ChiIV3 Acts as a Novel Target of WRKY40 to Mediate Pepper Immunity Against *Ralstonia solanacearum* Infection

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ChiIV3, a chitinase of pepper (*Capsicum annuum*), stimulates cell death in pepper plants. However, there are only scarce reports on its role in resistance against bacterial wilt disease such as that caused by *Ralstonia solanacearum* and their transcriptional regulation. In this study, the silencing of *ChiIV3* in pepper plants significantly reduced the resistance to *R. solanacearum*. The transcript of *ChiIV3* was induced by *R. solanacearum* inoculation (RSI) as well as exogenous application of methyl jasmonate and abscisic acid. The bioinformatics analysis revealed that the *ChiIV3* promoter consists of multiple stress-related *cis* elements, including six W-boxes and one MYB1AT. With the 5' deletion assay in the *ChiIV3* promoter, the W4-box located from –640 to –635 bp was identified as the *cis* element that is required for the response to RSI. In addition, the W4-box element was shown to be essential for the binding of the *ChiIV3* promoter by the WRKY40 transcription factor, which is known to positively regulate the defense response to *R. solanacearum*. Site-directed mutagenesis in the W4-box sequence impaired the binding of WRKY40 to the *ChiIV3* promoter. Subsequently, the transcription of *ChiIV3* decreased in WRKY40-silenced pepper plants. These results demonstrated that the expression of the defense gene *ChiIV3* is controlled through multiple modes of regulation, and WRKY40 directly binds to the W4-box element of the *ChiIV3* promoter region for its transcriptional regulation.

Keywords: plant responses to pathogens, pathogenesis-related proteins, resistance genes

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Host plants have developed their defense mechanisms to survive against microbial pathogens in natural environments (Jones and Dangl 2006; Yin et al. 2015; Zhong et al. 2018). The defense mechanisms comprised activation of the mitogen-activated protein kinase (MAPK) cascades and accumulation of reactive oxygen species and nitric oxide. A number of transcription factors, such as WRKY, bZIP, ERF, and NAC, have been involved in defense induction by regulating the expression of pathogenesis-related (*PR*) genes (Hwang et al. 2011; Jones et al. 2016; Kim and Hwang 2011; Mur et al. 2017; Park et al. 2013; Tsuda et al. 2013; Zhang and Feng 2014; Zhou et al. 2017). Plants are dependent on innate immunity to fight against pathogen attack, due to the lack of the adaptive immune system (Jones and Dangl 2006). Plants have evolved two characteristic interconnecting layers of immunity, namely, pattern triggered immunity (PTI) and effector-triggered immunity (ETI) (Hein et al. 2009; Jones and Dangl 2006). Both systems of immunity have been reported to share common signaling components and, possibly, similar sets of *PR* genes. In the PTI and ETI signaling pathways, *PR* genes are involved; however, they have varied expression levels and resistance duration (Thomma et al. 2011; Tsuda and Katagiri 2010).

Chitin, a polymer of *N*-acetyl-D-glucosamine, is a fungal cell-wall component that is not found in plant cells (Vanholme et al. 2014). Host plants containing chitin-degrading enzymes can destroy fungal cell walls and inhibit pathogen multiplication. Chitin fragments (chitoooligosaccharides) may induce PTI reaction in host plants (Wan et al. 2008a and b). Therefore, chitins are recognized as a kind of pathogen-associated molecular pattern for a large number of fungal pathogens. Even though the bacterial cell wall does not contain chitin, some plant chitinases showed suppression effects on bacterial growth. For example, *Brassica juncea* chitinase BjCHI1 exhibited antimicrobial properties against both fungal and bacterial pathogens (Guan and Chye 2008). As chitinases are generally encoded by a gene family, they constitute a group of *PR* proteins involved in plant immunity (Ahmed et al. 2012; Iseli et al. 1996; Lee et al. 2012; Nakahara and Masuta 2014). The expression of chitinases is regulated in plants and has exhibited an inducible pattern at the transcription level upon pathogen infection (Gao et al. 2014; Hong and Hwang 2006). A vast array of *cis* elements in promoters is responsible for perceiving environmental stress signals or pathogen invasion (Baykal et al. 2006; Chen et al. 2015; Gao et al. 2014; Hong and Hwang 2006; Liu et al. 2017). Since each chitinase promoter

harbors different *cis* elements, the transcription pattern of one single gene varies from the other genes. The transcriptional regulation of chitinase genes seems very complicated, a particular splicing pattern was recently found on a basic chitinase *PR3b* gene in Burley tobacco that caused an alternative splicing in low-nicotine *nic1* and *nic2* mutants and was regulated by jasmonate (JA) and ethylene (ET) (Tominaga et al. 2016). However, the transcription regulation and function of chitinase genes have not been fully explained, especially in nonmodel plants.

WRKY proteins represent one of the largest transcription factor (TF) families with one or two conserved WRKY domains that bind to W-box *cis* elements in the promoters of their targets (Agarwal et al. 2011; Eulgem 2006; Eulgem et al. 1999, 2000; Rushton et al. 2010, 2012). This specific regulation mode is not only essential for plant growth and development but also for response to environmental stressors, such as pathogen attack (Eulgem 2006; Eulgem and Somssich 2007; Ishihama and Yoshioka 2012; Pandey and Somssich 2009). In general, a set of WRKYs constitute a WRKY web within which one WRKY acts as a TF to regulate the transcription of other members or physically interacts with other members (Baldoni et al. 2013; Eulgem and Somssich 2007). Most WRKY TFs can arbitrarily bind to distinctive and conserved W-boxes [TTGAC(C/T)]. Residues in the conserved WRKYGQK motif interact with DNA bases mainly through extensive apolar interactions with thymine methyl groups. The importance of these contacts was verified by substituting relevant T bases with U and surface plasmon resonance analyses of DNA binding (Yamasaki et al. 2005; Yamasaki et al. 2012). However, knowledge about the specific targets of a given WRKY TF for it to fulfill the specific function and how it could determine its target specificity is very limited.

Pepper (*Capsicum annuum*) is a vegetable of great economic significance worldwide that often suffered various soil-borne diseases, such as bacterial wilt caused by *R. solanacearum* and phytophthora blight (Choi and Hwang 2011; Choi et al. 2012, 2013; Fu et al. 2015; Liu et al. 2016; Römer et al. 2007; Tang et al. 2007; Wang et al. 2014). A better understanding of the plant immune system will help improve the disease resistance of pepper at a genetic level. Fifteen putative chitinase genes have been identified in the published genome of pepper (Kim et al. 2014b). Among the chitinase family, a basic class II chitinase, CACHi2 (Hong and Hwang 2006), and class IV chitinase, ChitIV (Kim et al. 2015), have been found to be involved in response to pathogen infection. Overexpression of CACHi2 in *Arabidopsis* plants has shown an increased resistance against *Pseudomonas syringae* pv. *tomato* and increased tolerance to NaCl-induced osmotic stress (Hong and Hwang 2006). The class IV chitinase CaChitIV interacts with CaPIK1 and promotes the CaPIK1-triggered cell death and defense responses (Kim et al. 2015). However, the majority of these chitinases in pepper have not been functionally characterized so far.

Our previous study showed that WRKY40, a member of the WRKY subgroup IIa in pepper, is transcriptionally up-regulated by high temperature and high humidity or by *R. solanacearum* inoculation and it acts as a positive regulator in the response of pepper to *R. solanacearum* infection or in thermotolerance under high humidity (Dang et al. 2013). However, what defense genes targeted by WRKY40 and how they function remain to be elucidated. In the present study, we focused on the pepper *ChiIV3*, a cDNA-encoding class IV chitinase. The objective of this study was to uncover the role of *ChiIV3* in pepper immunity during *R. solanacearum* infection and how it is transcriptionally regulated. Our data showed that *ChiIV3* is transcriptionally regulated by WRKY40 via the W4-box within its

promoter and contributes positively to the response of pepper to the attack of *R. solanacearum*.

RESULTS

ChiIV3 is induced by *R. solanacearum*, methyl jasmonate (MeJA), and abscisic acid (ABA).

In our previous study, *ChiIV3*, a cDNA encoding a chitinase, was identified in pepper plant (Liu et al. 2017). To understand its transcription regulation, a 1,017 bp promoter sequence of *ChiIV3* was analyzed using the PlantCARE database. The results indicated that the *ChiIV3* promoter consisted of numerous regulatory *cis* elements, including six W-boxes, three MYB1ATs, two HSEs (heat-shock elements), three MYB1ATs, and two CGTCA-motifs. An ERELEE4 motif and TATA-box were detected at the 5' and 3' termini, respectively (Fig. 1A). Since W-box, MYB1AT, and CGTCA-motifs are pathogen-responsive and hormone-associated *cis* elements, transcription of *ChiIV3* was investigated after RSI and treatment with phytohormones. Upon RSI, the transcript level of *ChiIV3* was found to be significantly increased from 1 to 24 h postinoculation (hpi), with the highest level exhibited at 12 hpi (Fig. 1B). Transcript of *ChiIV3* showed diverse changes in response to the phytohormone treatments. *ChiIV3* transcripts were induced by exogenous application of MeJA and ABA and were reduced by application of ethephon (ETH), brassinolide (BR), and salicylic acid (SA) (Fig. 1C through G). These results demonstrated that the expression of *ChiIV3* was regulated by RSI and phytohormones.

ChiIV3-silencing pepper plants showed severe bacterial wilt symptoms.

To ascertain the role of *ChiIV3* in disease resistance, a loss-of-function of *ChiIV3* was produced by virus-induced gene silencing (VIGS) (Fig. 2A). The transcript levels of *ChiIV3* in roots and stems of silenced pepper plants were detected to assess the silencing efficiency. *ChiIV3* transcripts in roots and stems of *ChiIV3*-silenced pepper plants were, respectively, approximately one fifth and one half of those in the wild-type pepper plants (unsilenced), indicating that the expression of *ChiIV3* was effectively inhibited (Fig. 2B). Trypan blue staining indicated that *ChiIV3*-silencing mitigated the hypersensitive cell death induced by *R. solanacearum* infection in pepper leaves (Fig. 2C). Predominantly, *ChiIV3*-silenced plants developed more susceptibility to *R. solanacearum* infection. On comparison with unsilenced control plants, such susceptibility was demonstrated by more severe disease symptoms (Fig. 2D), a higher relative disease index (Fig. 2E), and lower transcriptional levels of defense-related marker genes, including jasmonic acid (JA)-dependent *DEF1* and ABA-responsive *ABR1* (Fig. 2F). These results suggest that silencing of *ChiIV3* significantly reduced the resistance of pepper plants against *R. solanacearum* infection.

Identification of *ChiIV3* promoter regions essential for RSI and phytohormone induction.

To determine which *cis* element was essential for perceiving signals of RSI, MeJA, or ABA, a 5' deletion assay was conducted on the *ChiIV3* promoter (Fig. 3A). The reporter systems linked with promoter deletions and plant transformation were successfully established in our previous study (Liu et al. 2017). β -glucuronidase (GUS) quantification data revealed that p1017, p891, and p712 deletions were sufficient to trigger GUS expression under RSI, while p459 and p276 deletions could not trigger GUS expression. This indicated that the *cis* elements between -712 bp and -459 bp might be the key domain required for RSI, comprising two CGTCA motifs, two MYB1ATs, and three noticeable W-boxes, i.e., W3-box (W^{-672 to -667}), W4-box

(W^{-640 to -635}), and W5-box (W^{-466 to -461}) (Fig. 3B). The *cis* elements associated with MeJA and ABA were different from those associated with RSI. The region between -459 and -276 bp comprising the W6-box was found to be associated with MeJA (Fig. 3C), while the region between -891 and -712 bp comprising the W2-box and a MYB1AT motif was associated with ABA signaling (Fig. 3D).

W4-box in the *ChiIV3* promoter is critical for RSI.

Three W-boxes were located from -712 bp to -459 bp in the *ChiIV3* promoter. To determine which W-box was responsible for perceiving RSI, *ChiIV3* promoters were constructed with a mutation in the W3-box, W4-box, or W5-box (Fig. 4A). *Agrobacterium* cells containing the *ChiIV3* promoter-GUS fusions and its mutants were infiltrated into the leaves of 8-week-old pepper plants. After 24 h, agroinfiltrated leaves were inoculated with *R. solanacearum*. At 48 hpi, the inoculated leaves were

tested for GUS activity. In comparison with the wild-type promoter, both W3-box and W5-box mutants showed similar induction effects with GUS activity (Fig. 4B), while the W4-box mutant could not induce the expression of GUS triggered by *R. solanacearum* inoculation. This showed that the W4-box element was critical for *ChiIV3* in response to *R. solanacearum* infection. In addition, the W6-box (W-box-339 to -334 bp) located between -339 and -334 bp and MYB1AT element located between -735 and -730 bp were essential for the response of pepper plants to the exogenous application of MeJA and ABA (Fig. 5A and B).

W4-box is required for WRKY40 binding to the *ChiIV3* promoter.

To clearly understand *ChiIV3* transcriptional regulation, a yeast one-hybrid system was performed to identify the putative upstream regulator involved in *ChiIV3* activation. As the

Fig. 1. Promoter and expression profile analysis of *ChiIV3* in response to *Ralstonia solanacearum* inoculation and exogenous phytohormones. **A**, Potential stress-related *cis*-acting elements present in the promoter of *ChiIV3* (*pChiIV3*). HSE: heat-shock element, MYB1AT: transcription factor MYB binding site, CGTCA-motif: methyl jasmonate (MeJA)-responsive element, ERELEE4: ethylene-responsive element, W-box: transcription factor WRKY binding site. **B**, Accumulation of *ChiIV3* transcripts in pepper leaves inoculated with *R. solanacearum*. Total RNA was extracted from leaves of 2-week-old pepper seedlings at different intervals of time postinoculation. **C** through **G**, Transcript level analysis of *ChiIV3* after treatments with phytohormones, including MeJA, abscisic acid (ABA), ethephon (ETH), brassinolide (BR), and salicylic acid (SA). Total RNA was extracted from 2-week-old pepper seedlings at different timepoints after treatments. For **B** through **G**, the expression level of *ChiIV3* in untreated plants was set to 1. Error bars indicate standard deviation of three individual replicates. Asterisks indicate significant differences determined by Fisher's protected least significant differences test (one asterisk [*] indicates $P < 0.05$, two [**] $P < 0.01$).

W4-box of the *ChiIV3* promoter was critical for RSI, a 22-bp oligonucleotide containing a W4-box was used as a bait. A total of 43 positive clones were obtained and 11 clones were successfully amplified by PCR for further sequencing. Sequencing results hit three pepper genes with duplicates, including three NAD-dependent glyceraldehyde 3-P dehydrogenase (NADP, XP_006347401.1), two CBS domain-containing protein (CBS, XP_006344361.1), and six WRKY40 (NM_001325081). The bait and prey plasmids extracted from positive yeast clones were cotransformed into yeast strain Y1HGOLD to confirm binding activity of the three genes (Fig. 6A). NADP and CBS could not demonstrate the growth ability on SD/-Leu medium and were not further investigated (Fig. 6A). The positive result of WRKY40 was exciting and validated our previous finding

that it also acts as a positive regulator in pepper resistance to *R. solanacearum* (Dang et al. 2013).

The chromatin immunoprecipitation (ChIP) procedure was performed to verify the binding of WRKY40 to the W4-box in *ChiIV3* promoter. The oligonucleotides of the W4-box linked to the core *CaMV35* promoter (-46 to +8 bp) was synthesized and fused to GUS reporter gene to create the reporter construct *W4-p35Score:GUS* (Fig. 6B). Similarly, *W4m-p35Score:GUS* was also constructed with a 4-bp replacement of -TGAC- by -CCTT-, resulting in a sequence mutation in the W4-box (Fig. 6B). GV3101 cells carrying *W4-p35Score:GUS* or *W4m-p35Score:GUS* were individually combined with *p35S:WRKY40-HA* (hemagglutinin) for chromatin isolation used for both ChIP and GUS activity measurements. ChIP-PCR results

Fig. 2. Increased susceptibility of *ChiIV3*-silenced pepper plants to *Ralstonia solanacearum* inoculation. **A**, Lineal structure of *ChiIV3*-silenced vector. LB, left border; RB, right border. **B**, Silencing efficiency detection in roots and stems of *ChiIV3*-silenced pepper plants infected by *R. solanacearum*. Pepper plants with white leaves were used as a positive control. The transcript level of *ChiIV3* in unsilenced pepper plants without treatment was set to 1. **C**, Trypan blue staining was performed to evaluate cell death in *ChiIV3*-silenced pepper leaves infected by *R. solanacearum*. **D**, Disease symptoms of *ChiIV3*-silenced and unsilenced pepper plants inoculated with *R. solanacearum*. **E**, *R. solanacearum*-inoculated *ChiIV3*-silenced and unsilenced pepper leaves were scored daily, using a disease index ranging from 0 to 4: 0 (no wilting), 1 (1 to 25% wilted), 2 (26 to 50% wilted), 3 (51 to 75% wilted), and 4 (76 to 100% wilted or dead). **F**, Quantitative reverse transcription PCR was performed to investigate the transcript accumulation of defense-associated marker genes, including jasmonic acid-dependent *DEF1* and abscisic acid-responsive *ABR1*, in *ChiIV3*-silenced and unsilenced pepper leaves 12 h after *R. solanacearum* inoculation. The transcript level of marker genes in unsilenced pepper plants without treatment was set to 1. In B, E, and F, error bars indicate standard deviation of three individual replicates. Asterisks indicate significant differences determined by Fisher's protected least significant differences test (one asterisk [*] indicates $P < 0.05$, two [**] $P < 0.01$).

showed that WRKY40 product could directly bind to the W4-box element of *pChiIV3* (Fig. 6C). In addition, GUS activities driven by the *W4-p35S core* promoter were induced to high levels by transient expression of *WRKY40*. However, the transient expression of *WRKY40* did not induce an increased GUS activity driven by the *W4m-p35S core* (Fig. 6D). In RSI, the binding of *WRKY40* to *pChiIV3* was significantly achieved when compared with the uninoculated control (Fig. 6E). These results verified that the W4-box was the key *cis* element required for *WRKY40* to activate *ChiIV3*.

To further confirm the binding of *WRKY40* to the W4-box in the *ChiIV3* promoter, microscale thermophoresis (MST) experiments were performed using *WRKY40*-GFP fusion protein that is transiently expressed in pepper leaves. *WRKY40*-GFP labeled with NT-647-NHS fluorescent dye and synthetic W4-box elements were subjected to thermophoresis experiments. Thermophoresis signal was plotted against ligand concentration to obtain a dose-response curve that facilitated precise evaluation of the binding affinity (Fig. 6F). The resultant binding curves showed that the wild-type W4-box was clearly associated with *WRKY40* protein and binding intensity increased with the increase in concentration of target DNA.

WRKY40 modulates *ChiIV3* promoter activity in pepper.

To examine whether *ChiIV3* is regulated by *WRKY40*, full-length cDNAs of *WRKY40* were fused to HA-tag driven by

CaMV35 promoter to generate *p35S:WRKY40-HA* (Fig. 7A). GV3101 cells containing *p35S:WRKY40-HA* were infiltrated into the leaves of 8-week-old pepper. The protein expression level of *WRKY40*-HA was confirmed by immunoblot analysis at 48 hpi (Fig. 7B). Additionally, the transcriptional level of *ChiIV3* was found to be significantly induced by *WRKY40* overexpression (Fig. 7C). *Agrobacterium*-mediated cotransformation of *WRKY40*-HA and *ChiIV3 promoter:GUS* revealed that *WRKY40* significantly activated GUS expression driven by the *ChiIV3* promoter (Fig. 7D). GUS activity was also determined on *WRKY40* knockdown pepper plants. In comparison with that in unsilenced plants, *ChiIV3* promoter-driven GUS activities were found to be significantly reduced in *WRKY40*-silenced pepper plants (Fig. 7E). Altogether, these results indicated that transcription of *pChiIV3* is mediated by *WRKY40*.

DISCUSSION

Plant PR proteins induced by pathogen infection play vital roles in disease resistance (Hadwiger 2015; Li et al. 2011; Murray et al. 2005; Vasavirama and Kirti 2012; Zhang and Mou 2009). Plant chitinases are comprised of a family of universal PR proteins that can catalyze the hydrolysis of fungal chitin to generate chitooligosaccharides. Released chitin fragments are found to be involved in PTI during fungal pathogen infection

Fig. 3. *ChiIV3* promoter activation in response to *Ralstonia solanacearum* infection and hormone treatments in tobacco plants. **A**, Schematic diagram of the vector of *ChiIV3* promoter (*pChiIV3*), its deletions (p1017, p891, p712, p459, and p276) and *cis*-element distribution in five deletions of *pChiIV3*. LB, left border; RB, right border; W1-W6 represent W-boxes found in *ChiIV3* promoter. **B** to **D**, *ChiIV3* promoter activation in response to *R. solanacearum* inoculation and exogenous methyl jasmonate (MeJA) and abscisic acid (ABA) in leaves of transgenic tobacco plants harboring 5'-*ChiIV3*-GUS chimeric constructs. The pathogen and exogenous MeJA- and ABA-treated transgenic tobacco leaves were harvested 24 h posttreatment for analyzing β -glucuronidase (GUS) activities. The GUS activities in transgenic tobacco leaves harboring a deletion of p1017 (full-length promoter) treated with mock solution (MgCl₂ or hormone solution) were set to 1. Error bars indicate standard deviation of three individual replicates. Asterisks indicate significant differences determined by Fisher's protected least significant differences test (one asterisk [*] indicates $P < 0.05$, two [**] $P < 0.01$).

(Chen et al. 2018). However, the transcription of plant chitinase genes is not strictly controlled by fungal pathogens, because certain bacteria, viruses, and oomycetes without chitin in their cell walls have been found to induce chitinase gene expression (Guan and Chye 2008; Hong and Hwang 2002; Hong et al. 2000; Mishra et al. 2012). This suggested that plant chitinases exert their roles in resistance to several kinds of pathogens, including bacteria. Recently, a pepper class IV chitinase showed that CaChitIV interacted with CaPIK1 and promoted CaPIK1-triggered cell death against *Xanthomonas campestris* pv. *vesicatoria* infection (Kim et al. 2015). Our previous study reported that ChiIV3 showed antifungal activity against *Phytophthora capsici* and a W5-box element in the promoter was involved in the response to *P. capsici* infection (Liu et al. 2017). This was consistent with the conclusion that chitinase can act as an antifungal protein and exhibit critical roles in response to fungal invasion (Maximova et al. 2006; Kashyap and Deswal 2017; Khan et al. 2017; Kim et al. 2015; Miyamoto et al. 2012; Richa et al. 2016; Tobias et al. 2007; Yang et al. 2014). In this study, we uncovered the role of ChiIV3 during *R. solanacearum* infection, which causes severe bacterial wilt disease in pepper plant. *ChiIV3*-silencing pepper plants showed high susceptibility to *R. solanacearum*, coupled by severe disease symptoms and low expression level of defense-associated marker genes. Subsequently, pepper ChiIV3 acted as a plant PR protein required for resistance against *R. solanacearum*.

The pepper *ChiIV3* gene was expressed in a multiple regulatory mode, especially when induced by *R. solanacearum* infection. The expression of chitinase genes have been highly regulated to coordinate with plant growth and development as well as the response to biotic and abiotic stress conditions (Filippi et al. 2016; Karmakar et al. 2016; Kim et al. 2014a; Mészáros et al. 2014; Wu et al. 2017). Transcriptional expression of a gene is commonly coordinated by transcription factors, which bind and recognize the promoter composed of multiple *cis*-regulatory elements (Kim et al. 1997; Zhao et al. 2007, 2012). The present study discovered that the expression of *ChiIV3* was significantly induced by *R. solanacearum*, MeJA, and ABA, which led us to study the *cis* elements present in the *ChiIV3* promoter. Using PlantCARE software, six W-boxes, three MYB1ATs, two HSEs, three MYB1ATs, and two CGTCA-motifs were predicted from the *ChiIV3* promoter. Through promoter GUS fusion experiments, the W4-box element was found to be critical in response to *R. solanacearum* infection. In addition, the W6-box located from -339 to -334 bp and the MYB1AT element located from -735 to -730 bp were found to be involved in the response to exogenous MeJA and ABA, respectively. Even though the function of *cis* elements in the *ChiIV3* promoter had not been completely elucidated, current findings suggested that the *ChiIV3* gene was controlled by complex regulatory modes. The phytohormones anchored various *cis* elements to modulate *ChiIV3* expression.

Fig. 4. Site-directed mutagenesis analysis of *ChiIV3* promoter in response to *Ralstonia solanacearum* infection in pepper plants. **A**, Locations of W-boxes in *ChiIV3* promoter (*pChiIV3*). Numbers above the bar indicate the position of five *pChiIV3* deletions. The underlined nucleotides represent W-boxes. **B**, The schematic diagram of *pChiIV3*:GUS and its W-box-mutant constructs (*pChiIV3*-W3m:GUS, *pChiIV3*-W4m:GUS, or *pChiIV3*-W5m:GUS) and their β -glucuronidase (GUS) activities initiated by *R. solanacearum* inoculation in pepper leaves. The mutated nucleotides of the W-box are shaded. The GV3101 cells containing *pChiIV3*:GUS or its W-box mutant constructs were penetrated into the pepper leaves and were maintained in the greenhouse for 24 h, followed by *R. solanacearum* inoculation. The pathogen-inoculated leaf tissues were harvested for GUS activity measurements at 48 h postinoculation. Values are means $\pm$ standard deviation (SD) ($n = 6$). Error bars indicate SD. Asterisks indicate significant differences determined by Fisher's protected least significant differences test (one asterisk [*] indicates $P < 0.05$, two [**] $P < 0.01$).

Additionally, the W5-box was involved in response to *P. capsici* infection (Liu et al. 2017), while the W4-box was essential for *R. solanacearum* induction.

WRKY proteins depend on the conserved WRKY domains to bind to W-box *cis* elements in the promoters of their targets (Agarwal et al. 2011; Eulgem 2006; Eulgem et al. 1999, 2000; Rushton et al. 2010, 2012). The specificity of targets is determined by WRKY domains of WRKY proteins and W-boxes in target gene promoters. Previous studies have reported that nucleotide sequences flanking W-box elements demonstrated a certain degree of binding specificity to WRKY proteins (Brand et al. 2013; Ciolkowski et al. 2008; Gao et al. 2016). The specificity might also be modulated through posttranslational modification of WRKY proteins. Various enzymes, such as MAPKs and MAPK kinases (Adachi et al. 2016; Giri et al. 2013; Ishihama et al. 2011, 2014; Kim and Zhang 2004; Liu et al. 2004; Miao et al. 2007; Pecher et al. 2014; Shen et al. 2012; Weyhe et al. 2014b; Yoo et al. 2014; Zhang et al. 2012), Ca²⁺-dependent protein kinases (Gao and He 2013), VQ-motif-containing proteins (Cheng et al. 2012; Lei et al. 2017; Pecher

et al. 2014; Wang et al. 2015; Weyhe et al. 2014a), calmodulin (Park et al. 2005), histone deacetylases (Kim et al. 2008; Singh et al. 2014a and b), and E3 ubiquitin ligase (Miao and Zentgraf 2010), have been found to interact with WRKY proteins. Some WRKY proteins can even interact with each other in plants (Chen et al. 2010). The interaction of WRKY with other proteins can often lead to the change in transcriptional activation activity or targeting specificity. Through yeast one-hybrid, ChIP, and MST assays, the W4-box was confirmed to be the critical element targeted by WRKY40. Moreover, the W4-box seemed to be a unique binding site for WRKY40, because site-directed mutagenesis in the W4-box completely impaired the induction of *ChiIV3* by WRKY40. It is still unclear whether the specificity of *ChiIV3* targeted by WRKY40 is determined through protein-protein interaction. The identification of interacting pepper proteins of WRKY40 would provide new insight into WRKY40-*ChiIV3* mediated pepper resistance to *R. solanacearum* infection.

Arabidopsis WRKY40 together with WRKY18 and WRKY60 are three paralogous WRKY transcription factors that regulate

Fig. 5. Site-directed mutagenesis analysis to confirm the *cis* elements of *pChiIV3* in response to exogenous methyl jasmonate (MeJA) and abscisic acid (ABA) treatments in pepper plants. **A** and **B**, The schematic representation of serial mutant constructs of *pChiIV3:GUS* (*pChiIV3-W4-M:GUS*, *pChiIV3-W6-M:GUS*, and *pChiIV3-MYB1AT-M:GUS*) and the measurement of β -glucuronidase (GUS) activity in response to *R. solanacearum* inoculation and exogenous MeJA and ABA treatments, respectively. The mutated nucleotides are underlined. At 24 h postinoculation or treatment, the infiltrated pepper leaves were harvested for measuring GUS activity. Error bars indicate standard deviation of six individual replicates. Asterisks indicate significant differences determined by Fisher's protected least significant differences test (one asterisk [*] indicates $P < 0.05$, two [**] $P < 0.01$).

plant defense in a complex pattern (Liu et al. 2012). Three WRKY proteins can form both homo- and hetero-complexes and exhibit physical interaction with themselves or with each other through a basic leucine zipper motif at their N-terminus (Xu et al. 2006). The transcription of WRKY40 was suppressed by ABA and negatively regulates ABA-dependent signaling by direct repression of ABA-responsive *ABI4* and *ABI5* genes (Liu et al. 2012). With transient expression in *Arabidopsis thaliana* protoplasts, WRKY40 localizes to phytochrome B-containing nuclear bodies (PNBs). However, WRKY40 can relocate from PNBs to nucleoplasm in the presence of ABA in a dynamic and phosphorylation-dependent manner (Geilen and Böhmer 2015). WRKY40 transcription in pepper was activated by WRKY6 before regulating the *R. solanacearum* resistance as well as tolerance to high temperature and high humidity. Numerous targets of WRKY40 have been identified in our previous study, including *CaC3H14* encoding a tandem CCCH zinc finger protein and *CaCDPK15* encoding a calcium-dependent protein kinase (Shen et al. 2016a; Qiu et al. 2018). In this study, we reported that *ChiIV3* was a novel target of WRKY40 involved in resistance to *R. solanacearum* infection. It can be concluded that WRKY40 in pepper could directly modulate several targets to execute its regulatory function. However, target genes involved in tolerance to high temperature or high humidity have not yet been experimentally proven.

Besides RSI and phytohormones, other unknown factors must be involved in *ChiIV3* transcription regulation because

its promoter contained multistress-responsive *cis* elements. In addition, roles of the W1-box, W2-box, and W3-box have not been explained. Considering that the W-box is the binding target of WRKY protein, other WRKY transcription factors, including WRKY40, might have jointly regulated *ChiIV3* expression. Moreover, transcription of *ChiIV3* was reduced by the application of SA, ETH, or BR. Negative regulation of these three phytohormones possibly occurred through the *cis* elements in the *ChiIV3* promoter. It will be interesting to determine which *cis* elements were involved in negative regulation of *ChiIV3* transcription in future studies.

Based on the comprehensive evidence in the present study, a proposed model was constructed to explain the transcription of *ChiIV3* modulated by WRKY40 (Fig. 8). When pepper plants are infected by *R. solanacearum*, signal is transmitted to the nuclei, thus promoting the transcription of *WRKY40* (Dang et al. 2013, 2014). WRKY40 protein directly binds to promoters of *ChiIV3* in a W-box-dependent manner. The enhanced expression of *ChiIV3* then induces defense response and expression of defense-associated marker genes, such as *ABR1* and *DEF1*. The pepper resistance to *R. solanacearum* was successfully elucidated through this regulatory mode.

In conclusion, the present study reported that pepper chitinase *ChiIV3* played an important role in resistance to bacterial wilt. The transcription of the *ChiIV3* gene was induced by *R. solanacearum*, JA, and ABA through *cis*-regulatory elements in the promoter region. The W4-box was found to be essential

Fig. 6. WRKY40 acts as a positive regulator of *ChiIV3*. **A**, Yeast one-hybrid analyses of the upstream regulators of *ChiIV3*. Bait plasmids coding *WRKY40*, *NADP*, and *CBS* were retransformed into yeast containing bait plasmids and were planted on SD/-Leu medium supplemented with 300 ng of Aureobasidin A (ABA^r) per milliliter. **B**, Schematic diagram of the *W4-p35S core:GUS* vector and *W4m-p35S core:GUS*. M, mutant; LB, left border; RB, right border. Mutated nucleotides are underlined. **C**, WRKY40 directly binds to W4-containing fragment of *pChiIV3* in nonstressed pepper plants analyzed by chromatin immunoprecipitation (ChIP) assay. Pepper leaves expressing WRKY40-HA (hemagglutinin) were harvested at 48 h postinoculation for the isolation of chromatin and the ChIP assay. **D**, The transient overexpression of *WRKY40* triggered the expression of β-glucuronidase (GUS) driven by *W4-p35S core* in a W4 dependent manner. GUS activity in pepper leaves coinfiltrated with GV3101 cells containing *pW4-p35S core:GUS* and *p35S:00* was set to 1. **E**, *R. solanacearum* inoculation improved the binding of WRKY40 to W4-box containing *pChiIV3*. **F**, An MST-based binding experiment was performed to confirm the binding of purified WRKY40 with W4-box and W4m-box mutation. The binding states of the molecule display different thermophoretic depletion profiles. The difference in thermophoresis can be plotted as a change in normalized fluorescence versus ligand concentration to obtain a binding curve. In D and E, data represent mean ± standard deviation (SD) of three independent experiments. Error bars indicate SD. Asterisks indicate significant differences determined by student's *t* test (one asterisk [*] indicates *P* < 0.05, two [**] *P* < 0.01).

for *R. solanacearum* induction and it was the binding site of WRKY40. The collective data supported that *ChiIV3* was a novel resistance gene directly regulated by WRKY40 to stimulate plant resistance against *R. solanacearum* infection. These results provide insight into the function and regulation of pepper *ChiIV3* during RSI.

MATERIALS AND METHODS

Plant materials and cultivation.

Seeds of pepper inbred line yanshan01 (*Capsicum annuum* cv. yanshan01) and two tobacco cultivars (*Nicotiana tabacum* K326 and *N. benthamiana*) were collected by the pepper breeding group in Fujian Agriculture and Forestry University (Fujian Province, China). Pepper and tobacco seeds were germinated in a steam-sterilized soil mix (peat moss: per liter [2:1, vol/vol]) in plastic pots. Following germination for 2 weeks, the seedlings were transferred to larger pots and were maintained in a growth room at 25°C, 60 to 70 mmol photons m⁻²s⁻¹, 70% relative humidity, and a 16-h light and 8-h dark photoperiod.

Pathogen inoculation and exogenous application of MeJA and ABA.

Ralstonia solanacearum was isolated and stored in the pepper breeding center of Fujian Agriculture and Forestry University. For preparation of *R. solanacearum*, stem exudates and stem vascular parts from pepper plants were purified using the tetrazolium chloride method (van Elsas et al. 2001). The strains were incubated overnight at 200 rpm, 28°C in SPA medium

(200 g of potato, 20 g of sucrose, 3 g of beef extract, and 5 g of tryptone in 1 liter of double-distilled [dd]H₂O). The cell pellet was collected using low-speed centrifugation and was suspended in sterile 10 mM MgCl₂. Suspension was then adjusted to an optical density at 595 (OD₅₉₅) of 0.8 (10⁸ CFU/ml). Ten microliters of *R. solanacearum* suspension was injected into the roots of transgenic tobacco or *ChiIV3*-silenced pepper leaves using a syringe and, later, the leaves were harvested at different time intervals for further assay. For exogenous application of phytohormones, plants were sprayed with 100 μM ABA and 100 μM MeJA dissolved in ddH₂O. Plants sprayed with ddH₂O were kept as a control.

Amplification of 5' deletions or site-directed mutagenesis of *pChiIV3* by PCR.

pChiIV3 and its 5' deletion derivatives (−1,017, −891, −712, −459, and 276 to +1; the first nucleotide of the initiation codon ATG was set as +1) were constructed using Gateway technology (Liu et al. 2017). Site-directed mutagenesis of *pChiIV3* was performed using the primers *pChiIV3*-W3m-F, *pChiIV3*-W4m-F, *pChiIV3*-W5m-F, *pChiIV3*-W6m-F, and *pChiIV3*-MYB1AT-F (Supplementary Table S1). The *attB*-containing PCR product of *pChiIV3* and its 5' deletion derivatives were cloned into the satellite vector pDONR207 via BP reaction. After confirmation by sequencing, the generated BP products were transferred into the destination vector pMDC163 via LR reaction to generate *pChiIV3*, *pChiIV3*-W3-M, *pChiIV3*-W4-M, *pChiIV3*-W5-M, *pChiIV3*-W6-M, and *pChiIV3*-MYB1AT-M, respectively.

VIGS of *ChiIV3* in pepper plants.

Loss-of-function of *ChiIV3* in pepper plant in response to *R. solanacearum* inoculation was performed using VIGS

Fig. 7. WRKY40 triggers the expression of β-glucuronidase (GUS) driven by *pChiIV3*. **A**, The schematic diagram of effector and reporter vectors. **B**, Immunoblot analysis of WRKY40-HA (hemagglutinin) transiently expressed in pepper leaves at 48 h postinoculation (an anti-HA antibody was used to detect WRKY40-HA proteins on immunoblot). IB, immunoblotting. **C**, Overexpression of *WRKY40* induced the transcript expression of *ChiIV3*, analyzed by quantitative reverse transcription PCR. The transcript level of *ChiIV3* in pepper leaves infiltrated with GV3101 cells containing p35S:00 (empty vector) was set to 1. **D**, The expression of GUS driven by *pChiIV3* was improved by transient overexpression of *WRKY40*. GUS activity of pepper leaves coinfiltrated with *pChiIV3*:GUS and p35S:00 was set to 1. **E**, *WRKY40*-silencing partially stops the transcript accumulation of *ChiIV3* in healthy pepper plants. The transcript level of *ChiIV3* in unsilenced pepper plants was set to 1. In C to E, asterisks indicate significant differences determined by Fisher's protected least significant differences test (one asterisk [*] indicates $P < 0.05$, two [**] $P < 0.01$).

Fig. 8. Proposed model for the role of *ChiIV3* modulated by WRKY40 in defense signaling. The pepper WRKY transcription factor WRKY40 regulates expression of *ChiIV3* via the W-box in the upstream promoter. *ChiIV3* products trigger defense response and the expression of defense-associated marker genes including *DEF1* and *ABR1* against *R. solanacearum*. RS, *Ralstonia solanacearum*. Arrows indicate positive regulation.

technology as described previously (Liu et al. 2016). Briefly, a specific 3' untranslated region fragment of *ChiIV3* that was approximately 200 bp in length was employed to construct the VIGS vector, the specificity of the region was confirmed by BLASTN against pepper genome databases (Pepper Genome Platform). The fragment was amplified and cloned into the VIGS vector pTRV2, using Gateway technology to yield pTRV2-*ChiIV3*. After confirmation by sequencing, pTRV2-*ChiIV3* with the pTRV1 vector or pTRV2 vector were separately transformed into GV3101 cells. A 5-ml culture of each strain was grown overnight in YEP medium (per milliliter, 10 mg of yeast extract, 10 mg of peptone, and 5 mg of NaCl) supplemented with appropriate antibiotics (per milliliter, 75 mg of kanamycin and 75 mg of rifampicin). GV3101 cells were harvested by centrifugation and were resuspended in infiltration buffer (10 mM MgCl₂, 10 mM morpholineethane sulfonic acid [MES], pH 5.7, and 200 μ M acetosyringone) and adjusted to OD₆₀₀ = 0.5. GV3101 strains containing the pTRV1 vector and pTRV2 or pTRV2-*ChiIV3* were mixed at a 1:1 ratio, were shaken at room temperature for 3 h, and were coinfiltrated into the cotyledons of pepper seedlings. The VIGS-treatment pepper plants were grown at 25°C with a 16-h light and 8-h dark photoperiod cycle.

Fluorometric assays and histochemical staining for GUS activity.

For GUS activity, total protein was extracted from stress-treated leaves in transgenic tobacco, *ChiIV3*-silenced pepper, or pepper transiently expressing the GUS-tag vectors and was quantified according to the method described by Mou et al. (2013), with a slight modification. The rate of *p*-nitrophenol release (γ = 415 nm) was measured using a microplate reader (Biotek) to confirm the GUS activity. For histochemical staining, infected pepper leaves were harvested and immersed in GUS staining solution (200 mM phosphate buffer, pH 7.0, containing 100 mM K₃[Fe(CN)₆], 100 mM K₄[Fe(CN)₆], 1,000 mM EDTA-Na₂, and 0.1% 5-bromo-4-chloro-3-indolyl- β -D-glucuronide) and were incubated at 37°C overnight, then, cleaned with 70% ethanol (EtOH).

Agrobacterium-mediated transient expression assay.

Agrobacterium-mediated transient expression assays were performed in pepper plants according to a previously described method, with a slight modification (Liu et al. 2015). *Agrobacterium* cells containing different constructs were grown in YEP medium (per liter, 10 g of yeast extract; 10 g of peptone; 5 g of sodium chloride; pH 7.0) overnight at 28°C, and the cell pellet was collected, by centrifugation for 5 min at 8,000 rpm, and was suspended in the infiltration medium (10 mM MgCl₂, 10 mM MES, pH 5.7, and 200 μ M acetosyringone). The adjusted cell suspension was penetrated into pepper leaves using a needleless syringe. The treated pepper plants were maintained at 25°C in greenhouse.

Histochemical staining.

3, 3'-Diaminobenzidine tetrahydrochloride (DAB) and trypan blue staining procedures were performed as described in our previous studies (Liu et al. 2015, 2016). For DAB staining, treated pepper leaves were harvested and immersed in DAB solution (1 mg of DAB per milliliter). At 8 h posttreatment, DAB-stained pepper leaves were boiled in absolute ethanol for destaining. For trypan blue staining, pepper leaves were harvested and boiled in trypan blue solution (10 ml lactic acid, 10 ml glycerol, 10 ml phenol, 40 ml ethanol, 10 ml ddH₂O, and 1 ml trypan blue) for 5 min, were kept at room temperature for 8 h, then, were transferred to chloral hydrate solution (2.5 g of chloral hydrate per milliliter dissolved in distilled water) and were boiled for 25 min, for destaining.

Yeast one hybrid.

Bait construct, pW4-AbAi containing a 22-bp *pChiIV3* fragment with a W4-box (ATAAGCTGTCAACTAAGTGAC GAGCTAT) was transformed into yeast strain Y1HGold according to the protocol handbook (Clontech). To determine the expression profile of the bait construct in yeast, the transformed yeast strain was subjected to grow on SD/-Leu medium supplemented with gradient concentrations of Aureobasidin A (AbA^r) (50, 100, 200, 300, and 400 ng/ml). For the W4-box, a concentration of 300 ng/ml was enough to suppress the background activity of the bait yeast strain. Pepper cDNA library fused with pGADT7-Rec AD was created from pepper leaves infected by *R. solanacearum* and was screened using the bait yeast strain. The transformed yeast cells were plated on SD/-Leu medium supplemented with 300 ng of AbA^r per milliliter and were incubated at 30°C for 3 to 5 days. PCR was performed to detect the insertion of the prey vector and was subjected to sequence analysis. Y1HGold strains transformed with p53-AbAi+pGADT7-p53 and p53-AbAi+pGADT7-WRKY40 were used as positive and negative controls, respectively.

Western blotting and ChIP assay.

Western blotting was performed following the method described in our previous study (Liu et al. 2017). Protein extraction buffer (10% glycerol, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2% Triton X-100, 10 mM dithiothreitol, 1 $\times$ complete protease inhibitor cocktail [Sigma-Aldrich] and 2% [wt/vol] polyvinylpyrrolidone) (Choi et al. 2012) was used to extract total proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed to separate the proteins of different sizes. Rabbit anti-HA-peroxidase antibody (Abcam) and goat antirabbit antibody were used to detect the protein level of HA-fused protein. For the ChIP assay, the pepper leaves transiently overexpressing WRKY40-HA, were harvested for chromatin isolation. The isolated chromatins were trimmed with micrococcal nuclease to an average length of 200 to 500 bp. Anti-HA antibodies were used to immunoprecipitate the DNA-protein complex of the clipped chromatin. The immunoprecipitated DNA was analyzed for enrichment of WRKY40 in the promoter of *ChiIV3* by PCR.

MST analysis.

Monolith NT. 115 system (Nano Temper) was used to perform MST experiments using 100% LED and 20% IR-laser power. Purified WRKY40-6HIS fusion protein was labeled using a monolith NT protein labeling kit RED-NHS (Nano Temper) that reacts with primary amines of targeted protein to form stable dye-protein conjugates. The labeling procedure was performed according to the aforementioned manual. The oligonucleotides of the W4-containing fragment (GAAGTGA TAAGCTGTCAACTAAGTGACGAGCTA) and its mutants (GAAGTGATAAGCTAAGGACTAAGTGACGAGCTA; mutated nucleotides are underlined) were synthesized by Convenience Biology Co., Ltd. and were dissolved in water (100 μ M). The samples were added into Premium Capillaries and were measured in 20 mM HEPES buffer, pH 7.5, 200 mM KCl, 10 mM TCEP, and 0.05% Tween 20. Data were analyzed during acquisition (on the fly data analysis) using NT-Analysis software provided with the system.

Total RNA isolation and real-time PCR analysis.

Trizol was used for the extraction of total RNA from the pepper leaves. Real-time quantitative reverse transcription (RT)-PCR was performed with specific primers (Supplementary Table S2), to determine the relative transcriptional levels of targeted genes, following the manufacturer's

instructions on a Bio-Rad Real-Time PCR system (Bio-Rad) and SYBR Premix Ex Taq II system (Takara). Total RNA isolation and real-time RT-PCR assays were performed following previously described procedures (Cai et al. 2015; Dang et al. 2013; Shen et al. 2016b). Three or six (transient overexpression) independent biological replicates were used for each treatment. Data were analyzed by the Livak method and were expressed as a normalized relative expression level ($2^{-\Delta\Delta CT}$) of the respective genes (Livak and Schmittgen 2001). The relative transcriptional level of each sample was normalized by *CaACTIN* (GQ339766) and *18S ribosomal RNA* (EF564281), respectively.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

PlantCARE database:
<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>

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