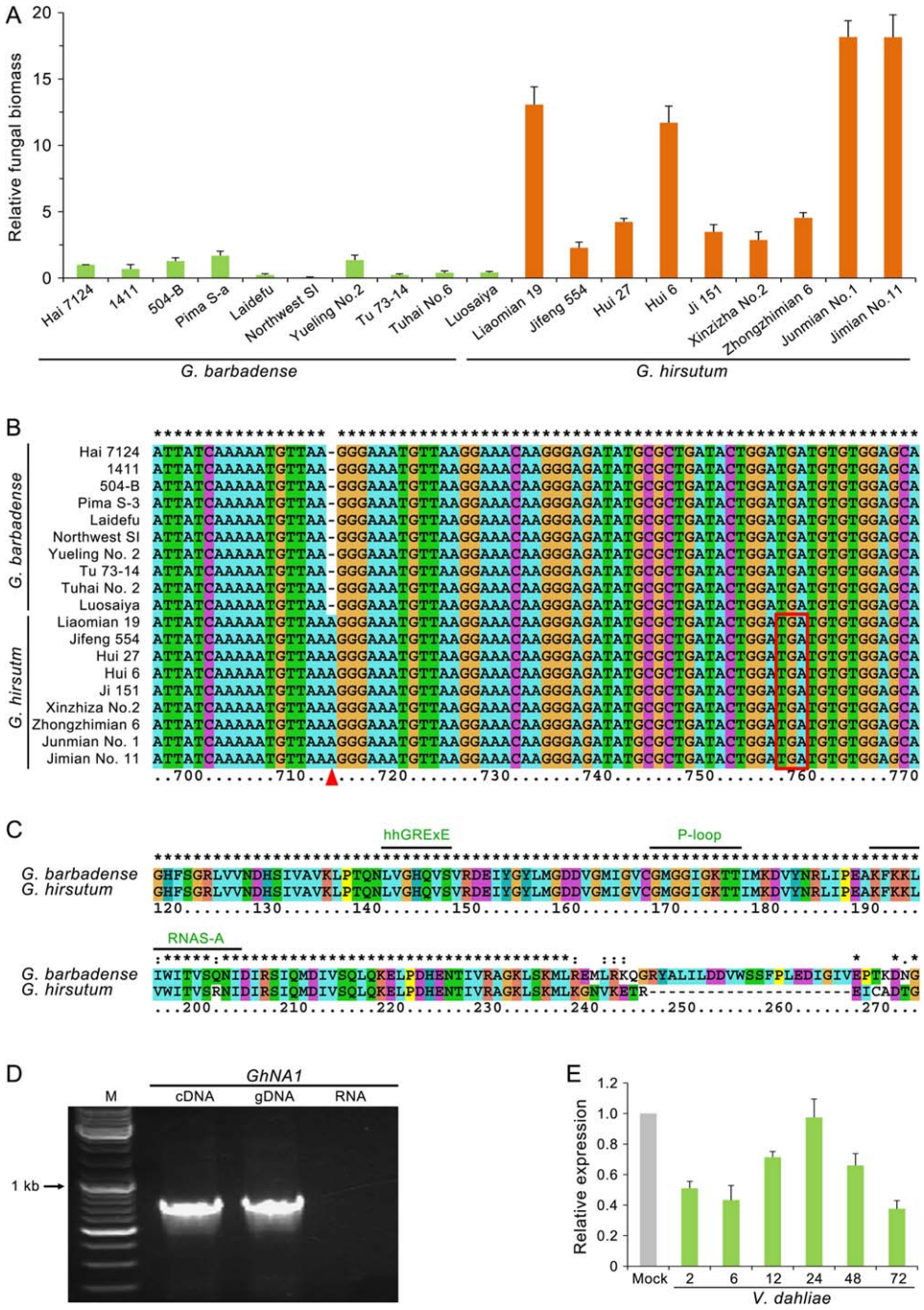


Fig. 4 Gene expression analysis of *GbaNA1* and its subcellular localization. (A–D) Expression analysis of *GbaNA1* in cotton over time after inoculation with *Verticillium dahliae* Vd991 or treatment with salicylic acid (SA), methyl jasmonate (MeJA) and ethephon (ETH). After RNA isolation and cDNA synthesis, relative expression analyses of *GbaNA1* were performed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), using the cotton *18S* gene as a reference, and compared with the expression of cotton plants treated with sterile water (Mock). Relative gene quantifications were calculated using the comparative threshold $2^{-\Delta\Delta CT}$ method, and values represent the averages of three independent biological replicates of three plants each. Error bars represent standard errors. For the expression analysis of *GbaNA1* in cotton after inoculation with *V. dahliae*, plants of 3-week-old cotton (cv. Hai 7124) were root-dip inoculated with 5 mL of *V. dahliae* strain Vd991 conidial suspension at 5×10^6 conidia/mL (A), 10 mM ETH (B), 10 mM MeJA (C) and 10 mM SA (D), and harvested at 2, 6, 12, 24, 48 and 72 h. (E) For the subcellular localization of GbaNA1, a *35S::GbaNA1:GFP* gene construct (P35S:GbaNA1) and an empty vector *35S::GFP* (P35S:GFP) were introduced into onion epidermal cells using microprojectile bombardment technology, and were viewed using laser scanning confocal microscopy with ×200 magnification, excitation at 488 nm and emission at 510 nm. GFP, green fluorescent protein.



DDBJ/ENA/GenBank under the accession NVYA00000000), *GbaNA1*-mediated resistance appears to involve a mechanism distinct from direct recognition of the fungal effector Ave1.

DISCUSSION

Cotton is one of the most economically important crops, but most cultivated varieties lack adequate innate immunity or resistance to Verticillium wilt. Over the past few decades, several genes involved in Verticillium wilt resistance have been identified in cotton (Duan *et al.*, 2016; Gao W *et al.*, 2013; Li *et al.*, 2016; Mo *et al.*, 2015; Munis *et al.*, 2010; Yang *et al.*, 2015, 2016; Zhang *et al.*, 2011, 2012, 2013; Zhao *et al.*, 2013), which have advanced our understanding of the molecular underpinnings of Verticillium wilt resistance. A receptor-like *R* gene has been described in cotton, but only confers resistance to *V. dahliae* isolates with a corresponding avirulence gene (race 1), which are currently less

Fig. 5 Functional divergence of the *GbaNA1* homologue in *Gossypium hirsutum*. (A) Quantification of fungal biomass in *G. barbadense* and *G. hirsutum* germplasm accessions. Three-week-old seedlings of nine *G. barbadense* and nine *G. hirsutum* accessions were inoculated by a root-dip method with 5 mL of *Verticillium dahliae* strain Vd991 conidial suspension (5×10^6 conidia/mL) or sterile water (mock). Three replicates of 12 plants each were arranged in a complete random block design. Relative fungal biomass *in planta* was determined using quantitative polymerase chain reaction (qPCR) 4 weeks after inoculation from shoot RNA extractions. Bars represent *V. dahliae* elongation factor 1α (*EF-1α*) transcript levels relative to cotton *18S* gene transcript levels. The development of fungal biomass in *G. barbadense* cv. Hai 7124 was set to 1.0. Green and orange bars represent fungal biomass in *G. barbadense* and *G. hirsutum* cultivars, respectively. Error bars represent standard errors in a sample of three plants. (B) Alignment of *GbaNA1* homologues isolated from *G. hirsutum* accessions (*GhNA1*). All sequences were compared with the reference *GbaNA1*. The red triangle indicates an insertion variation in *G. hirsutum* accessions: a 1-bp (adenine) insertion occurs at the 712-bp position compared with the allelic gene in *G. barbadense* accessions. The premature termination results from a stop codon (TGA) at 759 bp (red box). (C) Amino acid alignment of GbaNA1 and GhNA1. *GhNA1* encodes only 252 amino acids as a result of the premature termination, and GhNA1 lacks motifs in the NB-ARC domain found in GbaNA1. The remaining motifs are marked by green lines. (D) Cloning *GhNA1* from the Verticillium wilt-susceptible *G. hirsutum* cv. Junmian No. 1. RNA was isolated from cotton roots 48 h after inoculation with *V. dahliae* Vd991. *GhNA1* was cloned by reverse transcription-polymerase chain reaction (RT-PCR) using the cDNA template and genomic DNA, respectively. DNA contamination in the RNA sample was assayed by PCR (RNA lane). (E) Expression analysis of *GhaNA1* in *G. hirsutum* cv. Junmian No. 1 after inoculation with *V. dahliae* Vd991. Three-week-old cotton seedlings were root-dipped (5×10^6 conidia/mL) and RNA was extracted from roots over a time course. The relative expression levels of *GhNA1* were assessed by quantitative RT-PCR, using the cotton *18S* gene as a reference. Plants treated with sterile water were used as controls (Mock). Error bars represent standard errors of three biological replicates.

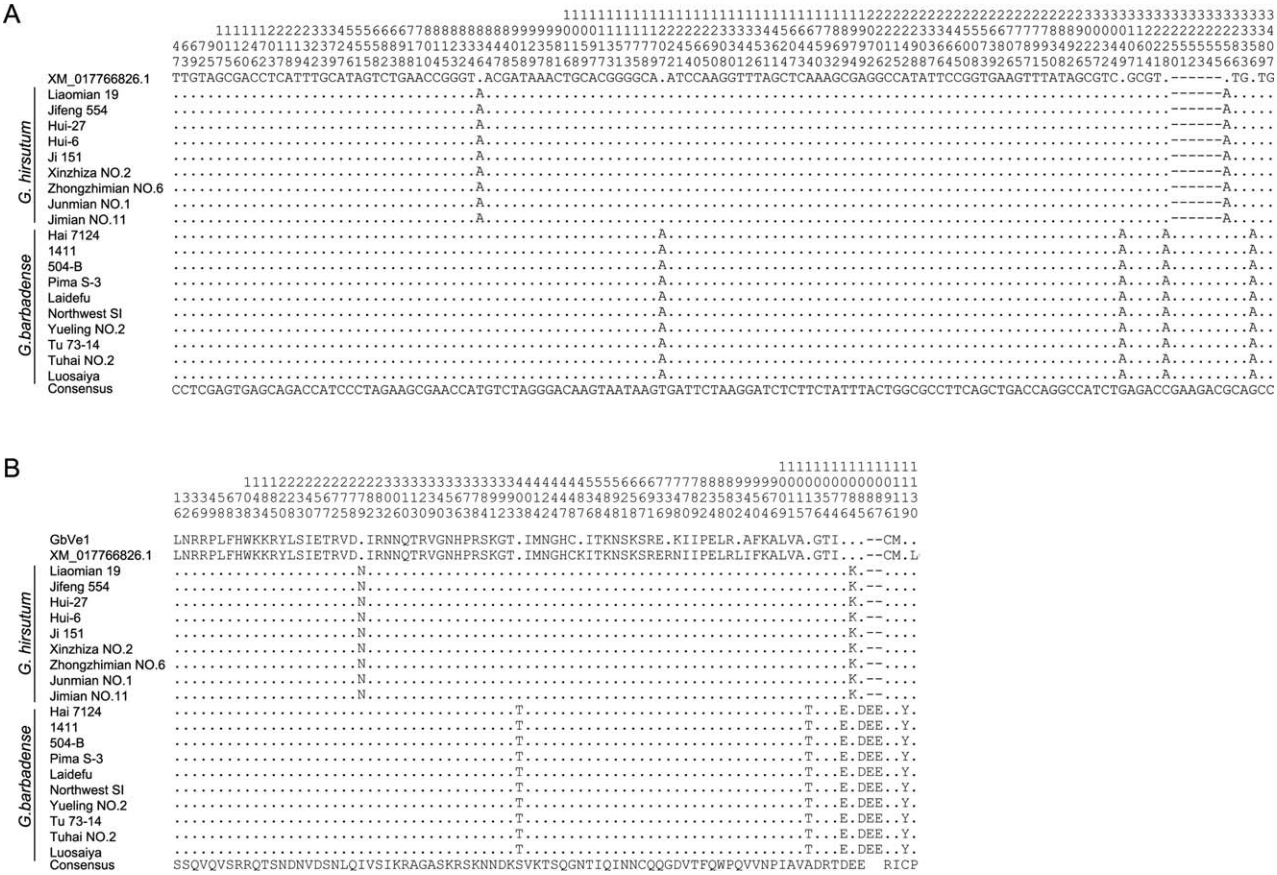


Fig. 6 Partial sequence polymorphisms of *GbVe1* allelic genes and peptide sequences in *Gossypium barbadense* and *G. hirsutum* germplasm accessions. DNA (A) and protein (B) sequences encoded by *GbVe1* homologue alleles in *G. barbadense* and *G. hirsutum* germplasm accessions. ClustalX 1.83 was used for multiple sequence alignments. All sequences are compared with the reference sequences XM_017766826.1 (*Gossypium arboreum*) and GbVe1 (only available at the peptide level; Zhang *et al.*, 2012). Only residues that deviate from the reference sequences are shown in the alignment; deletions are indicated by dashes (-).
The polymorphism positions are written vertically, i.e. the first polymorphism occurs at position 47 of the coding sequence.

frequent in cotton pathosystems (Short *et al.*, 2014). In this study, we identified *GbaNA1*, a gene that encodes an NBS-LRR domain-containing protein, and provided a functional characterization of GbaNA1 and its involvement in conferring resistance to the non-race 1 strain Vd991 in *G. barbadense*.

NBS-LRR proteins play a central role in the innate immune systems of plants (Tameling and Takken, 2008). NBS-LRR proteins function as intracellular receptors that detect pathogen effector proteins directly or indirectly by the recognition of effector-induced modifications to other host proteins, resulting in a suite of defence responses (Eitas and Dangl, 2010; Li *et al.*, 2015). To date, many NBS-LRR proteins involved in plant disease resistance have been identified (Anderson *et al.*, 1997; Ellis *et al.*, 1999; Feuillet *et al.*, 2003; Hinsch and Staskawicz, 1996; Periyannan *et al.*, 2013; Sanseverino *et al.*, 2013; Shen *et al.*, 2007; Wang *et al.*, 2015; Whitham *et al.*, 1994; Zhu *et al.*, 2017). In cotton, several NBS-LRR proteins involved in Verticillium wilt resistance have been reported previously (Yang *et al.*, 2016; Zhu *et al.*, 2013). One CC-NBS-LRR gene, *GbRVd*, plays an important role in protecting *G. barbadense* against infection by *V. dahliae* (Yang *et al.*, 2016). The micro-RNA (miRNA) regulation of NBS-LRR defence genes during fungal pathogen infection has also been associated with Verticillium wilt resistance in cotton (Zhu *et al.*, 2013).

The LRR domains of plant NBS-LRR proteins have long been hypothesized to be involved in the recognition of specific pathogen effector molecules. LRR proteins seem to be under rapid diversifying selection, and often contain highly imperfect motifs and repeats of varying lengths (Michelmore and Meyers, 1998; Qi and Innes, 2013; Takken and Goverse, 2012). GbaNA1 displays structural similarity to the CC-NBS-LRR proteins RPS2, Rpi-blb1, RPM1 and Rx (Fig. 3), and the LRR domains of GbaNA1 are highly variable (Fig. 3). Therefore, GbaNA1 probably belongs to the CC-NBS-LRR class.

In this study, we evaluated the role of *GbaNA1* in conferring resistance to the hemibiotrophic pathogen *V. dahliae* by *GbaNA1* silencing in the resistant *G. barbadense* (Fig. 2). GbaNA1 was clearly localized in cell nuclei by transient expression in onion epidermal cells (Fig. 4E), suggesting that nuclear localization is required for GbaNA1-mediated Verticillium wilt resistance. Different NBS-LRR proteins involved in defence are known to be localized to different subcellular positions. RPS4 translocates to the nucleus (Wirthmueller *et al.*, 2007), whereas RPS5 and RPM1 require plasma membrane localization to function (Gao Z *et al.*, 2011; Qi *et al.*, 2012). For other proteins, subcellular trafficking may be required to activate the full defence responses (Bai *et al.*, 2012; Heidrich *et al.*, 2011; Qi and Innes, 2013).

The activation of plant NBS-LRR proteins by pathogen effectors triggers cellular immune responses, including PCD, influx of extracellular Ca^{2+}, ROS production and transcriptional reprogramming (Andersson *et al.*, 2006; Gao *et al.*, 2013; Qi and Innes, 2013). Several studies have demonstrated that specific hormones are involved in defence responses mediated by plant NBS-LRR resistance proteins (Qi and Innes, 2013; Roberts *et al.*, 2013). For instance, auto-activation of mutations in the *Arabidopsis* CC-NBS-LRR protein ADR1-L2 causes an increase in the defence hormone SA (Roberts *et al.*, 2013). In the current study, gene expression analysis showed that the expression of *GbaNA1* in *G. barbadense* could be significantly induced by hormone treatment, including SA, MeJA and ETH (Fig. 4B–D), and the expression pattern after SA treatment was similar to the induction by *V. dahliae* Vd991 *in vivo* (Fig. 4A,D). Therefore, the defence response mediated by *GbaNA1* is probably associated with hormone signalling. Interestingly, *GbaNA1* was continually up-regulated up to 72 h after treatment with ETH (Fig. 4B), suggesting that the Verticillium wilt resistance function of GbaNA1 was most substantially linked to ET signalling.

Generally, the NBS-LRR class is the most represented group of resistance genes in plant genomes, and they often occur in clusters, resulting from gene duplication and amplification events at specific loci (Hulbert *et al.*, 2001; Meyers *et al.*, 2003). In cotton genomes, multiple NBS-LRR proteins are clustered in specific loci, and several may be involved in disease resistance (Chen *et al.*, 2015). In this study, we identified a gene cluster involved in Verticillium wilt resistance, which included eight NBS-LRR genes, but only one conferred resistance to the non-race 1 test strain *V. dahliae* Vd991 (Fig. 1). This finding is consistent with previous studies which have reported that resistance genes are generally located in clusters of tandemly repeated homologues, a subset of which encode functional resistance genes against pathogens; the others may represent a reservoir of variation that may be employed in the generation of novel functional resistance genes to adapt to pathogen variation (Kruijt *et al.*, 2005; Meyers *et al.*, 2003). Therefore, considerable evidence generated in this study suggests that GbaNA1 is the functional resistance gene in the VdRL08 locus. This conclusion is supported by the sequence divergence analysis of Verticillium wilt-susceptible *G. hirsutum GbaNA1* homologue alleles, all of which contained a single indel that resulted in a premature stop codon and truncated resistance proteins (Fig. 5A–C). This situation is consistent with what has been observed in other Verticillium wilt resistance genes, such as *Ve1* (Fradin *et al.*, 2009).

In addition, *GbaAGH3.2*-silenced Hai 7124 plants displayed severe symptoms of wilting leaves and stunting (Fig. S3), suggesting that *GbaAGH3.2*, which encodes an auxin-responsive GH3 (Gretchen Hagen3) protein, is also associated with some Verticillium wilt resistance in *G. barbadense*. The auxin-responsive GH3 family generally participates in auxin homeostasis by catalysing auxin conjugation and binding free indole-3-acetic acid (IAA) or other hormones to amino acids, and the biological functions of

the auxin-responsive GH3 family are involved in the control of plant growth and development, environmental stress responses and plant–pathogen interactions (Feng *et al.*, 2015; Kumar *et al.*, 2012; Zhang *et al.*, 2007). For example, GH3.5, a member of the auxin-responsive GH3 family in *Arabidopsis thaliana*, which possesses *in vitro* adenylation activity on both IAA and SA, acts as a bifunctional modulator in both SA and auxin signalling during pathogen infection (Zhang *et al.*, 2007). Therefore, *GbaAGH3.2* may be involved in Verticillium wilt resistance through hormone homeostasis.

In conclusion, we found that silencing of *GbaNA1*, an NBS-LRR class gene in the Verticillium wilt resistance locus VdRL08, compromised resistance to the non-race 1 *V. dahliae* strain Vd991 in *G. barbadense*. In addition, the *GbaNA1* homologue in *G. hirsutum* displays premature termination and is non-functional. Our study demonstrated that *GbaNA1* is a functional gene in the VdRL08 locus which is involved in conferring Verticillium wilt resistance in *G. barbadense*.

EXPERIMENTAL PROCEDURES

Plant, fungus and inoculation method

Cotton seeds were subjected to 37% formaldehyde fumigation for 24 h to ensure that the seeds were free of pathogens. The treated seeds were sown and grown in sterilized potting soil (PINDSTRUP, Ryomgaard, Denmark) with 20% vermiculite weight to weight (w/w) at 28 °C with a 14-h/10-h light/dark photoperiod for 3 weeks. The highly virulent *V. dahliae* strain Vd991 (used in all experiments) was cultured in potato dextrose broth (PDB) at 25 °C for 7 days with shaking. Conidia were harvested by centrifugation and washed with sterile water; the final concentration was adjusted to 5×10^6 conidia/mL with sterile distilled water using a haemocytometer. Cotton seedlings grown in potting soil with 20% w/w vermiculite for 3 weeks were uprooted and the roots were gently washed with sterile distilled water. The roots were then dipped in *V. dahliae* conidial suspension (5 mL per seedling) for 2 min, and the seedlings were replanted into potting soil with 20% w/w vermiculite. Uninoculated plants were uprooted and dipped in sterile distilled water for 2 min. No other procedures to artificially wound roots were performed.

Validation of the protein-coding gene in locus VdRL08 in *G. barbadense*

Genomic DNA was extracted from 3-week-old seedlings of *G. barbadense* cv. Hai 7124 using a DNAsecure plant kit (Tiangen, Beijing, China). To test for the presence of VdRL08 genes in *G. barbadense*, specific primers were designed for the amplification of about 500-bp segments of 21 different genes, based on the sequence of the *G. raimondii* VdRL08 locus (Table S1). PCRs consisted of 20-μL reaction volumes containing 7 μL of sterile distilled water, 10 μL of 2 × TransTaq High Fidelity (HiFi) PCR SuperMix I mix (Transgen Biotech, Beijing, China), 1 μL of template (~50 ng/μL) and 1 μL of each primer at a concentration of 10 μM. The PCR program consisted of an initial denaturation step of 10 min at 94 °C, followed by denaturation for 45 s at 94 °C, annealing for 45 s at 56 °C and extension for 30 s at 72 °C for 36 cycles. The PCR products were purified and cloned into pGEM-T vectors (Promega, Madison, WI, USA), and confirmed by sequencing.

VIGS in *G. barbadense*

For the VIGS assays, approximately 500-bp fragments were amplified from *G. barbadense* genomic DNA with primers designed from the *G. raimondii* reference sequence as described previously (Gao X *et al.*, 2011). Fragments were separately integrated into vectors and introduced into *Agrobacterium tumefaciens* GV3101. *Agrobacterium* strains harbouring the pTRV2*::GbaNA1* plasmid were combined with strains harbouring the pTRV1 vector in a 1 : 1 ratio and co-infiltrated into cotyledons of *G. barbadense* cv. Hai 7124 2 weeks after sowing seeds. The effectiveness of the VIGS assay was evaluated using the gene *CLA1* (cloroplastos alterados 1, essential for chloroplast development) as a control. Approximately 14 days later, white leaves were observed in plants in which the *CLA1* gene had been targeted by VIGS, at which point all plants were inoculated with 5 mL of *V. dahliae* Vd991 conidial suspension (5×10^6 conidia/mL) using the same root-dip method as described above. For each gene target, 12 plants separated into three replicates were tested. The silencing efficiency of selected genes was detected by qRT-PCR, which compared gene expression in treated plants with gene expression in untreated plants collected at the same time. Verticillium wilt symptoms were investigated 3 weeks after inoculation. For fungal biomass quantification, stems of three inoculated plants per gene target (one per replicate) were harvested at 21 days post-inoculation. The fungal biomass was determined by qPCR as described previously (Santhanam *et al.*, 2013). qPCR was performed using a SYBR premix Ex Taq II kit (TaKaRa, Kusatsu, Shiga, Japan), with primers specific to the cotton *18S* gene and *V. dahliae* elongation factor 1α (*EF-1α*) as controls (Table S1).

Gene cloning

To clone *GbaNA1*, 3-week-old seedlings of *G. barbadense* cv. Hai 7124 were inoculated with 5 mL of 5×10^6 conidia/mL suspension as described above, and root samples were collected 72 h after inoculation. Total RNA was extracted using a Plant RNA Purification Kit (Tiangen), and cDNA was synthesized using a RevertAid™ First Strand cDNA Synthesis Kit from MBI (Fermentas, Glen Burnie, MD, USA). Primers were designed according to the full ORF of the gene Gorai.007323100.1 in the *G. raimondii* reference genome (Paterson *et al.*, 2012; Table S1). Primers were used to amplify the target fragment from genomic DNA and cDNA. The PCR conditions consisted of an initial denaturation step at 94 °C for 10 min, followed by 36 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 3 min. All PCR products were cloned into the pGEM-T-Easy vector (Promega) and sequenced. *GbaNA1* from *G. hirsutum* was sequenced using the same method.

Sequence analysis

The ORFs of *GbaNA1* were determined using ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/), and the protein sequences were deduced on the basis of codon sequences. The conserved domains of GbaNA1 were predicted using the InterProScan database (Version 5.21). The primary protein structure (conserved motifs of the NB-LRR protein) was determined by protein sequence alignment with known NB-LRR proteins,

including potato Rpi-blb (Q7XBQ9), potato Rx (Q9XGF5), mouse-ear cress RPM1 (Q39214) and RPS2 (Q42484), and the homologue GraNA1 from *G. raimondii*. ClustalX 1.83 software was used for the multiple sequence alignment (Thompson *et al.*, 1997).

Subcellular localization analysis

To examine the subcellular localization of GbaNA1, the full-length *GbaNA1* coding region was inserted into the pRTL2 vector to generate a C-terminal fusion with the *GFP* gene under the control of the CaMV 35S promoter (*P*35S*:GbaNA1*). The CaMV35S*::GFP* construct (empty vector, *P*35S*:GFP*) was used as the control. All plasmid constructs were confirmed by sequencing. The *P*35S*:GFP* control and *P*35S*:GbaNA1* vectors were transiently expressed in onion epidermal cells using microprojectile bombardment technology with the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA, USA). The subcellular localization of the *P*35S*:GFP* fusion protein was observed with a laser scanning confocal microscope (LSM T-PMT) with excitation at 488 nm and emission at 510 nm.

Gene expression analysis

For the expression analysis of *GbaNA1* in cotton, 3-week-old seedlings of *G. barbadense* Hai 7124 were inoculated with 5 mL of conidial suspension (5×10^6 conidia/mL) of *V. dahliae* Vd991 using a root-dip method. The inoculated root samples were collected at six time points (2, 6, 12, 24, 48 and 72 h) after inoculation, with three seedlings for each sample. For the expression analysis of *GbaNA1* in hormone-treated cotton, 3-week-old seedlings of Hai 7124 were sprayed with 10 mM SA, 10 mM ETH or 10 mM MeJA. The leaves from hormone-treated plants were harvested at 2, 6, 12, 24, 48 and 72 h after spraying. Control plants were treated with sterile distilled water. qRT-PCR analyses were performed using a SYBR Premix Ex Taq kit (TaKaRa) and a QuantStudio 6 Flex Real Time PCR System (Applied Biosystems, Foster City, CA, USA). All comparisons of relative gene expression were between treated plants and untreated control plants collected at the same time points. The cotton *18S* gene was used as an endogenous control. Gene expression data represent the means of three independent biological replicates. The relative expression levels of genes were evaluated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Allelic gene cloning and sequence analysis of *GbaNA1*

GbaNA1 homologues were amplified in 18 cotton germplasm accessions, including nine germplasm accessions of *G. barbadense* and nine germplasm accessions of *G. hirsutum* (Table S2). PCR was conducted with the primers used to clone *GbaNA1*, with an initial denaturation step at 94 °C for 10 min, followed by 36 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 3 min. The ORFs of *GbaNA1* homologues were determined by ORF Finder and the protein sequences were deduced on the basis of gene sequences. Sequence alignments were carried out using ClustalX with default settings.

GbVe1 homologue sequencing of *G. barbadense* and *G. hirsutum* cotton cultivars

The allelic diversity of the race 1 resistance gene homologue was investigated in the nine *G. barbadense* and nine *G. hirsutum* cultivars mentioned above. The peptide sequence of Gbve1 (Zhang *et al.*, 2012) was used as a query to BLAST the *G. arboreum* genome, which returned a peptide with 99% sequence identity (XM_017766826.1). Primers GbVe1-F (ATTGATACTAATGAGGATGTCACTC) and GbVe1-R (TTTCATCACCCCTTTCCATGGT) were designed from the *G. arboreum* nucleotide sequence and used to amplify allelic genes from genomic DNA of *G. barbadense* and *G. hirsutum*. All PCR products were sequenced and aligned in order to study polymorphisms. Peptide sequences were predicted from the codon sequences and aligned using ClustalX with default settings.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Polymerase chain reaction (PCR) amplification of 21 genes from the VdRL08 locus in *Gossypium barbadense*.

Fig. S2 Expression levels of genes in the silenced cotton lines.

Fig. S3 Verticillium wilt symptoms in non-NB-ARC gene-silenced plants.

Fig. S4 Cloning *GbaNA1* from the resistant *Gossypium barbadense* cv. Hai 7124.

Fig. S5 Nucleotide sequence alignment of *GbaNA1* allelic genes in *Gossypium barbadense* and *G. hirsutum* germplasm accessions.

Table S1 Primers used in this study.

Table S2 Information on the cotton varieties used in this study.