The plant specific transcription factors CBP60g and SARD1 are targeted by a Verticillium secretory protein VdSCP41 to modulate immunity.

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ABSTRACT
The vascular pathogen *Verticillium dahliae* infects the roots of plants to cause *Verticillium* wilt. The molecular mechanisms underlying *V. dahliae* virulence and host resistance remain elusive. Here, we demonstrate that a secretory protein, VdSCP41, functions as an intracellular effector that promotes *V. dahliae* virulence. The *Arabidopsis* master immune regulators CBP60g and SARD1 and cotton GhCBP60b are targeted by VdSCP41. VdSCP41 binds the C-terminal portion of CBP60g to inhibit its transcription factor activity. Further analyses reveal a transcription activation domain within CBP60g that is required for VdSCP41 targeting. Mutations in both CBP60g and SARD1 compromise *Arabidopsis* resistance against *V. dahliae* and partially impair VdSCP41-mediated virulence. Moreover, Virus-induced silencing of GhCBP60b compromises cotton resistance to *V. dahliae*. This work uncovers a virulence strategy in which the *V. dahliae* secretory protein, VdSCP41, directly targets plant transcription factors to inhibit immunity, and reveals CBP60g, SARD1 and GhCBP60b as crucial components governing *V. dahliae* resistance.

**INTRODUCTION**

The vascular pathogen *Verticillium dahliae* infects a broad range of plants and
causes devastating diseases. *V. dahliae* can survive in the form of microsclerotia in soil for over ten years (Schnathorst, 1981). During *V. dahliae* colonization, microsclerotia germinate and develop hyphae that adhere tightly to the root surface upon the perception of plant roots (Zhao et al., 2014). A few hyphae form hyphopodia at the infection site and further differentiate into penetration pegs to penetrate into plant cells and colonize the vascular tissue (Fradin and Thomma, 2006; Schnathorst, 1981; Vallad and Subbarao, 2008; Zhao et al., 2014; Zhao et al., 2016). Although a few key steps mediating these infection processes have been elucidated, the molecular mechanisms underlying *V. dahliae* virulence remain largely unknown.

Progress has been made in isolating virulence factors that are crucial for *V. dahliae* virulence. Several genes that regulate *V. dahliae* development have been characterized as contributing to virulence. *VDH1* and *VdGARP1*, which regulate microsclerotial development, are required for *V. dahliae* virulence in cotton plants (Gao et al., 2010; Klimes and Dobinson, 2006). *VdRac1* and *VdPKAC1* regulate both the development and pathogenicity of the fungus in host plants (Tian et al., 2015; Tzima et al., 2010; Tzima et al., 2012). *VdEG1*, *VdEG3*, *VdCYP1*, and *VdSNF1* also function as factors that contribute to *V. dahliae* virulence on cotton (Gui et al., 2017; Tzima et al., 2011; Zhang et al., 2016).

In addition to development-associated virulence factors, fungal pathogens deliver effectors that act as virulence factors, inhibiting host defense to promote pathogenesis, which also occurs in bacterial and oomycete pathogens. The race 1 strain-specific effector Ave1 contributes to *V. dahliae* virulence on tomato plants not carrying *Ve1* (de
Jonge et al., 2012). Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPS) (NLP1 and NLP2) secreted by V. dahliae strain JR2 are required for its pathogenicity in tomato and Arabidopsis plants (Santhanam et al., 2013). VdSge1 encodes a transcriptional regulator that controls the expression of six putative effector genes. Deletion of VdSge1 in V. dahliae results in significantly impaired pathogenicity in tomato, suggesting an important role of secreted effectors in suppressing host immunity in V. dahliae (Santhanam and Thomma, 2013).

However, only two secreted effectors of V. dahliae have been indicated to function inside the plant cell to modulate host immunity thus far. VdIsc1, a V. dahliae effector lacking a known signal peptide, is thought to be delivered into host cells to hydrolyze a salicylic acid (SA) precursor and inhibit salicylate metabolism (Liu et al., 2014). The small cysteine-containing protein (SCP) VdSCP7 translocates into the nucleus of plant cells to either suppress or induce defense in plants through unknown mechanisms (Zhang et al., 2017).

Plants are equipped with immune components to counteract V. dahliae virulence. Tomato Ve1 was identified as an effective resistance locus that recognizes Ave1 secreted by Race 1 strains (Fradin et al., 2009; Schaible et al., 1951). Genetic analyses indicated that EDS1, NDR1 and SERK3/BAK1 are required for Ve1-mediated resistance in both tomato and Arabidopsis (Fradin et al., 2011; Fradin et al., 2009). Studies in cotton also showed that GhBAK1 and GhNDR1 are crucial components regulating defense against V. dahliae (Gao et al., 2013b; Gao et al., 2011), demonstrating the common requirements of NDR1 and SERK3/BAK1 in a conserved
defense mechanism against *V. dahliae* in these plants. *GbWRKY1, GhSSN, GbERF, GhMLP28, GhMKK2* and *GbNRX1* have also been shown to be required for cotton resistance against *V. dahliae* (Gao et al., 2011; Li et al., 2014a; Li et al., 2016; Qin et al., 2004; Sun et al., 2014; Yang et al., 2015). A comparative proteomic analysis indicated the involvement of both brassinosteroids and jasmonic acid signaling pathways in the regulation of cotton resistance to *V. dahliae* (Gao et al., 2013a).

Although several regulators have been identified, the mechanisms by which plants defend against *V. dahliae* remain obscure, and further investigation is required to isolate more host immune components governing *V. dahliae* resistance. Targeting key immune components is a common strategy employed by pathogenic effectors to promote pathogenicity (Boller and He, 2009; Cui et al., 2009; Dou and Zhou, 2012); thus, screening of host proteins targeted by pathogenic effectors provides an efficient way to identify crucial host defense components.

The calmodulin-binding proteins (CBPs) functions to bind calmodulin to transduce calcium signals (Bouche et al. 2005). The plant-specific CALMODULIN BINDING PROTEIN 60 (CBP60) protein family contains eight family members including CBP60a–g and SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1) (Bouche et al. 2005). CBP60g and SARD1 are two most closely related members that function partially redundantly in both SA signaling and bacterial resistance (Zhang et al. 2010; Wang et al., 2011). CBP60g contains a calmodulin-binding domain (CBD) which is essential for its function in defense, whereas SARD1 does not bind calmodulin (Wang et al., 2009; Wang et al. 2011;
Zhang et al. 2010). Both CBP60g and SARD1 function as transcription factors that
directly bind to promoters of genes controlling SA synthesis, such as

**ISOCHORISMATE SYNTHASE 1 (ICS1), ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5), NON-EXPRESSOR OF PATHOGENESIS RELATED GENES1 (NPR1)**

(Dong et al., 2004; Nawrath et al., 2002; Sun et al., 2015; Wildermuth et al., 2004).

Moreover, ChIP-seq analyses have revealed the binding of CBP60g and SARD1 to
the promoters of a number of genes regulating pathogen-associated molecular patterns
(PAMPs)-triggered immunity (PTI), effector-triggered immunity (ETI) and systemic
acquired resistance (SAR) (Sun et al., 2015), indicating their broad role in the
regulation of plant immunity. In this study, we identified VdSCP41 as a virulence
effector that suppresses plant immunity induced by PAMPs. VdSCP41 interacts with
the *Arabidopsis* CBP60g and SARD1 and modulates their transcription factor activity.

The contribution of VdSCP41 in *V. dahliae* virulence is significantly reduced during
the infection of the *cbp60g-1/sard1-1* double mutant. GhCBP60b, the closest protein
homolog of CBP60g in cotton, is also targeted by VdSCP41 and contributes to cotton
resistance against *V. dahliae*. Taken together, our findings revealed that CBP60g,
SARD1 and GhCBP60b are novel components governing *V. dahliae* resistance and
that these proteins are modulated by a secretory effector VdSCP41.

**RESULTS**

**VdSCP41 Contributes to *V. dahliae* Virulence in *Arabidopsis and Cotton Plants***

Secretome analyses revealed greater than 700 potential secreted proteins in *V.
*V. dahliae* (Klosterman et al., 2011). To date, relatively few secreted proteins carrying virulence function have been characterized in *V. dahliae*. We performed a reverse genetic screen to identify secreted proteins that are crucial for *V. dahliae* virulence. Using the newly developed USER-ATMT-DS binary vector (Wang et al., 2016), we constructed 56 gene deletion mutants in *V. dahliae* strain 592 (V592) that each targets an individual potentially secreted protein. The resulting mutants were subjected to virulence assessment in host plants, including upland cotton and the model plant *Arabidopsis*. A mutant carrying a targeted deletion of *VdSCP41* (VdΔscp41) was isolated (Figure 1A) and shown to display significantly reduced virulence compared with WT strain V592.

*VdSCP41* encodes a hypothetical protein in the secretome of the *V. dahliae* Ls.17 strain (VdLs.17) (Klosterman et al., 2011), and the expression of this gene is significantly up-regulated at 2 days post-inoculation (dpi) in *Arabidopsis* (Figure 1-figure supplement 1), suggesting a putative function of *VdSCP41* in *V. dahliae* infection. Targeted gene deletion of *VdSCP41* in the VdΔscp41 mutant was verified by Southern blotting (Figure 1B). The VdΔscp41 mutant displayed much weaker disease symptoms than WT V592 in both upland cotton (*Gossypium hirsutum*) (Figure 1C) and *Arabidopsis* (Figure 1D). Disease index analyses indicated significantly reduced virulence of the VdΔscp41 mutant compared with that of V592 in both upland cotton and *Arabidopsis* (Figure 1E-F, Figure 1—source data 1). The reduced virulence of the VdΔscp41 mutant was restored upon complementation with
GFP-tagged VdSCP41 (VdΔscp41/VdSCP41-GFP) (Figure 1C-F). Thus, VdSCP41 functions as a virulence effector that contributes to V. dahliae virulence on host plants.

VdSCP41 Acts as an Intracellular Effector

In V. dahliae, some signal peptide-containing SCPs are delivered to the septin-organized hyphal neck, which develops from the base of the hyphopodia and functions as a fungus-host penetration interface for the dynamic delivery of secretory proteins (Zhou et al., 2017). VdSCP41 contains an N-terminal signal peptide predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/) (Figure 2-figure supplement 1A). Therefore, we examined the localization of VdSCP41 in V. dahliae. GFP-tagged VdSCP41 (VdSCP41-GFP) was found to localize to the base of the hyphopodium and showed ring signals surrounding the hyphal neck when the VdΔscp41 mutant strain complemented with VdSCP41-GFP (VdΔscp41/VdSCP41-GFP) was cultured on a cellophane membrane for hyphopodium induction (Figure 2A). In contrast, VdSCP41 lacking signal peptide (ΔspVdSCP41-GFP) showed diffused signal at the base of the hyphopodium without clear ring signals surrounding the hyphal neck. The results demonstrate that VdSCP41-GFP was delivered to the penetration interface for secretion.

Nuclear localization signal (NLS) sequence prediction (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) identified a potential NLS in VdSCP41 (Figure 2-figure supplement 1A). We therefore took advantage of this NLS to examine the putative translocation of V. dahliae-delivered VdSCP41 into
the nucleus of plant cells. The VdΔscp41/VdSCP41-GFP and GFP-expressing V592 (V592-GFP) strains were separately inoculated onto onion epidermal cells. While GFP fluorescence from the V592-GFP strain was observed in conidial spores, VdSCP41-GFP secreted by V. dahliae was capable of translocating into plant cells and localizing to the nucleus, in addition to the pericellular space of onion epidermal cells (Figure 2-figure supplement 1B). The potential NLS sequence of SCP41 was mutated (SCP41-nls-GFP) and then complemented into VdΔscp41 to construct the VdΔscp41/VdSCP41-nls-GFP strain. In contrast to VdSCP41-GFP, VdSCP41-nls-GFP failed to translocate into the nucleus of onion epidermal cells (Figure 2-figure supplement 1B). These results suggested that VdSCP41 delivered by V. dahliae translocates into the nucleus of the plant cell, which requires the predicted NLS within its sequence.

We next transiently expressed mCherry-tagged VdSCP41 in plants to further verify its nuclear localization in plant cells. As the signal peptide located at the N terminus may guide secreted proteins into the plant extracellular space in some cases, we fused mCherry to VdSCP41 both with and without (ΔspVdSCP41) the signal peptide for analyses of subcellular localization. VdSCP41-mCherry and ΔspVdSCP41-mCherry were individually transiently expressed in either Arabidopsis protoplasts or Nicotiana benthamiana (N. b.) leaves. mCherry fluorescence imaging revealed that both VdSCP41 and ΔspVdSCP41 localized to the nucleus in Arabidopsis cells (Figure 2B). The protein expression level of VdSCP41-mCherry and ΔspVdSCP41-mCherry was detected by immunoblot (Figure 2-figure supplement 1C).
Similar nuclear localization was observed for both VdSCP41-mCherry and ΔspVdSCP41-mCherry at the nucleus of N. b. cells (Figure 2-figure supplement 1D). These results are consistent with the nuclear localization of the V. dahliae-delivered VdSCP41 in onion epidermal cells.

**VdSCP41 Expression in Arabidopsis Inhibits Plant Immunity**

It is believed that the initial function of a fungal effector protein is to suppress PTI. To investigate whether VdSCP41 is capable of inhibiting plant immunity when it is directly expressed in plants, Arabidopsis transgenic lines expressing ΔspVdSCP41 were constructed and assessed for PAMP-induced defense responses. Flg22 is the best-characterized PAMP derived from a bacterial pathogen, and it induces the expression of PTI-responsive genes in wildtype (WT) Arabidopsis plants. We observed reduced induction of flg22-induced ICS1 in two independent VdSCP41-expressing lines (Figure 2-figure supplement 1E), suggesting inhibition of PTI conferred by VdSCP41.

NLP proteins derived from bacterial, oomycete and fungal organisms have recently been characterized as PAMPs. A conserved 20-amino acid peptide (nlp20) within NLP represents the active immunogenic motif that induces PTI in plants (Albert et al., 2015; Böhm et al., 2014; Ottmann et al., 2009). We previously reported the immune-inducing activity of VdNLP1 and VdNLP2 derived from V592 in N. b., Arabidopsis, and cotton plants (Zhou et al., 2012). A corresponding peptide located in VdNLP2 derived from V592 (nlp20<sup>Vd2</sup>) was synthesized and shown to cause
up-regulation of cotton PR genes (Du et al., 2017), indicating the immunogenic activity of this peptide in cotton. Treatment of Arabidopsis with nlp20\textsuperscript{Vd2} significantly induced ICS1 and FMO1 expression (Figure 2C-D, Figure 2—source data 1), indicating that nlp20\textsuperscript{Vd2} also exhibits the active immunogenic activity characteristic of a PAMP in Arabidopsis. In transgenic lines expressing VdSCP41, but not transgenic lines expressing NLS mutated VdSCP41 (\textit{\textit{A}spVdSCP41}_{\text{nlk}}), suppression of nlp20\textsuperscript{Vd2}-induced ICS1 (Figure 2C) and FMO1 (Figure 2D) was observed, suggesting that VdSCP41 expression in plants inhibits nlp20\textsuperscript{Vd2}-triggered immunity. ICS1 encodes the key enzyme controlling SA production and is required for pathogen-induced SA accumulation (Wildermuth et al. 2001). We next quantified SA production in response to a nonpathogenic bacterial pathogen, \textit{Pst} DC3000 \textit{hrc}C, in both WT and \textit{\textit{A}spVdSCP41}-expressing plants. In consistent with the suppression of PAMP-induced ICS1 expression, transgenic lines expressing \textit{\textit{A}spVdSCP41} accumulated less free SA in response to \textit{Pst hrc}C than did WT plants (Figure 2-figure supplement 2A).

Inoculation of WT \textit{V. dahliae} strain V592 on Arabidopsis induced the expression of ICS1 and FMO1 (Figure 2-figure supplement 2B-C). This induced expression was further enhanced when the plants were inoculated with the Vd\textit{\textit{A}scp41} mutant compared with V592 (Figure 2-figure supplement 2B-C), indicating that VdSCP41 suppresses the induced expression of ICS1 and FMO1 during \textit{V. dahliae} infection. Taken together, these results reveal an inhibitory role of VdSCP41 in modulating plant immunity.
Modulating the activity of plant immune components is a common strategy used by effectors to suppress host immunity. To explore the virulence mechanisms employed by VdSCP41 in inhibiting plant immunity, we next searched for plant components that are targeted by VdSCP41. ΔspVdSCP41 was fused with a 3×FLAG tag and transiently expressed in Arabidopsis protoplasts followed by purification of VdSCP41-containing protein complexes. Protein lysates were immuno-precipitated using anti-FLAG-conjugated beads and subjected to tandem mass spectrometry. The plant CBP CBP60g was identified as a candidate interactor of VdSCP41 (Supplementary File 1-Table S1).

CBP60g was then fused with a 3×HA tag and used for reverse co-immunoprecipitation (Co-IP) analysis to verify its interaction with VdSCP41. FLAG-tagged VdSCP41 was transfected, either alone or together with HA-tagged CBP60g, into Arabidopsis protoplasts for transient expression. Anti-HA IP followed by an anti-FLAG immunoblot revealed that VdSCP41 was co-purified with CBP60g from plant cells (Figure 3-figure supplement 1A). VdSCP41 was further divided into an N-terminal portion (VdSCP41N, amino acid 1-213) and a C-terminal portion (VdSCP41C, amino acid 163-end), which exhibited an overlap of 50 amino acids, and was used to test interactions with CBP60g. Co-IP analysis revealed that VdSCP41C is sufficient for interacting with CBP60g (Figure 3A).
Quantitative luciferase complementation imaging assays were performed to further verify the interaction between VdSCP41 and CBP60g in N. b. Co-expression of the N-terminal region of luciferase (NLuc)-tagged BIK1 and the C-terminal region of luciferase (CLuc)-tagged XLG2 driven by the 35S promoter was performed as a positive interaction control (Liang et al., 2016). The co-expression of NLuc-VdSCP41 and CLuc-CBP60g driven by the 35S promoter in N. b. resulted in much higher luciferase activity than co-expression of NLuc-VdSCP41 and CLuc-XLG2, or CLuc-CBP60g and NLuc-BIK1 (Figure 3B-C, Figure 3-figure source data 1), confirming the interaction between VdSCP41 and CBP60g in the plants. The expression levels of the NLuc- and CLuc-fusion proteins were further detected via immunoblotting (Figure 3-figure supplement 1B).

The nuclear localization of VdSCP41 prompted us to examine whether CBP60g co-localizes with VdSCP41 in the nucleus. GFP-tagged CBP60g mainly localized in the nucleus when it was expressed alone in N. b. cells (Figure 3-figure supplement 1C). GFP-tagged CBP60g was co-expressed with mCherry-tagged VdSCP41 without signal peptide (ΔspVdSCP41-mCherry) in N. b. leaves via Agrobacterium-mediated transient expression. An overlay of the results of GFP and mCherry fluorescence imaging indicated co-localization of ΔspVdSCP41 and CBP60g in the nucleus in N. b. cells (Figure 3D). The protein expression level of GFP-CBP60g, ΔspVdSCP41-mCherry and ΔspVdSCP41-nls-mCherry was detected by immunoblot with the indicated antibodies (Figure 3-figure supplement 1D). In addition to co-localization, co-expression of ΔspVdSCP41-mCherry significantly increased the
nuclear accumulation of CBP60g-GFP (Figure 3D), which was not observed when
$\Delta p VdSCP41_{nls}$-mCherry with a mutated NLS was co-expressed with CBP60g-GFP
(Figure 3D).

CBP60g was induced by pathogen and PAMP treatments and required for full
resistance against bacterial pathogens (Wang et al., 2011; Zhang et al., 2010b). A
closely related protein in the CBP family, SARD1, functions partially redundantly
with CBP60g in bacterial resistance (Sun et al., 2015; Wang et al., 2011). We also
detected an interaction between VdSCP41 and SARD1 by Co-IP in Arabidopsis
protoplasts (Figure 3-figure supplement 2A). VdSCP41 co-localized with SARD1 in
$N. b$. leaves and increased its nuclear accumulation (Figure 3-figure supplement 2B),
indicating similar targeting of SARD1 by VdSCP41. It is unlikely an interaction
between CBP60g and SARD1 because the luciferase complementation assay did not
show interaction between CLuc-tagged CBP60g and NLuc-tagged SARD1 (Figure
3-figure supplement 3). Taken together, the above results demonstrated that both
CBP60g and SARD1 are targeted by VdSCP41.

VdSCP41 Interferes with the Transcription Factor Activity of CBP60g

CBP60g encodes a plant-specific transcription factor that regulates the
expression of a number of defense-related genes. The fact that CBP60g functions as
master transcription regulator (Sun et al., 2015; Wang et al., 2011) prompted us to
examine whether the targeting of CBP60g by VdSCP41 affects the induction of its
target genes. Dual reporter analyses revealed that $CBP60g$ expression in Arabidopsis
protoplasts significantly enhanced the expression of *ICS1::LUC* or *FMO1::LUC* (firefly luciferase) (Figure 4A-B, Figure 4—source data 1). Co-expression of *VdSCP41* inhibited CBP60g-induced *ICS1::LUC* (Figure 4A) and *FMO1::LUC* (Figure 4B) expression, whereas the co-expression of *VdSCP41N*, which is unable to bind CBP60g, was impaired in this inhibition (Figure 4A-B). CBP60g was next divided into an N-terminal portion (CBP60gN, amino acid 1-361) containing its DNA-binding domain and a C-terminal portion (CBP60gC, amino acid 211-end) lacking the functional DNA-binding domain (Zhang et al., 2010b), which exhibited an overlap of 150 amino acids. CBP60gN and CBP60gC were then used to test interactions with VdSCP41. Co-IP analysis showed that VdSCP41 binds to CBP60gC, but not CBP60gN (Figure 4C). The results indicated that VdSCP41 binds the C-terminal portion of CBP60g to interfere with its activity.

To test whether VdSCP41 targeting directly affects the DNA-binding activity of CBP60g, recombinant GST-tagged CBP60g and His-tagged VdSCP41C were purified and used for electrophoretic mobility shift assays (EMSAs). GST-CBP60g showed a specific binding to a 60-bp DNA fragment (*ICS1* promoter probe) within the *ICS1* promoter, which is reduced by the addition of unlabelled probe, as previously reported (Zhang et al. 2010) (Figure 4D). The preincubation of VdSCP41C with CBP60g significantly reduced the DNA-binding activity of CBP60g, whereas a soluble fragment of His-tagged VdSCP41 which does not contain its C-terminal portion (*VdSCP41*<sub>100-163</sub>) did not (Figure 4D). Another His-tagged *V. dahliae* protein (VDAG_01962) also did not affect the DNA-binding activity of CBP60g (Figure 4D).
Coexpression of VdSCP41 did not lead to cleavage or mobility shift of CBP60g (Figure 4-figure supplement 1), suggesting that VdSCP41 is unlikely to act as a protease to target CBP60g. The results proved that binding of VdSCP41C to CBP60g directly inhibits the DNA-binding activity of CBP60g.

CBP60gC Harbors a Transcription Activation Domain that is Required for Interacting with VdSCP41

The binding of VdSCP41 to the C-terminal portion of CBP60g prompted us to test the role of CBP60gC in CBP60g-mediated gene activation. Compared to the full induction of *ICS1::LUC* or *FMO1::LUC* by CBP60g (Figure 5A, Figure 5—source data 1), the deletion of the C-terminal portion (ΔC-CBP60g) dramatically compromised its activity to induce both *ICS1::LUC* and *FMO1::LUC* (Figure 5A), indicating that CBP60gC is required for CBP60g-mediated gene activation. The results suggest that putative transcription activator activity may be harbored within the CBP60gC. The basic helix-loop-helix (bHLH) transcription factor MYC2 directly binds to the G-box-like (CANNTG) element (Dombrecht et al., 2007; Godoy et al., 2011; Lian et al., 2017) via its bHLH domain to regulate the expression of its target genes, such as the *TERPENE SYNTHASE gene 10 (TPS10)* (Li et al., 2014b). We therefore took advantage of bHLH-mediated binding to the *TPS10* promoter to examine the putative transcription activator activity of CBP60gC. A fragment within the C-terminal portion of CBP60g (amino acid 211-440) activated *TPS10::LUC* reporter when it was fused with the bHLH domain of MYC2 (bHLH-CBP60gC211-440).
(Figure 5B, Figure 5—source data 1), compared with bHLH\textsubscript{MYC2} alone, indicating that the CBP60g\textsubscript{211-440} harbors transcription activator activity. Moreover, Co-IP analysis showed that CBP60g\textsubscript{211-end}, but not CBP60g\textsubscript{441-end}, co-purified with VdSCP41, indicating the requirement of CBP60g\textsubscript{211-440} for binding to VdSCP41 (Figure 5C). Thus, CBP60gC harbors a transcription activation domain, CBP60g\textsubscript{211-440}, that is required for VdSCP41 targeting.

The ΔC-CBP60g was further co-transfected with CBP60g, together with \textit{ICS1::LUC} or \textit{FMO1::LUC}. Dual reporter analyses indicated that co-expression of ΔC-CBP60g significantly suppressed CBP60g-induced \textit{ICS1::LUC} and \textit{FMO1::LUC} (Figure 5A), suggesting a dominant negative effect of ΔC-CBP60g on the activity of CBP60g.

\textbf{CBP60g and \textit{SARD1} Contribute to \textit{Arabidopsis} Resistance against \textit{V. dahliae}}

\textit{SARD1} functions both redundantly and differentially with \textit{CBP60g} (Sun et al., 2015; Wang et al., 2011). The finding that VdSCP41 targeted both CBP60g and SARD1 prompted us to assess the roles of \textit{CBP60g} and \textit{SARD1} in resistance to \textit{V. dahliae}. The WT and \textit{cbp60g-1/sard1-1} double mutant plants were inoculated with V592 for the assessment of disease symptoms. As shown in Figure 6, the \textit{cbp60g-1/sard1-1} double mutant displayed compromised resistance compared with the WT plants, demonstrating a contribution of \textit{CBP60g} and \textit{SARD1} to \textit{V. dahliae} resistance.

We next investigated the requirement of \textit{CBP60g} and \textit{SARD1} for
VdSCP41-mediated virulence. The WT and cbp60g-1/sardl-1 double mutant plants were inoculated with the VdΔscp41 mutant. The VdΔscp41 mutant displayed reduced virulence on the WT plants compared with V592. The reduced virulence arising from VdSCP41 deletion was partially impaired in the cbp60g-1/sardl-1 double mutant plants compared with than in the WT plants (Figure 6, Figure 6—source data 1). The results indicated that both CBP60g and SARD1 are required for full virulence conferred by VdSCP41. However, we still observed reduced virulence of the VdΔscp41 mutant compared with V592 on the cbp60g-1/sardl-1 double mutant plants, suggesting the existence of additional targets for VdSCP41 during V. dahliae infection.

VdSCP41 Interacts with and Co-localizes with GhCBP60b

As in Arabidopsis, we observed similar compromised virulence of VdΔscp41 mutant in upland cotton (Figure 1E) compared with V592. The results prompted us to test the putative VdSCP41 targeting of GhCBP60b, the closest protein homolog of CBP60g and SARD1 in cotton. Co-IP analysis revealed an interaction between VdSCP41 and GhCBP60b (Figure 7A). To examine the subcellular localization of GhCBP60b, GhCBP60b-GFP was constructed for transient expression in N. b. leaves. GhCBP60b-GFP localized to the nucleus of N. b. cells, and co-expression of GhCBP60b-GFP with VdSCP41-mCherry revealed co-localization of VdSCP41 and GhCBP60b in the nucleus of N. b. cells (Figure 7B), suggesting conserved targeting of Arabidopsis CBP60g and SARD1 and cotton GhCBP60b by VdSCP41.
**GhCBP60b is Required for Cotton Resistance against *V. dahliae***

To further examine whether *GhCBP60b* functions in resistance to *V. dahliae* in cotton, we generated a virus-induced gene silencing (VIGS) vector (Liu et al., 2002) targeting *GhCBP60b* (pTRV2-*GhCBP60b*). Cotton plants were infiltrated with pTRV1 together with pTRV2 or pTRV2-*GhCBP60b* and further inoculated with *V. dahliae*. Compared with pTRV2, pTRV2-*GhCBP60b*-infiltrated cotton plants exhibited higher ratios of wilting (Figure 7C, Figure 7—source data 1) and more severe disease symptoms (Figure 7D-E, Figure 7—source data 1), indicating a role for *GhCBP60b* in cotton resistance to *V. dahliae*. The reduced expression of *GhCBP60b* in pTRV2-*GhCBP60b*-infiltrated plants was verified via RT-PCR (Figure 7F, Figure 7—source data 1). The results support the targeting of GhCBP60b by VdSCP41 for virulence during *V. dahliae* infection in cotton.

**DISCUSSION**

In this study, we identified VdSCP41 as an intracellular effector that is crucial for *V. dahliae* virulence. VdSCP41 targets *Arabidopsis* CBP60g and SARD1 to interfere with the induction of their target genes. CBP60g and SARD1 are required for *Arabidopsis* resistance to *V. dahliae* and VdSCP41-mediated virulence. Silencing of *GhCBP60b* compromised cotton resistance to *V. dahliae*. The results revealed a conserved and important role for *Arabidopsis* CBP60g and SARD1 and cotton GhCBP60b, which are modulated by an intracellular effector, in plant resistance against *V. dahliae*. 
VdSCP41 is Secreted by *V. dahliae* and Translocates into Plant Cells

To actively suppress plant defense or modulate host physiology to benefit pathogenic fitness, oomycete and fungal pathogens deliver hundreds of effectors through specialized intracellular fungal structures, such as haustoria and infection hyphae, into the host apoplastic space or directly into plant cells (Lo Presti et al., 2015). Although haustoria have not been observed during infection, *V. dahliae* develops a penetration peg from a hyphopodium when infecting plant roots (Zhao et al., 2016). During *V. dahliae* infection, the penetration peg developed from the hyphopodium further develops a hyphal neck and forms a septin ring that partitions the hyphopodium and invasive hyphae. This septin-organized apparatus functions as a fungus-host interface for the dynamic delivery of secretory proteins, such as SCPs (Zhao et al., 2016; Zhou et al., 2017).

To date, relatively few *V. dahliae*-secreted effectors have been characterized as functioning inside host cells to modulate host immunity. Here, we provided evidence of the secretion and translocation of VdSCP41 secreted by *V. dahliae* into plant cells. VdSCP41 contains a signal peptide and localizes to the base of the hyphopodium to form a septin-like ring during infection (Figure 2A), indicating that it is secreted via the septin-organized apparatus at the fungus-host interface. The localization of VdSCP41 delivered by *V. dahliae* at the nucleus of onion epidermal cells (Figure 2-figure supplement 1B) suggested the translocation of VdSCP41 into plant cells. A few conserved motifs have been indicated to serve as signals for the uptake of fungal...
or oomycete effectors into plant cells. For example, a few effectors secreted by cereal powdery mildew and rust pathogens possess a Y/F/WxC motif that serves as a signal for translocation into the plant cell (Godfrey et al., 2010; Spanu et al., 2010). A set of oomycete effectors contain a RXLR-dEER motif that appears to assist in targeting effectors into plant cells (Dou et al., 2008; Whisson et al., 2007). Another set of oomycete effectors possess a FLAK motif for translocation (Schornack et al., 2010).

However, VdSCP41 lacks any of the above conserved motifs, and the mechanisms by which assist the uptake of VdSCP41 into plant cells remain unclear.

**VdSCP41 Targets CBP60g and SARD1 and Interferes with Plant Defense**

CBP60g and SARD1 are master regulators in immunity. ChIP-seq analyses allowed the identification of a large number of targets genes for CBP60g and SARD1 and support a model in which CBP60g and SARD1 accumulate in the plant nucleus and act as master regulators, to activate both positive and negative immune regulators (Sun et al., 2015; Wang et al., 2009; Zhang et al., 2010b). Genetic evidence revealed that CBP60g and SARD1 are positive immune regulators required for immune responses and bacterial resistance (Wang et al., 2009; Zhang et al., 2010b). We demonstrated that VdSCP41 targets CBP60g and SARD1 and interferes with their activity, thus supporting the virulence function of VdSCP41 for immune suppression.

Consistent with the inhibition of CBP60g and SARD1 activity, we observed less pathogen-induced SA accumulation in transgenic lines expressing *VdSCP41* than in WT plants (Figure 2-figure supplement 2A). As *CBP60g* and *SARD1* also directly bind to the promoters of both *ALD1* and *SARD4* (Sun et al., 2018), two major genes
encoding pipecolic acid (Pip) biosynthesis enzymes, to regulate Pip biosynthesis, a potential contribution of Pip to \textit{V. dahliae} resistance will be of interest for further investigation.

We showed that VdSCP41 binds the C-terminal portion of CBP60g to interfere with its transcription factor activity (Figure 4A-B). Coexpression of VdSCP41 did not lead to cleavage or mobility shift of CBP60g (Figure 4-figure supplement 1), suggesting that VdSCP41 is unlikely to act as a protease to target CBP60g. In addition, CBP60g\textsubscript{211-440}, a domain within the C-terminal portion of CBP60g, harbors transcription activator activity (Figure 5B) and is required for interacting with VdSCP41 (Figure 5C). It is likely that CBP60g\textsubscript{C} functions to promote transcriptional activation via recruiting additional activators, and binding of VdSCP41 interrupts the activity of this domain or the recruitment of associated activators via this domain. The dominant negative function of ΔC-CBP60g (Figure 5A) supports a dominant negative effect on CBP60g activity arising from increased nuclear accumulation of VdSCP41-impaired CBP60g. Thus, VdSCP41 mediated over-accumulation of CBP60g provides an additional strategy to further interfere with its transcription factor activity. The results suggest a novel virulence strategy in which a pathogenic effector directly targets host transcription factors to interfere with their activity and modulate plant immunity. However, it is equally possible that the increased nuclear accumulation of CBP60g results from the feed-back regulation as a result of impaired CBP60g function.

\textbf{VdSCP41 Functions to Suppress Immunity}
CBP60g and SARD1 are key components of the SA signaling pathway, which serves as an attractive target for bacterial and fungal effectors (DebRoy et al., 2004; Djamei et al., 2011; Nomura et al., 2011). Targeting of CBP60g and SARD1 by VdSCP41 may therefore interfere with plant resistance to *V. dahliae* via the manipulation of SA signaling. On the other hand, ChIP-seq analyses identified a number of SA-independent regulators that are directly targeted by CBP60g and SARD1 (Sun et al., 2015), revealing broader and SA-independent functions of CBP60g and SARD1 in immunity. It is likely that signaling pathways other than SA are also targeted by VdSCP41 through CBP60g and SARD1 to interfere with plant immunity. Consistent with this assumption, we showed that nlp20Vd2 functions to trigger the induction of both SA-dependent and SA-independent defense genes during *V. dahliae* infection (Figure 2C-D). Furthermore, VdSCP41 expression suppressed the induction of both SA-dependent *ICS1* and SA-independent *FMO1* by nlp20Vd2 (Figure 2C-D). Modulation of CBP60g and SARD1 may result in interference with their function as master transcription factors in PTI and in other defense pathways.

Taken together, our results support a model in which PAMPs from *V. dahliae*, such as NLPs and chitins, are recognized by plants to induce the expression of CBP60g and SARD1, which subsequently regulate the expression of a number of immune regulators to defend against pathogen infection (Figure 8A). VdSCP41 secreted by *V. dahliae* functions as an intracellular effector that targets the transcription activator domain of CBPs, interrupting the function of this domain, to interfere with their transcription factor activity, and thus modulates both
SA-dependent and SA-independent regulators to inhibit plant immunity against *V. dahliae* (Figure 8B).
### Key Resources Table

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**Fungal strains, plant materials and antibodies.**

The *Verticillium dahliae* strain V592 (Gao et al., 2010) was used in this study. *Verticillium dahliae* strains were grown on potato dextrose agar (PDA) medium at 25°C in the dark. To collect conidia, the mycelial plugs were cultured in potato dextrose broth (PDB) liquid medium with shaking at 200 rpm at 25°C for 3-5 days. Cotton plants (‘Xinluzao No. 16’) were used for virulence assessment in this study (Zhou et al., 2017). *Arabidopsis thaliana* plants used in this study includes Col-0 (wild-type), the *cbp60g-1/sard1-1* mutant (Zhang et al., 2010b). *VdSCP41* was amplified from the V592 cDNA and cloned into pCambia1300-35S-FLAG (Zhang et al., 2010a) to construct *Arabidopsis* transgenic lines expressing *VdSCP41*. Antibodies used in this study includes anti-FLAG (RRID:AB_259529), anti-HA (RRID:AB_514506), anti-CLuc (RRID:AB_439707), anti-GFP (RRID:AB_390913), and anti-mCherry (Easybio, BE2026).

**Generation of deletion and complementation fungal strains**

The upstream and downstream flanking sequences were PCR amplified from V592 genomic DNA and cloned into pGKO-HPT vector (Wang et al., 2016). The resulting construct was transformed into *Agrobacterium tumefaciens* EHA105, and used for *Agrobacterium tumefaciens*-mediated transformation (ATMT) to generate the *VdΔscp41* mutant strain (Wang et al., 2016). Genomic region, including 1.5 kb upstream from the start codon, of *VdSCP41* was amplified and cloned into pNat-Tef-TrpC vector (Zhou et al., 2017) to generate construct for complementation. The resulting construct was transformed into *Agrobacterium tumefaciens* EHA105,
and used for ATMT to generate VdΔscp41/VdSCP41 strain. Primers used in this study are listed in Supplementary File 2.

Protein complex purification and mass spectrum analysis

*Arabidopsis* protoplasts isolated from 10 gram leaves were transfected with ΔspVdSCP41-FLAG, ΔspVdSCP45-FLAG, or empty vector for protein expression. Transfected protoplasts were collected and total protein was extracted with extraction buffer containing 50mM HEPES, pH7.5, 150 mM KCl, 1 mM EDTA, 1 mM DTT, 0.2% Triton X-100, and 1×proteinase inhibitor cocktail. Total protein was incubated with 50μl anti-FLAG agarose beads (sigma) for 12 hours at 4°C. The immunocomplex was washed three times using the above buffer and eluted with 100μl 1μg/μl 3×FLAG peptide. The eluted proteins were run 10 mm into the separating gel and stained with Proteo Silver stain kit (Sigma). Total protein was destained and digested in-gel with sequencing grade trypsin (10 ng/mL trypsin, 50 mM ammonium bicarbonate [pH 8.0]) overnight. Peptides were sequentially extracted with 5% formic acid/50% acetonitrile and 0.1% formic acid/75% acetonitrile and concentrated to 20 μl. The extracted peptides were separated by an analytical capillary column packed with 5 mm spherical C18 reversed-phase material. The eluted peptides were sprayed into a LTQ mass spectrometer (Thermo Fisher Scientific) equipped with a nano-ESI ion source. The mass spectrometer was operated in data-dependent mode with one MS scan followed by five MS/MS scans for each cycle. Database searches were performed on an in-house Mascot server (Matrix Science Ltd., London, UK) against IPI (International Protein Index) *Arabidopsis* protein database.
Co-immunoprecipitation assay in protoplasts

Five-week-old *Arabidopsis* plants were used for protoplast isolation. pUC-35S-VdSCP41-FLAG, or its variants construct, was co-transfected with pUC-35S-CBP60g-HA, or its variants construct, pUC-35S-SARD1-HA, or pUC-35S-GhCBP60b-HA, into *Arabidopsis* protoplasts. Total protein was extracted with extraction buffer. For anti-HA IP, total protein was incubated with 2 μg of anti-HA antibody together with protein A agarose at 4°C for 4 hours. The agarose were collected and boiled for 5 minutes with 1× protein loading buffer. Immunoprecipitates were separated by 10% SDS-PAGE and the presence of VdSCP41-FLAG or its variants, CBP60g-HA or its variants, SARD1-HA, or GhCBP60b-HA was detected by anti-FLAG or anti-HA immunoblot respectively.

SA Measurement

SA was extracted and measured following the method described previously (Sun et al., 2015). Around 0.3 gram leaf tissue collected from 4-week-old plants was ground into powder in liquid nitrogen. Plant leaves were infiltrated with or without *Pst* DC3000 *hrcC* (OD600=0.1) 12 hours before sample collection. Three samples for each treatment were analysed. The samples were extracted with 0.8 mL 90% methanol and sonicated for 15 min, and the supernatant was transferred into a new tube. The pellet was re-extracted with 0.5 mL of 100% methanol, the supernatant was combined with the first-step supernatant and dried by vacuum. The pellet was resuspended in 500 μL 0.1 M sodium acetate (pH 5.5) in 10% methanol. An equal volume of 10% TCA was added and the samples were vortexed, sonicated for 5min. After centrifuged, the
supernatant was extracted three times with 0.5 ml of extraction buffer (ethylacetate/cyclopentane/isopropanol:100/99/1 by volume). After spinning, the organic phases were collected and dried by vacuum. The samples were then dissolved in 250 μL 100% methanol and filtered through a 0.22 μm filter. The samples were then assayed by HPLC-MS/MS analysis on AB SCIEX QTRAP 4500 system (AB SCIEX, Foster, CA, USA).

Luciferase complementation imaging assay

Agrobacterium tumefaciens GV3101 strain carrying CLuc or NLuc tagged construct were infiltrated into leaves of 4-week-old Nicotiana benthamiana. LUC activity in leaves was examined 2 days post infiltration. N. b. leaves were treated with 1 μM luciferin and kept in dark for 5 min to quench the fluorescence. LUC image was captured by CCD imaging apparatus and the quantitative LUC activity was determined by microplate luminometer. Expression of CLuc-tagged proteins or NLuc-tagged proteins was detected by anti-CLuc or anti-HA western blot, respectively.

Electrophoretic mobility shift assays (EMSAs)

The full-length CBP60g was cloned into the pGEX-6p-1 vector. VdSCP41C, VdSCP41_{100-163} or VDAG_01962 was cloned into the pET-28a vector. The resulting constructs were transformed into in E. coli BL21 (DE3) competent cells. BL21 (DE3) strains containing the expression vectors were cultured and induced by IPTG at 16°C for 16 hours. GST-tagged full-length CBP60g, His-tagged VdSCP41C or VdSCP41_{100-163}, and His-tagged VDAG_01962 recombinant protein were affinity
purified and used for EMSAs. A 60-bp probe within the DNA fragment 7 (Zhang et al., 2010) in the ICS1 promoter were labeled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase. Binding reactions were carried out in a 20μl volume of reaction buffer [10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1mM DTT, 1 μl 50 ng/μl poly(dI-dC)] for 30 min at room temperature. Labeled DNA probe (2 fmol) was incubated with 4 μg GST-CBP60g. 150× unlabeled DNA probe was used for competition. 1.5 μg His-tagged VdSCP41C\textsubscript{163-end}, VdSCP41\textsubscript{100-163}, or VDAG\textunderscore{}01962 was preincubated with GST-CBP60g for 30 min at room temperature before DNA binding. The reaction was stopped by adding DNA loading buffer and the samples were separated by a 5% native PAGE gel. After electrophoresis, the gel was autoradiographed.

RNA isolation and qRT-PCR

Total RNA was isolated with the TRIzol reagent (Invitrogen) and used for cDNA synthesis with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) following the instructions provided by the manufacturer. The quantitative PCR were performed with SYBR Premix Ex Taq kit (TaKaRa) following standard protocols. *Arabidopsis* col-0 or transgenic plants were treated with H$_2$O, 1μM flg22 or nlp20\textsuperscript{Vd2} (Du et al., 2017) as indicated for 3 hours. RNA was isolated and used for RT-PCR analysis for the expression of *ICS1* and *FMO1*. *AtTUB4* was used as internal control. To detect gene expression in cotton plants, leaves from cotton plants were collected 14 days post *Agrobacterium* infiltration. Quantitative RT-PCR was performed as described above. *G. hirsutum* HISTONE3 was used as internal control. For RT-PCR analyses of VdSCP41, V592 or VdΔscp41 strain was incubated with the roots of
7-day-old *Arabidopsis* col-0 plants for 2 days. Conidia were collected for RNA isolation and RT-PCR analysis for the expression of *VdSCP41*. For RT-PCR analyses of *V. dahliae*-infected *Arabidopsis* plants, *Arabidopsis* plants infected with or without V592 or VdΔscp41 strain were collected for RNA isolation and RT-PCR analysis for the expression of *ICS1* and *FMO1*. *VdELF1* was used as internal control for *VdSCP41*. *AtTUB4* was used as internal control for *ICS1* and *FMO1*.

**Fluorescence microscopy**

*Agrobacterium tumefaciens* EHA105 strain carrying pCambia1300-35S-CBP60g-GFP, pCambia1300-35S-SARD1-GFP, or pCambia1300-35S-GhCBP60b-GFP was infiltrated alone, or together with *Agrobacterium tumefaciens* EHA105 strain carrying pCambia1300-35S-VdSCP41-mCherry (or its variants mutants) into leaves of 4-week-old *N. b*. GFP and mCherry fluorescence were observed with Leica SP8 confocal microscopy 3 days post infiltration. The intensity of fluorescent signals was determined by Image J software. For fluorescence microscopy in *Arabidopsis*, *Arabidopsis* protoplasts were transfected with 35S-CBP60g-GFP alone, or together with 35S-VdSCP41-mCherry (or its variants mutants). The protoplasts were incubated overnight under faint light before GFP and mCherry fluorescence were observed. To examine the subcellular localization of VdSCP41, conidia were cultured on cellophane and incubated for 3-9 days before microscopy observation. The cellophane with mycelium were collected and observed as described (Zhou et al., 2017). The plasma membrane of the fungi was stained with FM4-64 (red).

**Reporter Assay in Arabidopsis Protoplast**
Arabidopsis protoplasts were co-transfected with ICS1::LUC or FMO1::LUC, and 35S::RLUC (Renilla luciferase), or together with VdSCP41, CBP60g, or their variants as indicated. 12 hours after transfection, protein of transfected protoplasts was isolated, and LUC activity was determined by using Dual-Luciferase® Reporter system (Promega) following manufacture’s instruction.

Virus-induced gene silencing in cotton plants

The VIGS was performed as described (Gao and Shan, 2013). Cotton plants were grown at 23-25°C in the growth room until two cotyledons have emerged. A 465 bp fragment of GhCBP60b cDNA was PCR amplified from G. hirsutum and cloned into pTRV2 plasmid (Liu et al., 2002). Agrobacterium strain carrying pTRV1 together with Agrobacterium strain carrying pTRV2 or pTRV2-GhCBP60b, as indicated, was infiltrated into the cotyledons of the cotton plants. The cotton plants were root-dip inoculated by V. dahliae V592 2 weeks post Agrobacterium infiltration.

Infection assay

Cotton or Arabidopsis plants were infected by the root-dip inoculation method (Gao et al., 2010). A conidial suspension of 10⁷/ml from the indicated stains was used as the inoculums. The disease grade was classified as follows: Grade 0 (no symptoms), 1 (0-25% wilted leaves), 2 (25-50%), 3 (50-75%) and 4 (75-100%). The disease index was calculated as 100 × [sum (number of plants × disease grade)] / [(total number of plants) × (maximal disease grade)] (Xu et al., 2014). The onion epidermis infection assay was performed as described (Zhang et al., 2017). A conidial suspension of 10⁷/ml from V592-GFP, VdΔscp41/SCP41-GFP or VdΔscp41/SCP41-nls-GFP strain
was inoculated onto the inner layer of onion epidermal cells and incubated on 1% water agar plates for 3-5 days before confocal imaging.

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**Figure Legends**

**Figure 1. VdSCP41 contributes to V. dahliae virulence on host plants.**

(A) Schematic description of the generation of the VdΔscp41 mutant. (B) Southern blot analysis of the VdSCP41 gene deletion in the VdΔscp41 mutant. Genomic DNA samples isolated from V592 and VdΔscp41 strains were digested by *BamHI* and subjected to Southern blot analysis. (C-D) Disease symptoms of upland cotton (C) and *Arabidopsis* (D) plants infected with the wildtype (V592), VdΔscp41, and VdΔscp41/VdSCP41-GFP strains, as indicated. (E-F) Disease index analyses of
upland cotton (E) and Arabidopsis (F) infected with the indicated strains. The plants were photographed and subjected to disease index analyses 3-4 weeks post inoculation. The disease indexes were evaluated with three replicates generated from 24 plants for each inoculum. Error bars indicate standard deviation of three biological replicates. Student’s t test was carried out to determine the significance of difference. * indicates significant difference at P value of <0.05. The experiments were repeated three times with similar results.

Figure 2. VdSCP41 functions to inhibit immunity in plants.

(A) VdSCP41 localizes to the base of hyphopodia and forms ring signals surrounding the hyphal neck. The VdΔscp41/VdSCP41-GFP strain and VdΔscp41/ΔspVdSCP41-GFP was cultured on a cellophane membrane for 5 days to induce the formation of hyphopodia. Localization of VdSCP41-GFP was visualized by Leica SP8 microscope. (B) Transiently expressed VdSCP41 preferentially localizes to the nucleus of Arabidopsis cells. Arabidopsis protoplasts were transfected with either VdSCP41-mCherry or ΔspVdSCP41-mCherry plasmid as indicated. mCherry fluorescence was visualized 16 hours post transfection. DAPI staining of nucleus was visualized under UV. (C-D) Expression of ΔspVdSCP41 in Arabidopsis inhibits nlp20Vd2-induced ICS1 (C) and FMO1 (D) expression. Wildtype (WT) and transgenic plants expressing ΔspVdSCP41 or ΔspVdSCP41-nls were treated with H2O or nlp20Vd2 as indicated. RNA was extracted for real-time PCR analyses. The experiments were repeated three times with similar results. Error bars indicate standard deviation.
Student’s t test was carried out to determine the significance of difference. * indicates significant difference at $P$ value of <0.05, whereas ** indicates significant difference at $P$ value of <0.01.

**Figure 3. VdSCP41 interacts with CBP60g in plants.**

(A) VdSCP41C interacts with CBP60g. *Arabidopsis* protoplasts were transfected with the indicated constructs. Protein was extracted 16 hours post transfection and immunoprecipitated with anti-HA or anti-FLAG. The presence of VdSCP41-FLAG or CBP60g-HA in the purified complex was detected by anti-FLAG or anti-HA immunoblot as indicated. (B) VdSCP41 interacts with CBP60g in *N. b.* Luciferase imaging of VdSCP41 and CBP60g interaction in *N. b.* leaves. *N. b.* leaves infiltrated with *Agrobacterium* strain carrying constructs as indicated were subjected to luciferase complementation imaging assay. (C) Quantitative luminescence of VdSCP41 and CBP60g interaction. *N. b.* leaves infiltrated with indicated constructs were sliced into strips, and relative luminescence was determined by microplate luminometer. Error bars indicate standard deviation of three technical repeats. ** indicates significant difference at $P$ value <0.01. The experiments were repeated three times with similar results. (D) VdSCP41 co-localizes with CBP60g and increases its nuclear accumulation. Representative confocal images of CBP60g-GFP subcellular accumulation were visualized by spin-disk microscope. *Agrobacterium* strain carrying CBP60g-GFP was *Agro*-infiltrated into *N. b.* leaves alone, or together with *Agrobacterium* strain carrying ΔspVdSCP41-mCherry, ΔspVdSCP41,ΔspVdSCP41,ΔspVdSCP41-mCherry or ΔspVdSCP41-ΔspVdSCP41,ΔspVdSCP41-mCherry or ΔspVdSCP41,ΔspVdSCP41-nls-mCherry.
NLS-VdSCP30-mCherry. An overlay of GFP and mCherry fluorescence imaging was visualized 48 hours post Agro-infiltration in *N. b.* leaves. The experiments were repeated three times with similar results.

**Figure 4. VdSCP41 binds the C-terminal portion of CBP60g to interfere with its activity.**

(A-B) Expression of *VdSCP41* inhibits the CBP60g-activated *ICS1::LUC* (A) and *FMO1::LUC* (B) reporter. *ICS1::LUC* or *FMO1::LUC* were transfected alone, or together with CBP60g, and VdSCP41, or its variants, as indicated. *35S::RLUC* was co-transfected as internal control. Error bars indicate standard deviation of three technical repeats. ** indicates significant difference between VdSCP41 and EV (empty vector) at *P* value of <0.01. (C) CBP60gC interacts with VdSCP41. *Arabidopsis* protoplasts were transfected with VdSCP41-FLAG alone or together with CBP60gN-HA, CBP60gC-HA, or CBP60g-HA. Protein was extracted 16 hours post transfection and immunoprecipitated with anti-HA antibody. The presence of VdSCP41-FLAG in the purified complex was detected by anti-FLAG immunoblot. The experiments were repeated three times with similar results. (D) VdSCP41 inhibits the DNA-binding activity of CBP60g. GST-CBP60g was incubated with [*γ-32P*]ATP-labeled 60-bp double-stranded DNA probe within the *ICS1* promoter, and subjected to EMSA. Unlabeled probe was used as competitors (150×) for binding. His-tagged VdSCP41C, VdSCP41100-163 or VDAG_01962 was preincubated with GST-CBP60g for 30 min at room temperature where needed as indicated before
EMSA. The experiments were repeated three times with similar results.

**Figure 5. CBP60gC harbors a transcription activation domain that is required for interacting with VdSCP41.**

(A) CBP60gC is required for CBP60g-mediated *ICS1* and *FMO1* activation. *ICS1::LUC* or *FMO1::LUC* was transfected alone, or together with CBP60g, or its variants, as indicated. ** indicates significant difference between ΔC-CBP60g or ΔC-CBP60g/CBP60g and CBP60g at P value of <0.01. (B) CBP60g\_211-440 harbors transcription activator activity. *TPS10::LUC* was transfected alone, or together with bHLH\_MYC2, bHLH-CBP60g\_211-440, as indicated. 35S::RLUC was co-transfected as internal control. ** indicates significant difference between bHLH-CBP60g\_211-440 and bHLH\_MYC2 at P value of <0.01. *LUC* reporter activity was determined 16 hours post transfection. The experiments were repeated three times with similar results. (C) CBP60g\_211-440 is required for interacting with VdSCP41. *Arabidopsis* protoplasts were transfected with the indicated constructs. Protein was extracted 16 hours post transfection and immunoprecipitated with anti-HA or anti-FLAG. The presence of VdSCP41-FLAG or CBP60g variants in the purified complex was detected by anti-FLAG or anti-HA immunoblot as indicated.

**Figure 6. CBP60g and SARD1 are required for VdSCP41-mediated virulence.**

Wildtype (WT) and *cbp60g-1/sard1-1* double mutant plants were inoculated with V592 and VdΔscp41 mutant strains. The plants were subjected to disease index
analyses 3-4 weeks post inoculation. The disease indexes were evaluated with three 
replicates generated from 24 plants for each inoculum. Error bars indicate standard 
deviation of three biological replicates. * indicates significant difference of V592 
virulence at \( P \) value of <0.05 between WT and \( cbp60g-1/sard1-1 \) double mutant 
plants. Student’s t test was carried out to determine the significance of difference of 
difference between indexes collected from three biological replicates. Lower case 
letters indicate significant difference of difference at \( P \) value of <0.05. The 
experiments were repeated three times with similar results.

Figure 7. \textit{GhCBP60b is targeted by VdSCP41 and required for cotton resistance} 
against \textit{V. dahliae}. 

(A) VdSCP41 is co-purified with GhCBP60b in \textit{Arabidopsis} protoplasts. \textit{Arabidopsis} 
protoplasts were transfected with VdSCP41-FLAG alone or together with 
GhCBP60b-HA. Protein was extracted 16 hours post transfection and 
immunoprecipitated with anti-HA. The presence of VdSCP41-FLAG in the purified 
complex was detected by anti-FLAG immunoblot. (B) VdSCP41 co-localizes with 
GhCBP60b. GFP-tagged GhCBP60b and mCherry-tagged VdSCP41 were 
co-expressed in \textit{N. b.} leaves. An overlay of GFP and mCherry fluorescence imaging 
was visualized 48 hours post \textit{Agro}-infiltration. The experiments were repeated three 
times with similar results. (C) Cotton VIGS-\textit{GhCBP60b} plants develop symptoms 
more rapidly than VIGS-\textit{vector} plants. Percentage of plants showing \textit{Verticillium} wilt 
phenotype at indicated time after infection was shown. The disease ratio was scored
with 15 plants per treatment and the assays were repeated for three times with similar results. (D-E) *GhCBP60b* is required for full resistance against *V. dahliae*. Cotton seedlings were infiltrated with *Agrobacterium* carrying pTRV1 together with pTRV2 or pTRV2-*GhCBP60b* as indicated. *V. dahliae* strain V592 was inoculated 10 days post *Agrobacterium* infiltration. Plants showing disease symptoms were photographed 30 days post *V. dahliae* inoculation (D). Disease index analyses of VIGS-vector or VIGS-*GhCBP60b* plants infected with V592 (E). The plants were subjected to disease index analyses 3-4 weeks post inoculation. The disease indexes were evaluated with three replicates generated from 24 cotton plants for each inoculum. The experiments were repeated 3 times with similar results.. Error bars indicate standard deviation of three technical repeats within one biological experiment. Student’s *t* test was carried out to determine the significance of difference. * indicates significant difference at *P* value of <0.05. (F) Expression of *GhCBP60b* is reduced in plants infiltrated with *Agrobacterium* carrying pTRV1 together with pTRV2-*GhCBP60b* (VIGS-*GhCBP60b*). Total RNA from infiltrated plants were extracted for RT-PCR analyses of *GhCBP60b* expression 20 days post *V. dahliae* inoculation. Error bars indicate standard deviation. * indicates significant difference at *P* value of <0.05.

**Figure 8. Model for VdSCP41–mediated suppression of defense in *Arabidopsis* during *V. dahliae* infection.**

(A) In the absence of VdSCP41, PAMPs derived from *V. dahliae*, such as NLPs and chitins, induce the expression of *CBP60g*, which in turn upregulates the expression of...
a number of immune regulators to activate defense. (B) In the presence of VdSCP41, VdSCP41 secreted from *V. dahliae* translocates into the nucleus of plant cell. VdSCP41 targets the transcription activation domain (TAD) of CBP60g, interrupting either the activity of this domain or the recruitment of associated co-activators via this domain, to interfere with their activity and plant immunity against *V. dahliae*. VdSCP41 mediated over-accumulation of CBP60g could provide an additional strategy to further interfere with its transcription factor activity.

**Supplemental Figure Legends**

**Figure 1-figure supplement 1. Expression of VdSCP41 in V. dahliae is induced by plant roots.**

*VdSCP41* is induced by *Arabidopsis* root in *V. dahliae*. *V. dahliae* conidia were co-cultured with (*V. dahliae*-induced) or without (*V. dahliae*) *Arabidopsis* roots for 2 days. RNA was extracted for real-time PCR analyses. The experiments were repeated three times with similar results. Error bars indicate standard deviation. ** indicates significant difference at *P* value of <0.01.

**Figure 2-figure supplement 1. VdSCP41 delivered by V. dahliae translocates into plant cells and inhibits immunity.**

(A) VdSCP41 contains a predicted signal peptide (SP) and nuclear localization signal (NLS) sequence. A predicted SP by SignalP is shown in red. A predicted NLS is shown in blue. (B) VdSCP41-GFP translocates into the nucleus of onion epidermal
cells. Conidia of the V592-GFP, VdSCP41-GFP or VdSCP41_nls-GFP strain were inoculated onto onion epidermal cells. GFP and DAPI staining of nucleus (UV) fluorescence was visualized 3 days post inoculation. The experiments were repeated three times with similar results. (C) Expression of mcherry-tagged VdSCP41 and ΔspVdSCP41 in Arabidopsis protoplasts. Arabidopsis protoplasts were transfected with either VdSCP41-mCherry or ΔspVdSCP41-mCherry plasmid as indicated. Total protein was extracted for anti-mcherry immunoblot. (D) Transiently-expressed VdSCP41 localizes to the nucleus of N. b. cells. N. b. leave cells were Agro-infiltrated with Agrobacterium strain carrying either VdSCP41-mCherry or ΔspVdSCP41-mCherry as indicated. mCherry fluorescence was visualized 48 hours post infiltration. (E) Expression of ΔspVdSCP41 in Arabidopsis inhibits flg22-induced gene expression. Wildtype (WT) and transgenic plants expressing VdSCP41 were treated with H2O, flg22 as indicated. RNA was extracted for real-time PCR analyses. The experiments were repeated three times with similar results. Error bars indicate standard deviation. Student’s t test was carried out to determine the significance of difference between WT and transgenic plants in the same treatment. * indicates significant difference at P value of <0.05, whereas ** indicates significant difference at P value of <0.01.

Figure 2-figure supplement 2. VdSCP41 inhibits pathogen-induced SA accumulation and gene expression.

(A) ΔspVdSCP41 transgenic lines accumulated less free SA than that in WT in
response to Pst hrcC. The plants were treated with or without Pst hrcC and subjected to free SA measurement. (B-C) VdΔscp41 mutant induces higher expression of ICS1 and FMO1 than V592 WT strain. Arabidopsis plants were inoculated with or without V592, or VdΔscp41 mutant as indicated. RNA was extracted for real-time PCR analyses of ICS1 (B) and FMO1 (C). Different letters indicate significant difference at P value of <0.05. The experiments were repeated twice with similar results.

**Figure 3-figure supplement 1. VdSCP41 interacts with CBP60g.**

(A) VdSCP41 is co-purified with CBP60g. Arabidopsis protoplasts were transfected with the indicated constructs. Protein was extracted 16 hours post transfection and immunoprecipitated with anti-HA or anti-FLAG. (B) Expression levels of NLuc- and CLuc-fusion proteins in N. b. All NLuc proteins were further fused with 3×HA tag in NLuc-vector. Anti-HA and anti-CLuc immuneblot were used to detect the expression levels of NLuc and CLuc-fusion proteins respectively. (C) CBP60g-GFP localizes in the nucleus of N. b. cells. N. b. leaves were infiltrated with Agrobacterium strain carrying either CBP60g-GFP, FLS2-GFP or GFP alone as indicated. GFP fluorescence was visualized 48 hours post infiltration. (D) Expression of CBP60g-GFP, ΔspVdSCP41-mCherry or ΔspVdSCP41-nls-mCherry in N. b. cells. N. b. leaves were infiltrated with Agrobacterium strain as indicated. Total protein was extracted for anti-GFP and anti-mcherry immunoblot.
Figure 3-figure supplement 2. VdSCP41 interacts and co-localizes with SARD1.

(A) VdSCP41 is co-purified with SARD1. Arabidopsis protoplasts were transfected with VdSCP41-FLAG alone or together with SARD1-HA. Protein was extracted 16 hours post transfection and immunoprecipitated with anti-HA antibody. The presence of VdSCP41-FLAG in the purified complex was detected by anti-FLAG immunoblot. The experiments were repeated three times with similar results. (B) VdSCP41 co-localizes with SARD1. Representative confocal images of SARD1-GFP subcellular accumulation were visualized by spin-disk microscope. Agrobacterium strain carrying SARD1-GFP was Agro-infiltrated into N. b. leaves alone, or together with Agrobacterium strain carrying VdSCP41-mCherry or VdSCP41-nls-mCherry. An overlay of GFP and mCherry fluorescence imaging was visualized 48 hours post Agro-infiltration in N. b. leaves.

Figure 3-figure supplement 3. Luciferase complementation assay did not reveal an interaction between CBP60g and SARD1.

(A) Luciferase imaging of VdSCP41 and CBP60g interaction in N. b. leaves. (B) Quantitative luminescence of VdSCP41 and CBP60g interaction. (C) Expression levels of NLuc- and CLuc-fusion proteins as indicated in N. b. All NLuc proteins were further fused with 3×HA tag in NLuc-vector. Anti-HA and anti-CLuc immuneblot were used to detect the expression levels of NLuc and CLuc-fusion proteins respectively.
Figure 4-figure supplement 1. VdSCP41 does not cleave CBP60g.

(A) Co-expression of VdSCP41 in *Arabidopsis* protoplasts does not cleave CBP60g. CBP60g-HA was transfected alone or together with VdSCP41-FLAG. CBP60g-HA and VdSCP41-FLAG were detected by anti-HA or anti-FLAG immunoblot. (B) Recombinant His-tagged VdSCP41$_{100}$ does not cleave GST-CBP60g. Purified GST-CBP60g was incubated with or without His-tagged VdSCP41$_{100}$ for 30 min at room temperature and subjected to anti-GST or anti-His immunoblot.

Supplementary File 1. Table S1. Data generated from mass spetrum experiments.

Data generated from mass spetrum experiment 1 (sheet 1) and mass spetrum experiment 2 (sheet 2). Information of protein IDs, scores, number of peptides for the identified proteins are listed. Matched peptides within CBP60g are shown in red.

Supplementary File 2. Primers used in this study.

Sequences of primers used in this study.

Source data files

Figure 1-source data 1. Source data for figure 1.

Figure 2-source data 1. Source data for figure 2.

Figure 3-source data 1. Source data for figure 3.

Figure 4-source data 1. Source data for figure 4.

Figure 5-source data 1. Source data for figure 5.
Figure 6-source data 1. Source data for figure 6.

Figure 7-source data 1. Source data for figure 7.
Figure 1
Figure 2
Figure 4

A

ICS1-LUC

Relative LUC Activity

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FMO1-LUC

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α-FLAG IB

α-HA IB

VdSCP41

CBP60g

CBP60gN/C

D

labeled probe (from ICS1 promoter )

GST-CBP60g          -  -  +  +  +  +  +
His-VdSCP41163-end  +  -  -  -  +  -  -
His-VdSCP41100-163  -  -  -  -  +  -  -
His-VDAG_01962      -  -  -  -  -  -  +
Unlabeled probe     -  -  -  -  150× -  -

Sample wells

Shift

Free probe

Figure 4
**Figure 5**

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**α-FLAG IB**

**α-HA IB**

**CBP60g<sub>g441-end</sub>/IgG**

**CBP60g<sub>211-end</sub>/IgG**
Figure 6

Disease index on Arabidopsis

- **V592**
- **VdΔscp41**

WT vs. *cbp60g-1/sard1-1*

Significance:
- **a**
- **b**
- *****
Figure 7

A. Immunoblot analysis showing VdSCP41 and GhCBP60b expression levels.

B. Fluorescence images of VdSCP41-mCherry and GhCBP60b-GFP in N. benthamiana cells.

C. Bar graph showing the percent wilting plants over time post inoculation for VIGS-vector and VIGS-GhCBP60b treatments.

D. Photographs of cotton plants inoculated with VIGS-vector and VIGS-GhCBP60b.

E. Disease index on cotton plants for VIGS-vector and VIGS-GhCBP60b treatments.

F. Relative normalized expression levels of GhCBP60b for VIGS-vector and VIGS-GhCBP60b treatments.
Figure 8
Figure 1-figure supplement 1

**VdSCP41**

![Graph showing relative normalized expression of VdSCP41 between V. dahliae and V. dahliae-induced samples.](image-url)
**A**

VdSCP41 protein sequence

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**E**

![Graph showing Relative Normalized Expression of ICS1](image)

**Figure 2-figure supplement 1**
Figure 2-figure supplement 2
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α-FLAG IB  
α-HA IB

VdSCP41
CBP60g

B

NLuc  VdSCP41  VdSCP41  BIK1  BIK1  
CLuc  CBP60g  XLG2  CBP60g  XLG2

130 KD
α-CLuc
90 KD

α-HA
90 KD

XLG2
CBP60g

BIK1  VdSCP41

C

BF  UV

N. b. cell

FLS2-GFP
CBP60g-GFP
GFP

D

Mock  EV  ΔpVdSCP41-mCherry  ΔpVdSCP41-mCherry

CBP60g-GFP

α-GFP IB

95 KD
55 KD

α-mCherry IB

72 KD
55 KD

Figure 3-figure supplement 1
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α-FLAG IB  

α-HA IB  

VdSCP41  

SARD1

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</tr>
<tr>
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<td>ΔspVdSCP41-nls</td>
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Figure 3-figure supplement 2
Figure 3-figure supplement 3
Figure 4-figure supplement 1