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Cotton *GhBAK1* Mediates *Verticillium* Wilt Resistance and Cell Death[F]

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Abstract

Virus-induced gene silencing (VIGS) offers a powerful approach for functional analysis of individual genes by knocking down their expression. We have adopted this approach to dissect gene functions in cotton resistant to *Verticillium* wilt, one of the most devastating diseases worldwide. We showed here that highly efficient VIGS was obtained in a cotton breeding line (CA4002) with partial resistance to *Verticillium* wilt, and *GhMKK2* and *GhVe1* are required for its resistance to *Verticillium* wilt. *Arabidopsis* AtBAK1/SERK3, a central regulator in plant disease resistance, belongs to a subfamily of somatic embryogenesis receptor kinases (SERKs) with five members, AtSERK1 to AtSERK5. Two BAK1 orthologs and one SERK1 ortholog were identified in the cotton genome. Importantly, *GhBAK1* is required for CA4002 resistance to *Verticillium* wilt. Surprisingly, silencing of *GhBAK1* is sufficient to trigger cell death accompanied with production of reactive oxygen species in cotton. This result is distinct from *Arabidopsis* in which AtBAK1 and AtSERK4 play redundant functions in cell death control. Apparently, cotton has only evolved SERK1 and BAK1 whereas AtSERK4/5 are newly evolved genes in *Arabidopsis*. Our studies indicate the functional importance of BAK1 in *Verticillium* wilt resistance and suggest the dynamic evolution of SERK family members in different plant species.

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Keywords

Cell death; *Gossypium hirsutum*; *Verticillium dahliae*; virus-induced gene silence

Cotton (*Gossypium* spp.) is one of the most important crops around the world because of the significant economic value of its textile fiber, feed, foodstuff, oil, and biofuel products. There are 50 species with 45 diploid and five allotetraploid species in the *Gossypium* genus, among which *Gossypium* hirsutum, a tetraploid species, produces more than 95% of the annual cotton yield worldwide (Chen et al. 2007). Diploid *Gossypium* species can be classified into eight subgenome types designated as A to G and K. The D genome of *G. raimondii* represents the smallest genome size among *Gossypium* species (880 Mb for the haploid), and possesses high levels of synteny or collinearity with other Gossypium *genomes* (Wendel 2000). The recent release of the draft *G. raimondii* D genome sequence provides a reference for the assembly of the *G. hirsutum* genome and a foundation for the functional genomic analysis of cotton genes in the post-genomic era (Wang et al. 2012).

With the availability of the cotton genome sequence, largescale molecular and genetic approaches are needed to understand cotton gene functions at the genome-wide level. We have developed an *Agrobacterium*-mediated virus-induced gene silencing (VIGS) assay in cotton to study individual gene functions by knocking down the expression of endogenous genes (Gao et al. 2011a, 2011b). VIGS is a type of RNA-mediated post-transcriptional gene silencing and functions as an antivirus defense mechanism in plants (Hamilton and Baulcombe 1999). Through *Agrobacterium* infiltration, the T-DNA containing the partial viral genome and gene of interest is delivered into host cells. The production of double-stranded RNAs between the endogenous gene and DNA fragment from the T-DNA vector results in the silencing of endogenous genes both locally and systemically throughout the plant tissues (Burch-Smith et al. 2004; Becker and Lange 2010). This rapid and efficient loss-of-function approach bypasses plant stable transformation and overcomes functional redundancy (Burch-Smith et al. 2004; Becker and Lange 2010). The *Agrobacterium* carrying the gene of interest is inoculated in cotton cotyledons at the 2-w-old seedling stage, and the silencing will be observed within 2 weeks after inoculation (Gao and Shan 2013). We further used the VIGS approach to study the genetic and molecular mechanisms of cotton resistance to *Verticillium* wilt, one of the most devastating cotton diseases worldwide (Gao et al. 2011b). Cotton *Verticillium* wilt is caused by the soil-borne pathogen *Verticillium dahliae*. This pathogen is particularly difficult to control by fungicides because the fungi reside in the woody vascular tissues and can be transmitted systemically in cotton plants (Fradin and Thomma 2006). By using a VIGS approach, we have shown that silencing cotton *GhNDR1* (Nonrace-specific Disease Resistance 1), *GhMKK2* (MAPK kinase 2), or *GhVe1* compromised its resistance to *V. dahliae* infection (Gao et al. 2011b). Recently, the tobacco rattle virus (TRV)-based VIGS assay has been extended to study cotton gene function in cotton fiber development (Qu et al. 2012). Thus, the TRV-VIGS system provides a powerful tool for rapid functional analysis of cotton genes involved in biotic and abiotic stresses and the development of seedlings to reproductive organs.

Being sessile, plants have evolved a large number of membrane-resident receptor-like kinases (RLKs) to cope with potential microbial invasions and maintain active growth and development (Shiu and Bleecker 2001, 2003; Shan et al. 2007; Boller and Felix 2009). *Arabidopsis* BAK1, a leucine-rich-repeat RLK (LRR-RLK) originally identified as a BRI1-associated receptor kinase mediating the signaling of plant growth hormone brassinosteroid (BR) signaling (Li et al. 2002; Nam and Li 2002), has emerged as an important player in plant disease resistance by association with bacterial flagellin sensor FLS2 and other immune sensors (Chinchilla et al. 2007; Heese et al. 2007; Schulze et al. 2010; Roux et al. 2011). *Arabidopsis* BAK1 is also known as somatic embryogenesis receptor kinase 3 (SERK3), belonging to a subfamily of RLKs with five members, SERK1 to SERK5 (Chinchilla et al. 2009). SERKs were named because overexpression of SERK1 enhanced the somatic embryogenesis ability of suspension cells (Schmidt et al. 1997; Hecht et al. 2001). Further phenotypic analyses of *serk1serk2* double mutants suggest that SERK1 and SERK2 play crucial and redundant functions in anther development and male gametophyte maturation (Colcombet et al. 2005). In addition, SERK1, BAK1/SERK3, and SERK4 (also known as BKK1) play a redundant role in BR signaling by association with BRI1 (Karlova et al. 2006; He et al. 2007; Gou et al. 2012). Remarkably, BAK1 and SERK4 also exhibit redundant functions in plant disease resistance via association with multiple immune sensors (Roux et al. 2011). In addition to their roles in BR signaling and plant disease resistance, BAK1 and SERK4 negatively regulate plant cell death because the *bak1serk4* double mutant exhibits seedling lethality accompanied with constitutive defense gene activation and spontaneous cell death (He et al. 2007). How BAK1 and SERK4 are involved in cell death control is still not clear.

In this study, we first established the *Agrobacterium*-mediated VIGS assay in a newly developed and released cotton germplasm line CA4002 that is partially resistant to *Verticillium* wilt (Dever et al. 2013). Consistent with our previous report that VIGS is not variety-specific, the VIGS efficiency reaches 100% in this line, and *GhMKK2* and *GhVe1* are required for its resistance to *Verticillium* wilt. An examination of the *G. raimondii* D genome and *G. hirsutum* unigene database identified two BAK1 orthologs and one SERK1 ortholog in cotton. Interestingly, silencing of *GhBAK1* by VIGS caused the compromised resistance to *Verticillium* wilt, pronounced cell death, and the production of reactive oxygen species (ROS) in cotton. Our results suggest that cotton has only evolved SERK1 and BAK1, and lacks SERK4/5 orthologs, whereas AtSERK4 and AtSERK5 are newly evolved genes in *Arabidopsis*, which play redundant roles with AtBAK1 in plant disease resistance and cell death control.

Results

Involvement of *GhMKK2* and *GhVe1* in cotton CA4002 resistance to *Verticillium* wilt

We have shown that VIGS of cotton *Cla1* (*GhCla1*), a gene involved in chloroplast development, results in an albino phenotype on the newly emerging leaves (Gao et al. 2011b). This phenotype is readily observed. Thus, we employed silencing of *GhCla1* as a visual marker to monitor the VIGS efficiency in cotton germplasm CA4002. The newly emerging true leaves from the infiltrated plants with *Agrobacterium* carrying *GhCla1*

exhibited an albino phenotype (Figure 1). Consistent with our previous report, the albino phenotype was observed on the leaves of the *GhCla1*-silenced plants approximately 10 d post-infiltration, and this phenotype was further developed and uniformly distributed over the entire leaves along with plant growth (Figure 1). Thus, we have established an efficient *Agrobacterium*-mediated VIGS assay in the cotton germplasm line CA4002 for transient silencing of interested genes. This result also substantiated our previous finding that VIGS is independent of cotton cultivars.

We have recently shown that silencing of *GhMKK2*, *GhNDR1*, or *GhVe1* in a commercial cotton cultivar (Fibermax 9160B2F) compromised its resistance to *V. dahliae* infection (Gao et al. 2011b). The newly released breeding line CA4002 confers partial resistance to *Verticillium* wilt (Dever et al. 2013). Yet, the molecular basis of this resistance remains unknown. Here, we tested whether a similar genetic requirement exists in CA4002 resistance to *Verticillium* wilt and examined the functional importance of *GhVe1* and *GhMKK2* genes for resistance to the *V. dahliae* King isolate, a “defoliating” type and aggressive cotton pathogen isolated in TX.

The *Agrobacterium* cultures carrying the recombinant pTRV vectors with either vector control, *GhMKK2* or *GhVe1*, were hand-infiltrated into the cotyledons of cotton CA4002 seedlings, and then *V. dahliae* was inoculated into the stems of cotton seedlings. The progressive wilting of the true leaves was scored. Consistent with an earlier report, CA4002 exhibited lower incidence of *Verticillium* wilt and less defoliation compared to most other cultivars and breeding lines (Dever et al. 2013). Silencing of *GhVe1* or *GhMKK2* enhanced plant susceptibility to *V. dahliae* infection, and plants exhibited a more severe wilting phenotype than the vector control-inoculated plants (Figure 2A, B). In particular, at a later infection stage, almost 100% of *GhMKK2* or *GhVe1*-VIGSed plants were severely infected by *V. dahliae* and most of the leaves showed the wilting phenotype compared to approximately 60% of control vector-inoculated plants (Figure 2C). Together, our loss-of-function assays indicated that *GhMKK2* and *GhVe1* partially contribute to cotton CA4002 resistance to *V. dahliae* infection.

Cotton SERK gene family

By using a VIGS approach, tomato BAK1 was shown to be required for *Verticillium* wilt resistance in tomato (Fradin et al. 2009). It remains unknown how many SERK family members exist in cotton and what their biological functions are. Here, we systematically characterized the SERK gene subfamily in cotton. We used the full-length AtBAK1 amino acid sequence as a query and Blasted against the *G. raimondii* D genome database at http://cgp.genomics.org.cn/page/species/blast.jsp. Three genes (10025422 in chromosome (Chr.) 12; 10029586 in Chr. 5; 10039700 in Chr. 10) with an e value of 0.0 were retrieved. These three genes encode proteins with more than 70% identity to AtBAK1 (Figure 3A). The gene 10025423, next to 10025422 in Chr. 12, encodes a protein with 68% identity with AtBAK1. The 10025423 and 10025422 may be a result of local gene duplication. However, the protein encoded by 10025423 lacks the intact transmembrane domain and juxtamembrane domain (Figure S1). Thus, this gene is unlikely to be functional. The gene next to these three genes (10005636) encodes a protein with only 49% identity to AtBAK1 full length and 39%

identity to the AtBAK1 LRR domain (Figure S2), suggesting that 10005636 does not belong to the SERK subfamily anymore. When any of the AtSERK1 to AtSERK5 SERKs were used as a query for Blast search, similar sequences with three genes (10025422, 10029586, and 10039700) were retrieved as top hits compared to those using AtBAK1 as a query. Sequence analysis indicates that 10025422 and 10029586 share 90% identity and belong to the same clade as AtBAK1 based on the phylogenetic tree (Figure 3). Thus, we named 10025422 as GrBAK1.1 and 10029586 as GrBAK1.2. The gene 10039700 belongs to the same clade as AtSERK1 and AtSERK2, and was named GrSERK1. Similarly, when searching the *G. hirsutum* unigene database at http://www.cottondb.org/blast/blast.html, we also retrieved three SERK family members, GhSERK1 (ADR00582.1), GhSERK2 (AEA76434.1), and GhBAK1 (AEG25668.1), in *G. hirsutum* (Figure S2). Sequence and phylogenetic analysis indicates that GhSERK2 (AEA76434.1) is almost identical to GhBAK1, and they all belong to the same clade as AtBAK1 (Figure S2). Therefore, we renamed GhBAK1 (AEG25668.1) as GhBAK1.1 and GhSERK2 (AEA76434.1) as GhBAK1.2, which correspond to GrBAK1.1 and GrBAK1.2 in *G. raimondii*. Thus, there are two BAK1 orthologs and one SERK1/SERK2 ortholog in the cotton genome, and we did not find a cotton SERK4/SERK5 ortholog.

Conserved function of BAK1 in plant resistance to *Verticillium* wilt

We further tested whether *GhBAK1* was required for cotton resistance to *Verticillium* wilt. We designed the primers to amplify the most identical region of *GhBAK1.1* and *GhBAK1.2* into TRV2 vector pYL156, which will likely silence both genes at once (Figure S2 A). Notably, this region shows the limited similarity with *GhSERK1* at the nucleotide level. The wilting phenotype in CA4002 plants silenced with *GhBAK1* by VIGS was observed and the percentage of wilting plants was scored after *V. dahliae* infection. Clearly, plants silenced with *GhBAK1* showed a more severe wilting phenotype than plants infiltrated with the vector control (Figure 4A). The percentage of wilting plants silenced with *GhBAK1* was also higher than those with control infiltration (Figure 4B). Thus, *GhBAK1* is an essential component for cotton resistance to *V. dahliae* infection.

The *V. dahliae* strain (King isolate) that we used to infect cotton is also able to infect *Arabidopsis* (Figure 5). Using a rootdipping inoculation assay, the newly emerging leaves of infected *Arabidopsis* plants showed a glassy and water-soaking phenotype 20 d post-inoculation and plant growth became retarded (Figure 5A). The water-soaking phenotype further progresses into the older leaves, which leads to the similar leaf wilting phenotype as observed in cotton at the later time point of infection. Importantly, two *bak1* null mutants, *bak1-3* and *bak1-4*, showed a more sensitive and severe wilting phenotype to *V. dahliae* infection than wild-type (WT) Col-0 plants (Figure 5B). The mutation in *SERK2* did not affect the *Arabidopsis* resistance to *V. dahliae*, indicating the involvement of specific SERK family members in *Verticillium* wilt resistance (Figure 5B). BAK1 has been shown to be required for multiple pathogen recognition receptor (PRR)-mediated responses, basal defense, and restriction of pathogen growth in *Arabidopsis*, tomato, and *Nicotiana benthamiana*. Cotton possesses highly conserved orthologs of *BAK1* genes which are required for *Verticillium* resistance (Figures 4, 5), implying the potential conserved signaling mechanisms mediating fungal *Verticillium* resistance.

Function of GhBAK1 in cell death control

Surprisingly, we also observed certain developmental defects in plants silenced with *GhBAK1*. The *GhBAK1* VIGSed plants showed wrinkled true leaves that hardly fully expanded (Figure 6A). Trypan blue staining indicated that silencing of *GhBAK1* triggered pronounced cell death in the newly emerging true leaves compared with the vector control inoculated leaves (Figure 6B). 3,3′-Diaminobenzidinetetrachloride (DAB) staining further revealed elevated ROS production as detected by the massive brown precipitates in *GhBAK1*-silenced leaves (Figure 6C). This result is distinct from *Arabidopsis* in which AtBAK1 and AtSERK4 play redundant functions in the control of cell death (He et al. 2007). However, this result is consistent with our genomic analysis of the cotton SERK family, in which cotton lacks SERK4 and SERK5 orthologs. Thus, cotton has only evolved orthologs of SERK1 and BAK1 (Figure 3) whereas AtSERK4 and AtSERK5 are newly evolved genes in *Arabidopsis*.

Discussion

The development of *Agrobacterium*-mediated VIGS assay and the availability of the whole cotton genome sequence provide a cornerstone for systematic characterization of cotton gene functions at a genome-wide level and an understanding of the conserved and unique functions of individual cotton genes. An examination of the *G. raimondii* and *G. hirsutum* database identified two BAK1 orthologs and one SERK1 ortholog in the cotton genome. Silencing of *GhBAK1* compromised the cotton CA4002 line's resistance to *Verticillium* wilt. Consistent with our previous report, silencing of *GhMKK2* or *GhVe1* also compromised *Verticillium* wilt resistance in the cotton CA4002 line. The variety Fibermax 9160B2F (Bayer CropSciences, Lubbock, TX, USA) and breeding line CA4002 were developed from different parents and so the effects of these genes may be sufficiently broad as to be used in a marker selection program. This study provides a mechanism to partially explain the resistance phenotype in this newly developed breeding line (Dever et al. 2013). Interestingly, *AtBAK1* is also required in *Arabidopsis* resistance to *Verticillium* wilt, suggesting the conserved *Verticillium* resistance mechanisms in different plant species. Surprisingly, we did not find any SERK4/5 orthologs in the cotton genome, suggesting that *Arabidopsis SERK4* and *SERK5* are newly evolved genes. However, SERK1/2 and BAK1 have evolved before the divergence of cotton and *Arabidopsis*. Notably, cotton has evolved two copies of *BAK1*. It is also possible that *GhBAK1.1* and *GhBAK1.2* are homologous genes in the *G. hirsutum* genome. Genome-wide characterization of SERK family members in different plant species will provide information about the dynamic evolution of this important RLK subfamily. Our study also suggests that cotton may be a better model to study the biological functions of SERK family members due to less functional redundancy. Consistent with this hypothesis, silencing *GhBAK1* is sufficient to induce cell death and ROS production in cotton.

BAK1 functions in plant disease resistance through association with FLS2 and other PRRs (Chinchilla et al. 2007; Heese et al. 2007; Schulze et al. 2010; Roux et al. 2011). In addition, BAK1 is involved in plant hormone BR signaling via hetero-dimerization with receptor BRI1 (Li et al. 2002; Nam and Li 2002). With a relatively short extracellular LRR domain,

BAK1 does not directly bind to ligands but instead functions as a regulatory partner to positively modulate FLS2 and BRI1 signaling via trans-phosphorylation. Rather than being involved in direct ligand binding (Kinoshita et al. 2005; Chinchilla et al. 2007), BAK1 more likely functions as a signaling partner for the regulation of receptors FLS2 and BRI1 (Chinchilla et al. 2009). Interestingly, *GhVe1* encodes a receptor-like protein, which might function in the receptor complex to perceive an unknown elicitor from *V. dahliae*. The requirement of both *GhVe1* and *GhBAK1* in *Verticillium* wilt resistance suggests that GhBAK1 and GhVe1 may associate with each other in mediating resistance to *V. dahliae* infection. Future examination of GhBAK1 and GhVe1 interaction *in vivo* and *in vitro* will shed light on the mechanisms underlying plant resistance to this devastating disease.

AtBAK1 and AtSERK4 redundantly control *Arabidopsis* cell death (He et al. 2007). The *Arabidopsis bak1 serk4* double mutant exhibited spontaneous cell death with constitutive defense gene expression and ROS accumulation (He et al. 2007), which is similar to the silencing of *GhBAK1* in cotton. Despite the unclear mechanism of BAK1/SERK4 in the control of cell death, BAK1 interacts with another RLK BIR1 (BAK1-interacting receptor-like kinase) constraining cell death and defense activation. Interestingly, a suppressor of BIR1-mediated cell death SOBIR also encodes an RLK, suggesting a BAK1-mediated receptor complex in the control of cell death (Gao et al. 2009). It will be interesting to test whether cotton GhBIR1 also interacts with GhBAK1 in the control of cell death, and which one functions as a bona fide receptor to perceive the death signal. Nevertheless, the multiple functions of BAK1 and other SERKs in plant growth, development, and disease resistance raise the question of how this important RLK subfamily dictates the bifurcate cellular outputs in response to distinct extrinsic and intrinsic signals.

Materials and Methods

Plant materials and growth

Cotton (*Gossypium hirsutum*) CA4002 line (Reg. No. GP-956, PI 665226) was grown in pots containing Metro Mix 900 (SunGR, Beavile, WA, USA) in a growth room at 23 °C, 60% relative humidity and 75 μE/m^2 s^1 light with a 12 h photoperiod. *Arabidopsis* WT Col-0, and mutants *bak1-3*, *bak1-4*, and *serk2* (all in Col-0 background), were obtained from the Arabidopsis Biological Resource Center (ABRC) as previously reported (Shan et al. 2008) and grown in soil Metro Mix 366 (SunGR, Beavile, WA, USA) in a growth room at 23 °C, 60% relative humidity, and 75 μE/m^2 s^1 light with a 12 h photoperiod.

Construction of VIGS vectors and *Agrobacterium*-mediated VIGS

GhCLA1 was cloned into the pYL156 (pTRV-RNA2) vector as previously described (Gao et al. 2011b). *GhBAK1* was amplified by polymerase chain reaction from a cDNA library of *G. hirsutum* leaf tissues with primers *GhBAK1-F*, 5′-CGGAATTCGCA-CACTCGGAGCTGCAAGG-3′, GhBAK1-R, 5′- GGGGTACC-GAGTGCACAACAGAGCC-3′, and inserted into the pYL156 vector with restriction enzymes *Eco*RI and *Kpn*I digestion. VIGS constructs of pYL156-*GhMKK2* and pLY156-*GhVe1* were conducted as previously described (Gao et al. 2011b).

The plasmids containing binary TRV vectors pTRV-RNA1 and pTRV-RNA2 (pYL156) vector, pYL156-*GhBAK1*, pYL156-*GhMKK2*, or pLY156-*GhVe1*, were transformed into *Agrobacterium tumefaciens* strain GV3101, respectively. *Agrobacterium* cultures were harvested and infiltrated into two fully expanded cotyledons of 2-w-old plants as previously described (Gao and Shan 2013). VIGS experiments were repeated at least three times with more than 15 plants for each construct per repeat.

Verticillium infection and disease scoring

Verticillium dahliae (isolate King) was maintained and cultured, and suspension spores were prepared as described previously (Gao et al. 2011b). Spores of *V. dahliae* were collected from mycelium growing on potato dextrose agar in a plate by scratching the surface using sterile H_2O and filtering through two layers of cheese cloth. For stem inoculation of cotton, spore suspension at a concentration of 1×10^6/mL containing 0.001% Tween-20 was injected slowly into the stem site approximately 1 cm below the cotyledons with a syringe needle (20 G) until the suspension dripped from the injection site. The plants were covered with a transparent plastic dome to maintain a high humidity at room temperature overnight. The disease phenotype was observed and wilting was scored at designated time points. The experiments were repeated three times with similar results.

For root inoculation of *Arabidopsis*, 2-w-old *Arabidopsis* seedlings were uprooted from the soil and extra soil was removed by washing with water and gentle tapping without damaging the roots. The roots were slightly blotted with a paper towel to remove excessive water, and subsequently inoculated by immersing the roots in the conidial suspension for 5 min. The seedlings were immediately transplanted into fresh soil, and covered with the dome at room temperature overnight. At least 10 seedlings were inoculated per treatment. The disease ratio was calculated as the percentage of wilting plants to the total infected plants.

Histological detection of ROS production and cell death in leaves

Histological ROS production in cotton plants was examined using a DAB staining method (ThordalChristensen et al. 1997) with modifications. Briefly, the leaves from plants approximately 2 w post-VIGS inoculation were excised and subsequently immersed in 1 mg/mL DAB (Sigma-Aldrich, St Louis, MO, USA; pH 3.8) solution with low vacuum pressure for 30 min, followed by overnight incubation at room temperature in the dark. The stained leaves were fixed and cleared in alcoholic lacto-phenol (95% ethanol: lactic acid: phenol = 2:1:1) at 65 °C, rinsed once with 50% ethanol, and twice with H_2O. The destained leaves were stored in 50% glycerol or subjected to microscope observation.

To examine cell death, the leaves were collected and stained with Trypan blue in lactophenol (lactic acid : glycerol : liquid phenol : distilled water = 1:1:1:1) solution. The stained leaves were destained with 95% ethanol/lactophenol solution, washed with 50% ethanol, and mounted in 50% glycerol for microscope observation.

Bioinformatics analysis of the cotton SERK gene family

To retrieve the cotton SERK genes, the full length AtBAK1 and AtSERK1, 2, 4, or 5 amino acid sequences were used as a query to Blast against the *G. raimondii* D genome database

(http://cgp.genomics.org.cn/page/species/blast.jsp) and the *G. hirsutum* unigene database (http://www.cottondb.org/blast/blast.html), respectively. Three genes (10025422 in Chr. 12; 10029586 in Chr.5; 10039700 in Chr.10) from the *G.raimondii* D genome with top hits with an e value of 0.0 were identified and analyzed. Three SERK genes from the *G. hirsutum* unigene database, GhSERK1 (ADR00582.1), GhSERK2 (AEA76434.1), and GhBAK1 (AEG25668.1), were retrieved. The amino acid sequence alignment between cotton and *Arabidopsis* SERKs was performed using the Multalin website (http://multalin.toulouse.inra.fr/multalin/) with a hierarchical clustering approach, and the phylogenetic tree was constructed using CLUSTALW and a rooted phylogenetic tree (http://www.genome.jp/tools/clustalw/).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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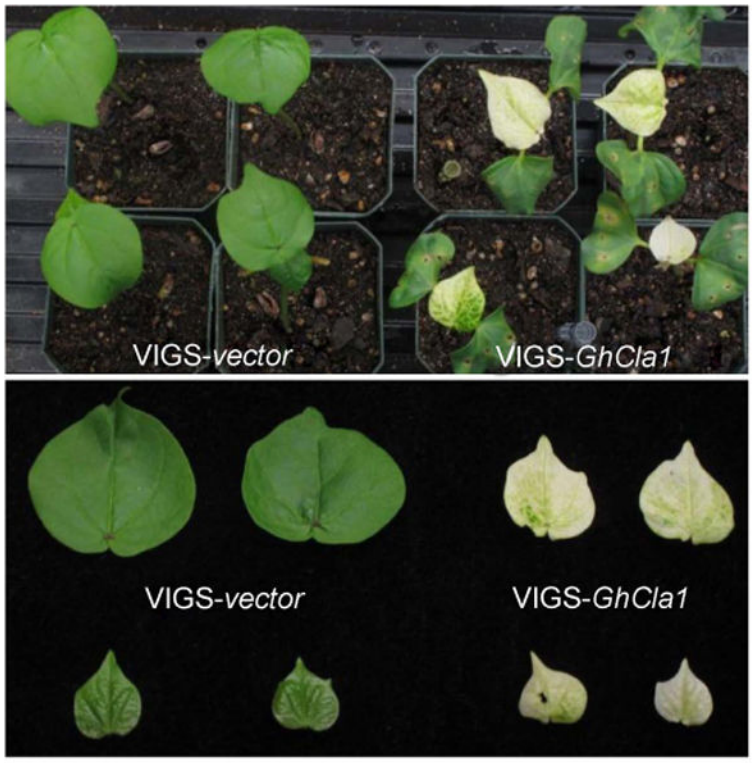

Figure 1. Establishment of *Agrobacterium*-mediated virus-induced gene silencing (VIGS) assay in the cotton CA4002 line

The cotyledons of 10-d-old CA4002 seedlings were hand-infiltrated with *Agrobacterium* carrying either pYL156-*GhCla1* or VIGS-vector control (pYL156-*GFP*). The albino phenotypes were observed from the newly emerging true leaves and pictures were taken at approximately 2 w after infiltration.

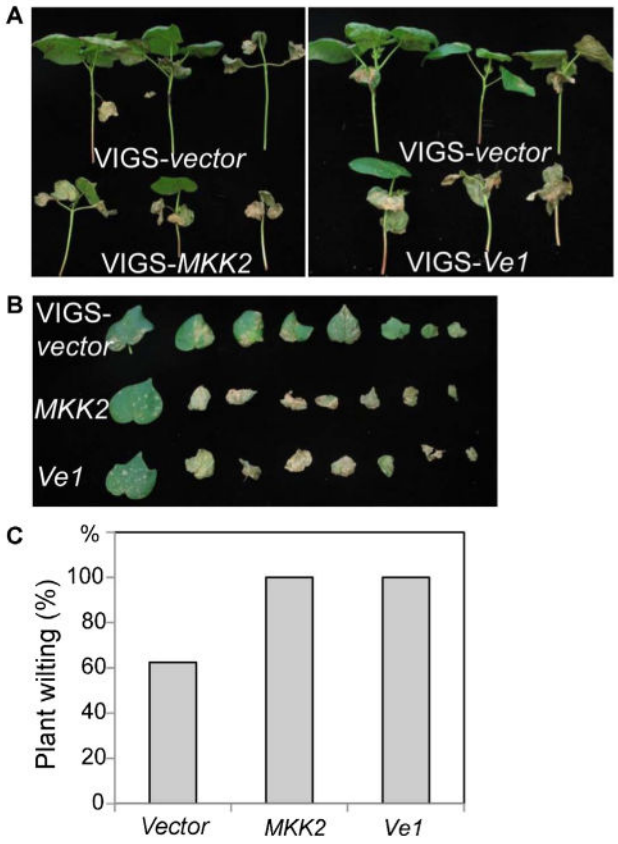

Figure 2. *GhMKK2* and *GhVe1* are required for resistance to *Verticillium dahliae* in the cotton CA4002 line

Ten-d-old CA4002 seedlings were hand-infiltrated with *Agrobacterium* carrying individual genes in the virus-induced gene silencing (VIGS) vector. When *GhCla1*-silenced plants showed albino phenotype (~2 w after infiltration), the seedlings that silenced *MKK2* or *Ve1* were stem-inoculated with *V. dahliae* (isolate King) suspension spores at a concentration of 1×10^6/mL

(A) Whole plants and **(B)** detached leaves are shown at 34 d after *V. dahliae* infection.

(C) Percentage of plants showing *Verticillium* wilt phenotype at 34 d after infection. The disease ratio was scored with at least 15 plants per treatment and the assays were repeated for three times with similar results.

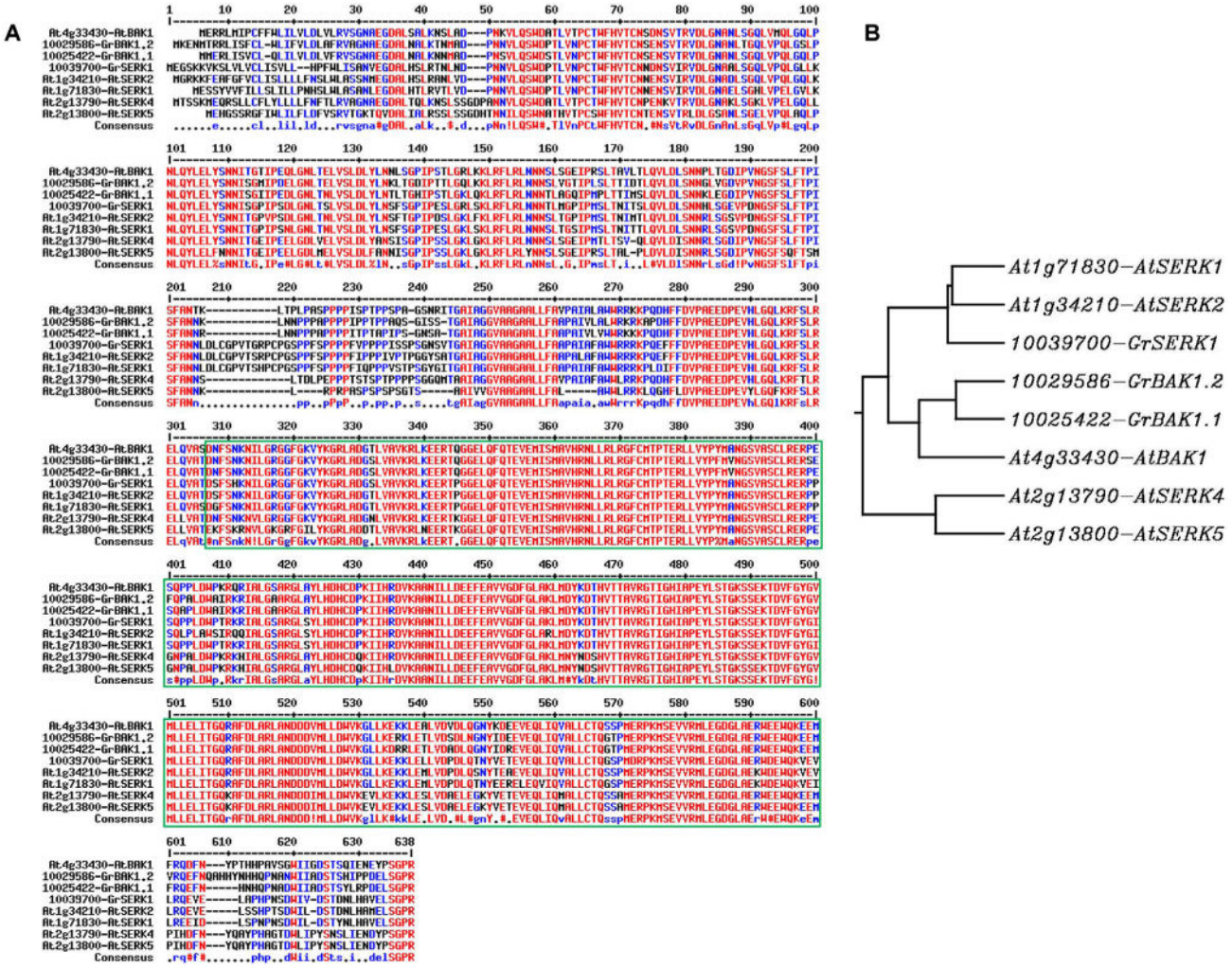

Figure 3. (A) Sequence alignment and (B) phylogenetic analysis of *Arabidopsis* SERK (AtSERK) and cotton *Gossypium raimondii* SERK (GrSERK) family members

The amino acid sequences were used for the alignment. The sequences squared in green are the kinase domain conserved among AtSERKs and GrSERKs. The alignment was performed using the Multalin website (http://multalin.toulouse.inra.fr/multalin/) with a hierarchical clustering approach, and the phylogenetic tree was constructed using CLUSTALW and rooted phylogenetic tree (http://www.genome.jp/tools/clustalw/).

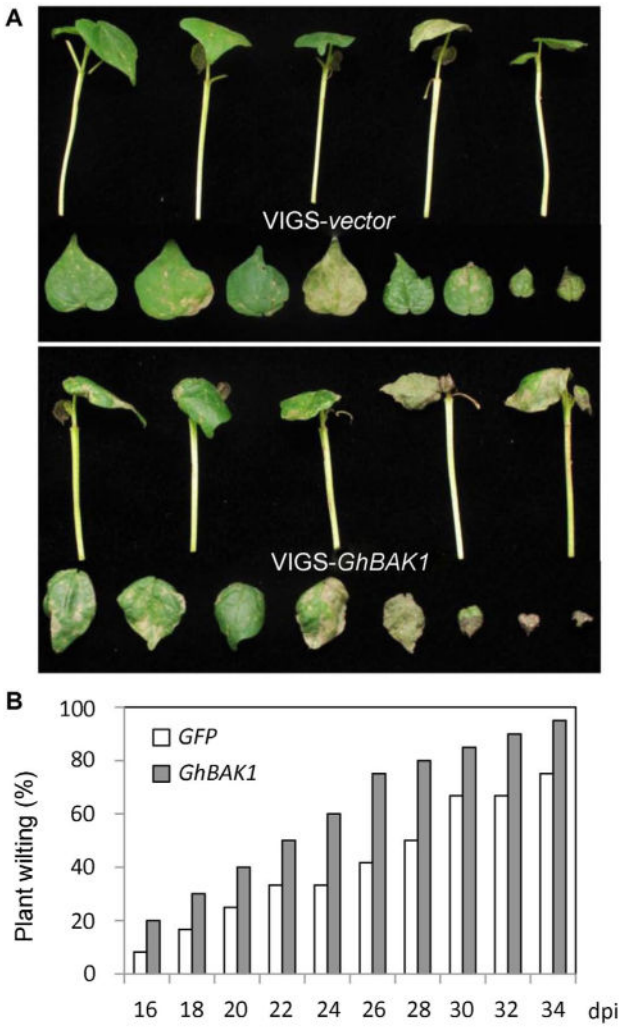

Figure 4. Silencing of *GhBAK1* compromises cotton resistance to *Verticillium dahliae* infection

Ten-d-old CA4002 plants were first infiltrated with *Agrobacterium* carrying pYL156-*GhBAK1* or vector control (pYL156-*GFP*), and then inoculated with *V. dahliae* (isolate King) suspension spores at a concentration of 1×10^6/mL.

(A) Whole plants (top panel) and detached leaves (bottom) are shown at 14 d after *V. dahliae* infection.

(B) Percentage of plants showing *Verticillium* wilt phenotype at the indicated days post-infection. The disease ratio was scored using 21 plants per treatment and the assays were repeated three times with similar results.

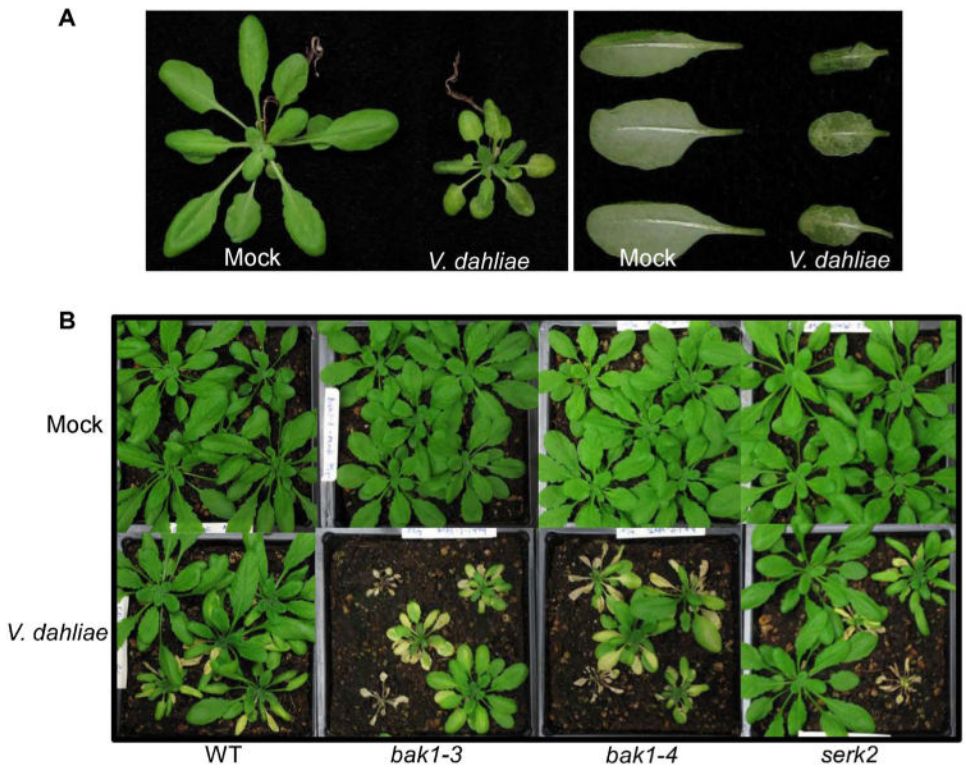

Figure 5. *Arabidopsis bak1* mutants are more sensitive to *Verticillium dahliae* infection

(A) Four week old *Arabidopsis* wild-type (WT) Col-0 plants were inoculated with *V. dahliae* using the root-dipping method. Photos were taken 20 days post-inoculation. The left panel shows intact plants and the right panel shows detached leave.

(B) The whole plant disease phenotype of WT, *bak1-3*, *bak1-4*, and *serk2* mutant plants infected with *V. dahliae*. Photos were taken 20 days post-inoculation.

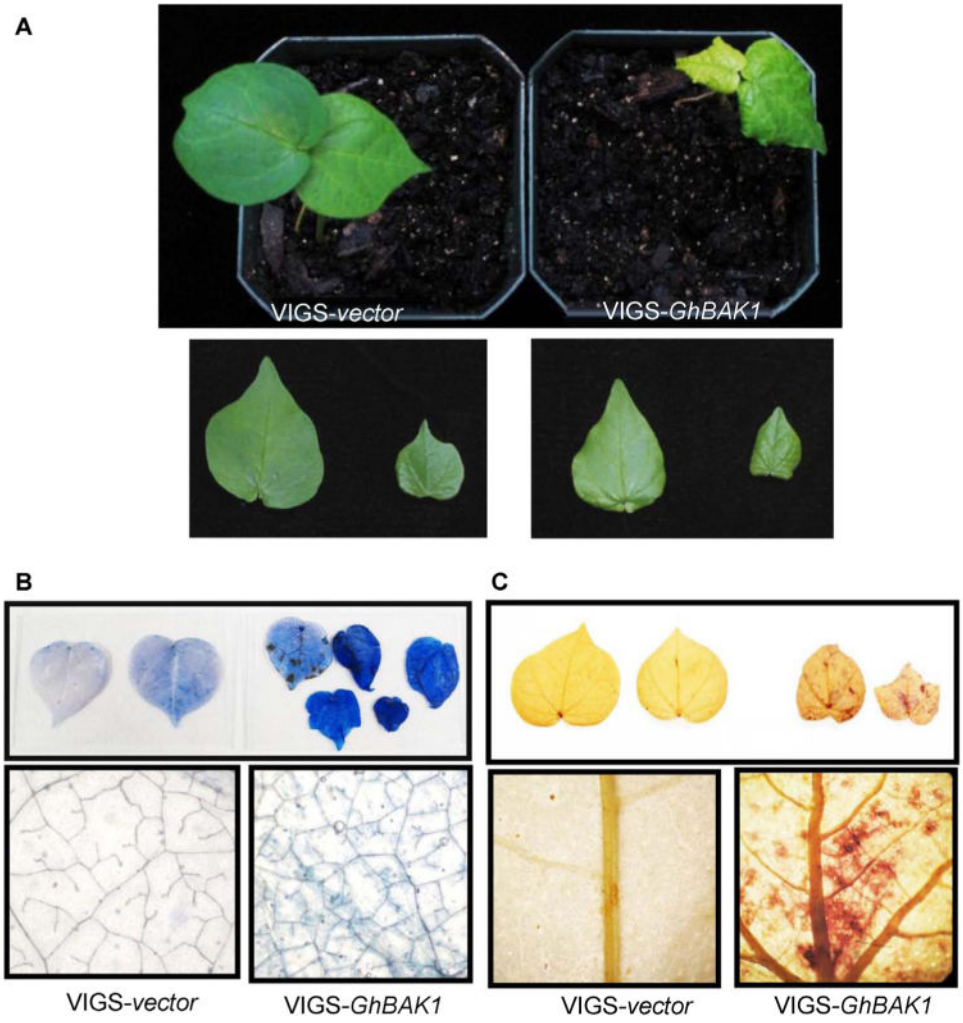

Figure 6. Silencing of *GhBAK1* triggers cell death and reactive oxygen species (ROS) production

(A) The *GhBAK1*-silenced plants display abnormal growth phenotype (top panel) and the true leaves do not fully expand (bottom panel). The pictures were taken at 28 d post-virus-induced gene silencing (VIGS) infiltration.

(B) Silencing of *GhBAK1* triggers cell death in true leaves as shown by blue-stained cells by Trypan blue staining. The leaves were detached and stained at 28 d post-VIGS infiltration. The bottom panels showed the close-up view of one of the leaves in the corresponding top panels.

(C) Silencing of *GhBAK1* induces ROS accumulation as shown by 3,3′-diaminobenzidine-tetrachloride (DAB) staining. The leaves were detached and stained at 28 d post-VIGS infiltration.