Title: Suppression of the homeobox gene *HDTF1* enhances resistance to *Verticillium dahliae* and *Botrytis cinerea* in cotton

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Running title: HDTF1 mediates cotton resistance to fungal pathogens

Abstract

Development of pathogen-resistant crops, such as fungus-resistant cotton, has significantly reduced chemical application and improved crop yield and quality. However, the mechanism of resistance to cotton pathogens such as *Verticillium dahliae* is still poorly understood. In this study, we characterized a cotton gene (*HDTF1*) that was isolated following transcriptome profiling during the resistance response of cotton to *V. dahliae*. *HDTF1* putatively encodes a homeodomain transcription factor, and its expression was found to be down-regulated in cotton upon inoculation with *V. dahliae* and *Botrytis cinerea*. To characterize the involvement of *HDTF1* in the response to these pathogens, we used virus-induced gene silencing (VIGS) to generate *HDTF1*-silenced cotton. VIGS reduction in *HDTF1* expression significantly enhanced cotton plant resistance to both pathogens. *HDTF1* silencing resulted in activation of jasmonic acid (JA)-mediated signaling and JA accumulation. However, the silenced plants were not altered in the accumulation of salicylic acid (SA) or the expression of marker genes associated with SA signaling. These results suggest that HDTF1 is a negative regulator of the JA pathway, and resistance to *V. dahliae* and *B. cinerea* can be engineered by activation of JA signaling.

Introduction

Verticillium wilt in cotton is a vascular disease caused by the soil-borne fungal pathogen *Verticillium dahliae*, which greatly affects cotton yield and quality (Zhang et al. 2012). The fungus can also infect more than 200 plant species, including many food and cash crops, such as potato, pepper, grapevine, olive, flax and sunflower (Fradin and Thomma 2006). The most common symptoms on *V. dahliae* infected leaves include wilt, discoloration, necrosis and defoliation. In cotton, infected leaves become discolored and form V-shaped lesions (Xu et al. 2011a). The pathogen can produce and secret toxic, elicitor-like substances and cell wall-degrading enzymes to suppress plant defense responses (Fradin and Thomma 2006). Resistant and tolerant plant species have been found to be less sensitive to Verticillium toxin, suggesting that

the toxin plays an important role in the pathogenicity of *V. dahliae* (Fradin and Thomma 2006).

In tomato, a locus was identified conferring race-specific resistance against *V. dahliae* and *V. albo-atrum* (Kawchuk et al. 2001). The *Ve1* gene at this locus encodes a receptor-like protein that mediates disease resistance to race 1 of *V. dahliae* and *V. albo-atrum* in tomato (Fradin et al. 2009). Correspondingly, the avirulence protein, Ave1, from race 1 of *V. dahliae* and *V. albo-atrum* is thought to be recognized by Ve1 and contributes to *Verticillium* virulence (de Jonge et al. 2012). The brassinosteroid insensitive 1 (BRI1)-associated receptor kinase 1 (BAK1) and suppressor of BIR1 (SOBIR1) are required for Ve1 to activate downstream signaling (Liebrand et al. 2013). However, Ve1 cannot confer resistance in plants infected with *V. dahliae* from cotton or race 2 of *V. dahliae* and *V. longisporum* from tomato, since these do not express the cognate avr protein (Fradin et al. 2011; Liu et al. 2014).

Although a number of molecular tools are being used to study plant-Verticillium interaction, very little is known about the molecular mechanism of plant defense responses to *V. dahliae* (Xu et al. 2011a; Xu et al. 2011b; Wang et al. 2012; Gao et al. 2013; Zhang et al. 2013; Konig et al. 2014). The analysis of mutants involved in the RNA-silencing and plant immunity pathways suggests that posttranscriptional gene silencing affects the regulation of the basal defense against *Verticillium* in Arabidopsis (Ellendorff et al. 2009). Among phytohormones, ethylene (ET) has been suggested to play a role in defense against *V. dahliae* in cotton (Zuo et al. 2007; Xu et al. 2011a). In addition, salicylic acid (SA), jasmonic acid (JA) and brassinosteroid (BR) signaling pathways may also be associated with resistance to *V. dahliae* (Gao et al. 2013; Zhang et al. 2013). Secondary metabolism, such as terpenoid metabolism and phenylpropanoid metabolism, also plays an important role in host resistance to *Verticillium spp.* (Townsend et al. 2005; Xu et al. 2011b; Gao et al. 2013; Knoig et al. 2014). Although several candidate genes involved in plant resistance to *Verticillium spp.* are known, only a few have been fully characterized (Vrain et al. 1987; Dung et al. 2013).

Homeobox proteins act as transcription factors and play important roles in

developmental processes and response to environmental stimuli in all major eukaryotic lineages, including fungi, plants and animals (Derelle et al. 2007; Brandt et al. 2014). Homeobox family members are characterised by the presence of a homeodomain (HD), a DNA-binding domain of 60 amino acids that folds into three alpha helices (Mukherjee et al. 2009). Some HD proteins have been found to function as key regulators in phytohormone-mediated signaling. For example, ATHB6 acts as a negative regulator in the abscisic acid (ABA) signaling pathway (Himmelbach et al. 2002). *H52*, a gene encoding a HD protein transcription factor of the HD-Zip class, is up-regulated after pathogen infection in tomato and involved in cellular protection by limiting the spread of programmed cell death (Mayda et al. 1999). HOS9 is also a HD protein, and is involved in plant development and freezing tolerance (Zhu et al. 2004).

In this study, we provide genetic and molecular evidence for the involvement of the homeobox protein *HDTF1* in resistance of cotton to fungal pathogens *V. dahliae* and *B. cinerea*. Reduced expression of *HDTF1* via virus-induced gene silencing (VIGS) was found to enhance cotton resistance to *V. dahliae* and *B. cinerea*, associated with JA accumulation and activated JA-mediated signaling, suggesting that HDTF1 negatively regulates JA signaling. We propose that *HDTF1* could be used in breeding new cotton varieties for resistance against fungal pathogens.

Results

HDTF1 isolation and sequence analysis

Differentially expressed genes have previously been identified in cotton following inoculation with *V. dahliae* strain ‘V991’ (Xu et al. 2011a; Xu et al. 2011b). Among them, *HDTF1* was found to be down-regulated upon *V. dahliae* infection, and putatively encodes a HD transcription factor (Xu et al. 2011b). The *HDTF1* gene has an open reading frame of 1044 bp in length and encodes a predicted protein of 347 amino acids molecular mass of 40.22 kDa and an isoelectric point of 5.04 (http://web.expasy.org/compute_pi/). Sequence alignment revealed that HDTF1 has a

60 amino acids HD predicted to fold into three alpha-helices. It also contains a plant-specific PINTOX domain (a highly conserved basic domain of about 70 aa) and an acidic domain. HDTF1 protein is structurally homologous to AtOCP3 (*Arabidopsis thaliana*, GI:30984585), OsGF14c-int (*Oryza sativa*, GI:50725038), VvSS0AEB28YD18 (*Vitis vinifera*, GI:349715058) and SlLEFL1032DD01 (*Solanum lycopersicum*, GI:225316088) (Figure 1). Based on its structural features and sequence homologies to known HD domain containing proteins, *HDTF1* was predicted to encode a HD transcription factor belonging to the PINTOX class (Mukherjee et al. 2009).

HDTF1 is primarily localized to the nucleus

Nuc-PLoc prediction analysis indicated that HDTF1 should be localized to the nucleus. To confirm this, yellow fluorescent protein (YFP) was fused to the N-terminus of HDTF1. The YFP and YFP-HDTF1 constructs were transiently expressed in three-week-old tobacco leaves and analyzed for protein expression using confocal microscopy. Consistent with Nuc-PLoc predictions, the YFP:HDTF1 fusion protein preferentially accumulated in the nucleus, whereas YFP alone was distributed throughout the cell (Figure 2A).

***HDTF1* is preferentially expressed in leaves and down-regulated upon *V. dahliae* and *B. cinerea* infection**

Next, we used qRT-PCR analysis to determine the *HDTF1* expression pattern in upland cotton (*Gossypium hirsutum* L. cv. ‘YZ1’). The roots, stems, leaves, ovules and fibers were harvested for RNA extraction. The qRT-PCR results indicated that *HDTF1* was preferentially expressed in the leaves, with low levels of expression seen in other tissues (Figure 3A).

HDTF1 expression was previously found to be down-regulated in cotton roots upon *V. dahliae* infection (Xu et al. 2011b). To evaluate *HDTF1* expression in leaves upon pathogen infection, four-week-old cotton seedlings were sprayed with water or a spore suspension of *V. dahliae* strain ‘V991’ (10^7 conidia per ml) or *B. cinerea* (10^5 conidia per ml). As shown in Figure 3B, the *HDTF1* expression in the leaves was

significantly reduced after inoculation with either *V. dahliae* and *B. cinerea*.

As a soil-borne pathogen, *V. dahliae* usually invades cotton through the roots and spreads through the vasculature before causing brown stems, yellow and wilting leaves and sometimes even death (Sink and Grey 1999). To confirm the effects of infection on root gene expression, we used a root dipping method to inoculate cotton seedlings with *V. dahliae*. As in the leaves, the *HDTF1* transcripts were also down-regulated in the inoculated roots compared to the control (Figure 3C), consistent with previous data (Xu et al. 2011b).

Silencing *HDTF1* with VIGS enhances cotton resistance to *V. dahliae*

VIGS is a rapid and effective way to verify gene function in cotton seedlings (Gao et al. 2011). We therefore used VIGS (construct *TRV:HDTF1*) to generate *HDTF1*-silenced cotton cultivar 'YZ1' plants; the empty vector was introduced into 'YZ1' as a control (*TRV:00*). RT-PCR was performed three weeks after infiltration to analyze gene expression in *TRV:00* and *TRV:HDTF1* roots (35 cycles of PCR amplification) and leaves (32 cycles of PCR amplification). The results revealed that *HDTF1* was successfully knocked down in three week-old cotton seedlings after VIGS (Figure 4A).

To investigate the role of *HDTF1* in cotton, *TRV:00* and *TRV:HDTF1* VIGS plants were inoculated with *V. dahliae* on the leaves and roots. Necrosic symptoms appeared on leaves three days after inoculation, and the *TRV:00* and *TRV:HDTF1* leaves showed different responses seven days later (Figure 4B). In the *TRV:00* leaves, the fungus infected the plants through wounds and resulted in serious necrosis. However, only minor disease symptoms appeared on the *TRV:HDTF1* leaves. The lesions on the *TRV:00* leaves were also significantly larger than those on the *TRV:HDTF1* leaves (Figure 4C).

TRV:00 and *TRV:HDTF1* VIGS plants were also inoculated using the root dipping method. Yellow wilted leaves appeared eight days after inoculation in control *TRV:00* plants. After 12 days there were severe symptoms of verticillium wilt in the *TVR:00* while fewer leaves showed wilting on *TRV:HDTF1* plants (Figure 4D). The

vascular tissue in *TRV:00* stems exhibited more brown coloration than that of *TRV:HDTF1* (Figure 4E). The percentage of plants showing discolored and wilting leaves were 33.25 and 7.57% for *TRV:00* and *TRV:HDTF1*, respectively (Figure 4F). The plant disease index reflects the disease incidence and degree in the plant population, and the disease index of *TRV:00* was significantly higher than that of *TRV:HDTF1* (Figure 4F). To further quantify the fungal colonization of *TRV:00* and *TRV:HDTF1*, the level of *V. dahliae* in the same parts of the *TRV:00* and *TRV:HDTF1* stems was measured by qRT-PCR, using fungus-specific primer (ITS1-F)in combination with *V. dahliae*-specific reverse primer (ST-VE1-R) (Ellendorff et al. 2009). The measured *V. dahliae* levels indicated that fungal colonization was nearly four times higher in *TRV:00* than it was in *TRV:HDTF1* (Figure 4G). These results show that reduced expression of *HDTF1* in VIGS cotton was associated with improved resistance to *V. dahliae*.

HDTF1-silenced cotton shows enhanced resistance to _B. cinerea_

As *HDTF1* expression was also suppressed in cotton upon infection with the necrotrophic fungus *B. cinerea* (Figure 3B), the role of *HDTF1* in the cotton response to *B. cinerea* was also investigated. Detached leaves from *TRV:00* and *TRV:HDTF1* VIGS plants were inoculated with *B. cinerea*. As shown in Figure 5, the control *TRV:00* leaves were highly susceptible to *B. cinerea* and exhibited severe necrosis, while only slight necrosis could be found in the *TRV:HDTF1* leaves (Figures 5A and 5C). Pathogen invasion and growth were also detected by lactophenol-trypan blue, which stained the hyphae as well as the dead cells of the infected leaves. There was a significant inhibition of *B. cinerea* mycelium invasion and growth in the *TRV:HDTF1* leaves, as indicated by the lower plant cell death and necrosis in the *HDTF1*-silenced cotton (Figure 5B). Therefore, *HDTF1* silencing improved cotton resistance to *B. cinerea.*

HDTF1 expression patterns under diverse hormone treatments

Phytohormones play pivotal roles in regulating plant signaling networks in response to biotic stresses (Bari and Jones 2009). The roles of JA, ET and SA as

primary signals in plant immunity have been well established in recent decades, and other phytohormones such as gibberellic acid (GA) and auxin (IAA) have also been found to regulate plant responses to biotic stresses (Pieterse et al. 2009). To determine whether *HDTF1* expression was related to phytohormone signaling, *HDTF1* expression was analyzed in cotton leaves and roots after the application of different phytohormones (Figure 6). *HDTF1* transcript abundance was slightly induced following leaf treatment with IAA, GA or ethephon (2-chloroethylphosphonic acid, ETH, which is metabolised to ethylene). After spraying leaves with 1 mM SA, *HDTF1* transcripts accumulated significantly within 0.5 h. However, *HDTF1* expression was slightly reduced 1 and 3 h after treatment following treatment with methyl jasmonate (MeJA). Similarly, *HDTF1* expression changes in cotton roots were also observed following phytohormone treatments; however, the *HDTF1* induction was not as dramatic in the roots as in the leaves (Figure 6).

HDTF1 silencing activates JA biosynthesis and the JA-related signaling pathway

SA and JA are the primary plant hormones that respond to fungal pathogens (Bari and Jones 2009). To investigate whether *HDTF1* suppression affects the synthesis of these defense-related phytohormones in cotton, we measured the endogenous SA and JA contents of *TRV:00* and *TRV:HDTF1* plants 48 h after inoculation with *V. dahliae*. The results showed that low SA and JA levels were detected in the *TRV:00* and *TRV:HDTF1* roots. The SA contents in *TRV:HDTF1* and *TRV:00* exhibited little difference under either the control or pathogen treatments. However, endogenous JA levels in *TRV:HDTF1* plants increased significantly in comparison to controls, not only after pathogen inoculation but also after the control treatment (Figure 7), suggesting that it is *HDTF1* silencing that is responsible for activating JA biosynthesis. These results were confirmed by a qRT-PCR expression analysis of genes that were involved in SA or JA synthesis. The transcript of *NDR1*, the gene involved in pathogen recognition that causes SA accumulation (Shapiro and Zhang 2001), was not altered in the control or *HDTF1*-silenced plants (Figure 8). The expression levels of genes involved in JA biosynthesis, such as *LOX1* and *OPR3*,

225 increased when *HDTF1* expression was suppressed (Figure 8). These results are
226 consistent with the hormone measurements. All of these findings demonstrated that
227 silencing *HDTF1* activated JA but not SA biosynthesis in cotton.

228 Phytohormones regulate plant defensive responses through a series of
229 downstream components (Pieterse et al. 2009). To elucidate the possible mechanisms
230 of enhanced resistance to *V. dahliae* in *HDTF1*-silenced cotton and the involvement of
231 *HDTF1* in the SA- or JA-mediated cotton immunity system, the expression levels of
232 several well-characterized SA- and JA-related defense genes were determined. The
233 expression levels of *WRKY46* and *PR1*, which are involved in SA-related defense
234 responses, were not influenced by *HDTF1* suppression in cotton after inoculating with
235 *V. dahliae* or following the control treatment. However, both treatments resulted in the
236 up-regulation of the expression levels of genes related to the JA-signal pathway, such
237 as *ERF1* and *JAZ1*. Meanwhile, the expression of *WRKY70*, a transcription factor
238 participating in SA-related biotrophic pathogen resistance that is inhibited by JA (Ren
239 et al. 2008), was suppressed in the *TRV:HDTF1* roots under the control treatment, but
240 no obvious change was found in cotton after inoculation with *V. dahliae* (Figure 8).
241 These results revealed that *HDTF1* silencing activated the expression of genes that
242 were involved in JA biosynthesis and signal transduction.
243

244 **Discussion**

245 We have demonstrated that a putative cotton homeobox protein, HDTF1,
246 participates in regulating JA signaling and plant disease resistance to *V. dahliae*.
247 Homeobox proteins form a large family and are known to play major role in many
248 different aspects of plant development and defense (Chen et al. 2014), including
249 responses to biotic and abiotic stresses (Cooper et al. 2003; Coego et al. 2005;
250 Ramirez et al. 2009; Ramirez et al. 2010). Plant HD genes have been classified into
251 14 subfamilies, including HD-Zip I, HD-Zip II, HD-Zip III, WOX, PHD and ZF-HD
252 (Chen et al. 2014). *HDTF1* was identified as being differentially expressed following
253 pathogen infection(Xu et al. 2011b), and classified as belonging to the PINTOX class

of HD proteins, which only exists in green plants and might function differently from other homeobox family members (Chen et al., 2014).

Homeobox proteins function as transcriptional regulators in hormone signaling, adaptive responses to environmental extremes and microbe-related signaling (Zhu et al. 2004; Ni et al. 2008; Ramirez et al. 2009). OCP3, a member of the PINTOX homeobox proteins from *Arabidopsis*, localizes to the nucleus and acts as a transcription factor in COI1-dependent JA signal transduction (Coego et al. 2005; Garcia-Andrade et al. 2011). An analysis of the subcellular localization of YFP-tagged HDTF1 protein revealed that that it also preferentially localized to the nucleus, consistent with its predicted function as a transcription factor.

A convenient method for gene function characterization is the silencing of endogenous genes through VIGS, and this approach was employed to determine the role of *HDTF1* in cotton. *HDTF1* silencing enhanced *V. dahliae* and *B. cinerea* resistance of cotton seedlings, as determined by pathogen inoculation assays. This suggested that *HDTF1* was involved in negatively regulating disease resistance in cotton. However, the exact regulatory mechanism of *HDTF1* remains to be discovered, especially in *V. dahliae* resistance. Despite efforts in the past decades, little is known about the genetic mechanism of cotton resistance to *V. dahliae*. Plant receptor-like kinases and receptor proteins, which are involved in detecting potential pathogens and activating downstream immunity signaling, have been shown to play important roles in the defense response (Wu and Zhou 2013). A receptor protein of tomato called Ve1 has been well characterized and provides resistance specifically to race 1 of *V. dahliae*, and it is the only *Verticillium* resistance gene reported so far (Fradin et al. 2009). *NDR1* is required for Ve1-mediated resistance in tomato and *Arabidopsis* (Fradin et al. 2009; Fradin et al. 2011), but *NDR1* expression levels did not change in *HDTF1*-silenced cotton. These results imply that *HDTF1*-mediated cotton resistance is independent of the Ve1-related signal pathway or that *HDTF1* might be located downstream of *NDR1* in the defense-related signal pathway.

The expression pattern of a gene partly reflects its function. Notably, when inoculated with the fungal pathogens *V. dahliae* and *B. cinerea, HDTF1* was repressed.

Interestingly, *HDTF1* expression was suppressed by MeJA but induced by SA. This suggests that, in cotton, *HDTF1* is responsive to pathogen infection and phytohormones. SA and JA are important phytohormones in regulating plant disease resistance (Vlot et al. 2009; An and Mou 2011; Wasternack and Hause 2013). Plants optimize cross-talk between SA- and JA-dependent defenses against pathogens with different lifestyles (Spoel et al. 2007). Given the expression pattern of *HDTF1* under the phytohormone treatments, we analyzed the effect of *HDTF1* silencing in SA/JA accumulation and SA-/JA-related gene expression under *V. dahliae* inoculation. Silencing of *HDTF1* did not affect the accumulation of SA in cotton roots, and minimal changes were identified in levels of expression of genes associated with the SA-signal pathway, such as *WRKY46* and *PR1*. Interestingly, *WRKY70*, which is induced by SA and considered to be a repressor of JA-responsive genes, was suppressed under the control treatment but not by *V. dahliae* inoculation. These results suggest that complex regulation occurs between the SA signal and *HDTF1*.

JA has also been well documented as playing a major role in regulating the defense response against necrotrophic pathogens (Bari and Jones 2009; Pieterse et al. 2009). In *Arabidopsis*, plants given a MeJA pre-treatment had increased resistance to the necrotrophic fungi *Alternaria brassicicola*, *B. cinerea* and *Plectosphaerella cucumerina* compared to untreated plants (Ren et al. 2008). In addition to regulating JA-responsive genes such as *PDF1.2*, *OCP3* also has a role in the perception of JA and in mediating resistance signaling in response to necrotrophic pathogens (Coego et al. 2005). The salient feature of *HDTF1*-silenced plants was the accumulation of JA, which was accompanied by the up-regulation of genes involved in JA biosynthesis, including *LOX1* and *OPR3*. This implies a negative regulatory role for *HDTF1* in JA biosynthesis and signaling.

Although the molecular basis of the interaction between plants and *V. dahliae* is still poorly understood, the *V. dahliae* symptoms are consistent with a switch from a biotrophic to necrotrophic life style (Reusche et al. 2013). Further evidence has suggested that JA, but not ET, signaling is required in Ve1-mediated resistance in tomato and *Arabidopsis* (Fradin et al. 2011). JA signaling was also activated in cotton

following *V. dahliae* infection. *GbSSI2* is involved in SA and JA signaling and can be induced by *V. dahliae*, and when it was silenced plants had greater susceptibility to *V. dahliae* (Gao et al. 2013). Therefore, *V. dahliae* resistance in *HDTF1*-silenced cotton might be explained by the activation of JA signaling, which would be partly similar to Ve1-mediated resistance to *V. dahliae* in tomato. *HDTF1* could be a candidate gene for cotton disease resistance breeding, and identifying the mechanism of HDTF1 function in regulating JA signaling is an interesting challenge for the future.

Materials and methods

Plant material, growth conditions and treatments

Cotton (*G. hirsutum* L. cv. 'YZ1') and tobacco (*Nicotiana benthamiana*) seedlings were grown in soil-filled pots under greenhouse conditions of 22/25 °C (night/day). The roots, stems, leaves, ovules and fibers of 'YZ1' were collected to analyze *HDTF1* expression of different cotton tissues. Leaves from four week-old 'YZ1' plants were used to investigate *HDTF1* expression changes under different treatments. Hormone treatments were performed by spraying the plants with 5 μM IAA, 0.5 μM GA, 200 μM ETH, 100 μM MeJA, 1 mM SA, or double-distilled water as control (Xu et al. 2011a; Gao et al. 2013). IAA, GA, ETH, MeJA and SA were dissolved in water. For *HDTF1* expression analysis in cotton roots, 'YZ1' seedlings were cultured in Hoagland's solution for 18 days and then treated with Hoagland's solution containing the corresponding concentrations of hormones (Long et al. 2014). Plant-pathogen interaction analyses were performed by spraying spore suspensions of *V. dahliae* strain 'V991' (10^7 conidia per ml) or *B. cinerea* (10^5 conidia per ml) on the leaves or dipping the roots into a 'V991' conidia suspension (2×10^5 conidia per ml). Water was used as a control treatment.

Isolation and characterization of *HDTF1*

The *HDTF1* expressed sequence tag (EST) was cloned on the basis of the results of our previous study (Xu et al. 2011b). The full-length *HDTF1* cDNA sequence was

obtained through the 5’- and 3’-rapid amplification of the cDNA ends (RACE). A sequence similarity analysis was performed with the DNAMAN software (http://www.lynnon.com). Homologous protein sequences were acquired from GenBank (http://www.ncbi.nlm.nih.gov/genbank).

Subcellular localization of HDTF1 protein

The HDTF1 protein subcellular localization was predicted using the Nuc-PLoc prediction program (http://www.csbio.sjtu.edu.cn/bioinf/Nuc-PLoc/). To study the localization of the HDTF1 protein, the *HDTF1* cDNA was inserted into an N-terminal YFP-fusion expression vector, pGWB442, and the 35S-YFP vector was constructed as a control. Both vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 for the transformation of three week-old tobacco leaves to determine the subcellular localization of HDTF1. Yellow fluorescence expression was observed 48 h post-transformation with a confocal microscope (Leica Microsystems TCS SP2 AOBS, Germany).

Expression analysis

To analyse gene expression levels, total RNA was extracted from ‘YZ1’ using the guanidine thiocyanate method (Zhu et al. 2005). The first strand cDNA was synthesized from 2 μg of total RNA using the M-MLV reverse transcript system (Promega, USA). RT-PCR was performed at 95 °C for 3 min followed by 28-35 cycles of amplification (95 °C for 20 s, 55-60 °C for 20 s and 72 °C for 20 s). The qRT-PCR was performed on an ABI 7500 Real Time PCR system (Applied Biosystems, USA) with SYBR green (Bio-Rad, USA). The relative changes were calculated with $2^{-\Delta Ct}$ and the cotton *UBIQUITIN 7* gene (*UB7*) was amplified as the reference gene (Livak and Schmittgen 2001). The primers used for the PCR amplification are listed in Supplemental Table S1.

Vector construction and genetic transformation

A 278 bp fragment from the ORF (open reading frame) of *HDTF1* was inserted into the *TRV:00* plasmid and it was digested with the restriction enzymes *Bam*HI and

*Kpn*I to generate the *TRV:HDTF1* construct (Liu et al. 2002). The primers used for PCR amplification and vector construction are listed in Supplemental Table S1. *TRV1*, *TRV:HDTF1* and *TRV:00* were then introduced into *A. tumefaciens* strain GV3101. *A. tumefaciens* containing *TRV1* and *A. tumefaciens* containing *TRV:HDTF1* or *TRV:00* were mixed in equal amounts and infiltrated into the cotyledons of 10-day-old 'YZ1' seedlings by syringe infiltration to generate the control (*TRV:00*) and *HDTF1*-silenced (*TRV:HDTF1*) cotton. *TRV:CLA1* (*cloroplastos alterados 1*) was used as a positive control as previously described (Gao et al. 2011). As shown in Figure S1, the leaf bleaching phenotype was expressed in the *TRV:CLA1* plants two weeks after infiltration.

Fungal pathogen inoculation

V. dahliae and *B. cinerea* strains were taken from storage at 4 °C and transferred onto a Potato-Dextrose Agar (PDA) medium for four days, and then high-activity hyphae were transferred onto fresh PDA medium for another seven days to enable spores to form. The colonies on the medium surface were flooded with 5 ml water and the surface was agitated to form spore suspensions.

Cotton 'YZ1' seedlings were infected with *V. dahliae* and *B. cinerea* three weeks after VIGS treatment. The infection of detached leaves with *V. dahliae* was performed following Munis et al. (2010). Small holes were made in the leaves, and 3 μl of the 'V991' conidial suspensions (10^7 conidia per ml) were applied. The area of infection was measured with ImageJ software (http://rsbweb.nih.gov/ij/) seven days after infection. At least eight lesions were measured in each experiment, and the experiment was repeated at least three times. Whole-plant inoculation assays were performed using the root dipping method with the 'V991' conidial suspension (2×10^5 conidia per ml) (Xu et al. 2011b). Roots were harvested for the measurement of the hormones and for RNA extraction 48 h after inoculation with *V. dahliae*. The rate of diseased plants and the disease index were recorded 12 days after inoculation, and they were scored from at least 16 plants per treatment and repeated at least three times. The counting methods were performed as in Xu et al. (2012). qRT-PCR of the fungal

colonization was performed by comparing the *V. dahliae* internal transcribed spacer (ITS) DNA levels (as a measure of fungal biomass) to the cotton *UB7* DNA levels at 12 days post-inoculation in representative *TRV:00* and *TRV:HDTF1* cotton stems above the cotyledons (Fradin et al. 2011). *TRV:00* and *TRV:HDTF1* leaves were inoculated with *B. cinerea* on an area 5 mm in diameter. Lesion sizes (of eight lesions per experiment) were recorded four days after infection, and this was done at least three times.

Trypan blue staining

30 h after inoculation with *B. cinerea*, leaves from the *TRV:00* and *TRV:HDTF1* plants were stained by boiling in lactophenol-trypan blue and subsequently destained with chloral hydrate as described by Choi and Hwang (2011) and Feng et al. (2013). Stained hyphae and dead cells were observed with a light microscope (Leica Microsystems TCS SP2 AOBS, Germany).

JA and SA measurements

TRV:00 and *TRV:HDTF1* plants were inoculated with *V. dahliae* or treated with water as a control inoculation. 48 h after treatment, cotton plant roots were harvested and JA and SA were extracted. Samples (0.1 g) were ground into a powder with liquid nitrogen, 1 ml of extraction buffer was added and mixed at 4 °C for 16 h. The supernatants were collected and analyzed on an HPLC-MS/MS (1200L LC-MS system, Varian, USA) (Bowling et al. 1994).

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Laboratory of Plant Pathology, Huazhong Agricultural University, China) for providing the *Botrytis cinerea* strain.

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Figure legends:

Figure 1. Multiple alignments of deduced HDTF1 amino acids with homologous proteins from other species. Black shading denotes the amino acids that were conserved in all sequences, and the gray shading denotes amino acids with similar physicochemical characteristics. The homeodomain (HD), PINTOX motif and acidic domain were shaded in boxes. Multiple alignments were performed using DNAMAN. The sequences of the following species were aligned: AtOCP3 (*Arabidopsis thaliana*, GI:30984585), OsGF14c-int (*Oryza sativa*, GI:50725038), VvSS0AEB28YD18 (*Vitis vinifera*, GI:349715058) and SlLEFL1032DD01 (*Solanum lycopersicum*, GI:225316088).

Figure 2. Subcellular localization of HDTF1 protein in tobacco cells. (A) Diagram of the *35S-YFP* and *35S-YFP:HDTF1* fusion constructs. (B) Transient expression of YFP and YFP:HDTF1 fusion proteins in tobacco leaf cells. A confocal microscope was used to observe the yellow fluorescence 48 h after infiltration by *Agrobacterium.* Scale bars: 5 μm.

Figure 3. *HDTF1* expression pattern in cotton tissue and upon fungal pathogen infection. (A) Tissue-specific expression of *HDTF1*. qRT-PCR analysis was performed with the reverse transcription products of the total RNA extracted from

different tissues (root, stem, leaf, ovule and fiber) of ‘YZ1’. (B) qRT-PCR analysis of *HDTF1* expression in pathogen-infected leaves. Total RNA was extracted from four-week-old ‘YZ1’ leaves at the indicated times. Treatments were applications of a spore suspension of *Verticillium dahliae* (10^7 conidia per ml), *Botrytis cinerea* (10^5 conidia per ml) or water as a control treatment. (C) qRT-PCR analysis of *HDTF1* expression from roots that were responsive to *V. dahliae*. The roots of ‘YZ1’ seedlings were harvested for RNA extraction at the indicated times after inoculation. Standard deviations were calculated from the results of three independent experiments.

Figure 4. *HDTF1* knock-downs in cotton with enhanced resistance to *Verticillium dahliae*. Ten-day-old ‘YZ1’ seedlings were infiltrated with *Agrobacterium* carrying *TRV:HDTF1* or the control vector *TRV:00* and were then inoculated with *V. dahliae*. (A) *HDTF1* expression levels in the leaves and roots of control plants (*TRV:00*) and *HDTF1*-silenced plants (*TRV:HDTF1*). Total RNA was extracted three weeks after virus-induced gene silencing (VIGS). The cotton *UB7* gene was amplified as the internal control. (B) Photograph of representative leaves from *TRV:00* and *TRV:HDTF1* plants seven days after *V. dahliae* (10^7 conidia per ml) inoculation. The black areas around the wounds were necrotic tissue caused by fungal infection. Scale bars: 800 μm. (C) Lesions cause by *V. dahlia* had their sizes recorded seven days after inoculation. The standard deviations were calculated from the results of three independent experiments ($n \geq 8$ lesions, ** $P < 0.01$, *t*-test). (D) Photograph of representative *TRV:00* and *TRV:HDTF1* plants 12 days after inoculation with a conidial suspension of *V. dahliae* (2×10^5 conidia per ml) via the root dipping method. (E) Sections of representative *TRV:00* and *TRV:HDTF1* cotton stems cut 1 cm above the cotyledons after *V. dahliae* inoculation. The brown areas at the cross-sections are diseased vascular bundles. Scale bars: 300 μm. (F) Diseased plants and disease index of *TRV:00* and *TRV:HDTF1* plants 12 days after *V. dahliae* inoculation. The standard deviations were calculated from the results of three independent experiments ($n \geq 16$ plants, ** $P < 0.01$, *t*-test). (G) qRT-PCR was used to analyze fungal colonization by comparing the *V. dahliae* internal transcribed spacer (ITS) DNA levels (as a measure

for fungal biomass) to the cotton *UB7* DNA levels 12 d post-inoculation. The standard deviations were calculated from the results of three independent experiments ($n \geq 3$, ** $P < 0.01$, *t*-test).

Figure 5. *HDTF1* silencing enhanced cotton resistance to *Botrytis cinerea*. (A) Representative leaves from control plants (*TRV:00*) and *HDTF1*-silenced plants (*TRV:HDTF1*) four days after *B. cinerea* inoculation (5 mm in diameter). Scale bars: 1 cm. (B) *TRV:00* and *TRV:HDTF1* leaves were stained with lactophenol-trypan blue 30 h after *B. cinerea* infection, and the stained hyphae and dead cells were observed under a microscope. Scale bars: 400 μm. (C) The lesions generated by *B. cinerea* were measured four days after inoculation. The standard deviations were calculated from the results of three independent experiments ($n \geq 8$ lesions, ** $P < 0.01$, *t*-test).

Figure 6. *HDTF1* expression pattern in cotton under hormone treatments. Leaves from four-week-old seedlings were sprayed with 5 μM IAA, 0.5 μM GA, 200 μM ETH, 100 μM MeJA, 1 mM SA or double-distilled water. Tissues were harvested at various time points. For the root treatments, the 'YZ1' seedlings were treated with Hoagland's solution that contained the corresponding hormone concentrations. The cotton *UB7* gene was employed as an internal control in the qRT-PCR analysis. The standard deviations were calculated from the results of three independent experiments. The values are presented as means and the error bars indicate the standard deviations of triplicate samples.

Figure 7. SA and JA measurements in *TRV:00* and *TRV:HDTF1* plants. Four-week-old *TRV:00* and *TRV:HDTF1* seedlings were inoculated with *Verticillium dahliae* or treated with water as a control inoculation. Then 48 h after the inoculation the contents of the free SA and JA were determined. The standard deviations were calculated from the results of three independent experiments ($n \geq 8$, ** $P < 0.01$, *t*-test).

Figure 8. qRT-PCR analysis of SA and JA-related genes. Four-week-old *TRV:00*

and *TRV:HDTF1* seedlings were inoculated with *Verticillium dahliae* or treated with water as a control inoculation. The roots were harvested 48 h after inoculation for RNA extraction. As shown, the expression levels of the genes involved in SA and JA synthesis, including *NDR1*, *LOX1* and *OPR3*, and the genes in the SA- and JA-signal pathways, including *WRKY46*, *WRKY70*, *PR1*, *ERF1* and *JAZ1*, were normalized with the *UB7* expression. The standard deviations were calculated from the results of three independent experiments. The values are presented as means and the error bars indicate the standard deviations of triplicate samples (* $P < 0.05$; ** $P < 0.01$, *t*-test).