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ORIGINAL ARTICLE

RNAi induced silencing of pathogenicity genes of *Fusarium* spp. for vascular wilt management in tomato

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Abstract The necessity to develop new strategies for the control of *Fusarium* wilt of tomato signifies the identification of pathogencity genes and ascertaining their role to use them as molecular tools for fungicide development or to develop transgenics. Semi-quantitative gene expression studies have identified two pathogenicity genes, *FOW2* and *chsV*, reported as ZnII)2Cys6-type transcription regulator and class V chitin synthase, respectively, as potential ones for being secreted all the time. The roles of these genes in the pathogenicity of *Fusarium oxysporum* and *F. solani* have been established by RNA interference (RNAi)-induced silencing (knockdown). The silencing vector encoding hairpin RNA of each of the gene fragment was constructed in a two-step PCR-based cloning, and introduced into the fungal genomic DNA. Silencing of either of the genes resulted in less virulent fungal phenotypes with altered physiological characteristics like sporulation and growth on solid media and a reduction in mRNA expression. The results therefore demonstrate the applicability of these pathogenicity genes as useful molecular targets for exploitation in *Fusarium* wilt control in tomato.

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Introduction

Species of *Fusarium*, *F. oxysporum* and *F. solani* are serious soilborne pathogens that incite vascular wilt of tomato worldwide (Ajilogba and Babalola 2013). In India, although vascular wilt of tomato caused by *F. oxysporum* has been widely reported (Shanmugam et al. 2011, 2015), association of *F. solani* with vascular wilt appears to be limited (Chandra et al. 1983). Management practises have little impact on vascular wilt incidence mainly due to the ability of *Fusarium* species to produce highly resistant chlamydospores, volatile inhibitors, and antibiotics (Shanmugam et al. 2015). These signify the necessity to relook at the aetiology of *Fusarium* wilt of tomato and to develop new strategies for its control. Recently, fungal genes have become of considerable interest for disease control either to create hypovirulent isolates by manipulating them for use in biocontrol or through host-induced gene silencing (HIGS). Candidate fungal genes have been discovered by the analyses of genome sequences and transcriptomes during host invasion by the pathogens and are thus of particular interest for identifying virulence factors (Sutherland et al. 2013).

Targeted disruption of several fungal genes have enhanced our knowledge on molecular mechanisms involved in pathogenesis (Nakayashiki and Nguyen 2008). RNA-mediated gene silencing or RNA interference has been exploited as a tool for functional analysis of eukaryotic genes by expression of hairpin-forming transcripts, and is now reliably used to elucidate gene function in many animal and plant species. Such hairpin RNA (hpRNA) technologies are the method of choice for a number of fungal species, for which gene knock-

out mutants can be difficult to obtain (Schumann et al. 2013). Also, the utility of the conventional approach of generating gene knock-out strains is limited for exploitation in fungal disease control. Effective RNA silencing has been demonstrated in several phytopathogenic fungi (Schumann et al. 2010) including *F. oxysporum*, though with contrasting effects (Schumann et al. 2013; Hu et al. 2015), and in *F. solani* (Ha et al. 2006; Liu et al. 2010; Koch et al. 2013) and *F. graminearum* (McDonald et al. 2005).

Fungal genes involved in pathogenicity may be employed as molecular tools for fungicide development or to develop transgenics. With the advent of sequencing of the pathogenic *Fusarium* spp. (Coleman et al. 2009; Ma et al. 2010), several hypothetical pathogenicity genes have been identified. These genes are poorly annotated and hence could not be associated with specific functions. Nevertheless, these genes may be important under unusual conditions that are not evaluated under laboratory conditions (Pena-Castillo and Hughes 2007). Among other genes, *FOW2* encodes a putative transcription regulator belonging to the Zn(II)2Cys6 family and has been speculated for regulation of the expression of several pathogenicity genes in *F. oxysporum* (Imazaki et al. 2007). *chsV* is one of the nine chitin synthase (CHS) genes that has been demonstrated to play a significant role in virulence (Kong et al. 2012). CHS plays a significant role in the polymerization of chitin, a β-1,4-linked polysaccharide made of *N*-acetylglucosamine, a major structural component of fungal cell walls. In fungi, al though all seven CHS classes have CHS domains and transmembrane domains in common, the class V and class VI CHS additionally contain the myosin motor domain (MMD) at their N-terminal. Myosins are known as mechanoenzymes that convert chemical energy released by ATP hydrolysis into a mechanical force that is directed along actin filaments (Kong et al. 2012). CHS genes have also been shown to be important for virulence in *Botrytis cinerea* (*BcCHS1* and *BcCHSIIIa*), *F. graminearum* (*GzCHS5* and *GzCHS7*), *F. verticillioides* (*CHS5* and *CHS7*), *Colletotrichum graminicola* (*CgCHSV*) and *Magnoporthe oryzae* (CHS1–7) (Kong et al. 2012). In *F. solani*, the roles of *FOW2* and *chsV* have yet to be established.

Fungal genes induced by plant hosts may be potential ones, if also constitutively expressed. These genes may serve as right RNAi targets to contain disease development in plants because of improvements in efficiencies of transformation technologies and of gene mutation in fungi. Hence, in this study, we investigated the aetiology of vascular wilt of tomato in India and targeted the *in planta*-induced genes of the fungal pathogens, *F. solani* and *F. oxysporum* for their in vitro expressions. The identified genes, *FOW2* and *chsV*, were then silenced to understand their roles in the pathogenesis of the pathogenic fungi for exploitation in vascular wilt control in the near future.

Materials and methods

Isolation and characterization of *Fusarium* isolates causing tomato wilt

Field surveys were conducted in the tomato-growing areas of Himachal Pradesh (9 locations) and Delhi (2 locations) during 2012–2014, and *Fusarium*-infected plant samples were collected from each of the locations. Isolation of the fungi was carried out from the infected tissues by the direct plating method. Briefly, the tissues were surface-sterilized in 2% sodium hypochlorite, rinsed repeatedly in distilled water, dried by blotting, and then plated directly onto potato dextrose agar (PDA) medium (Hi-media, India). After incubating the plates at 28 ± 2 °C for 24–48 h, colonies around the tissues were selected from each plate and further purified by sub-culturing onto PDA medium. Monoconidial cultures of the isolates were obtained through a single spore isolation technique. Pathogenicity tests were done twice in a screen house on 4-week-old plants of cvs. DVRT1 and Pusa Rohini. Wounded roots were submerged for 10 min in a conidial suspension (1×10^6 conidia mL^{-1} in sterile tap water), while control plants were dipped in sterile tap water. Seedlings were transplanted into 50-ml centrifuge tubes containing 50 g sterile soil. The pathogen was re-isolated from the infected tissues to confirm Koch's postulates. The control plants did not exhibit symptoms. Single-spore isolates of the pathogens were identified on the basis of morphological characters (Nelson et al. 1983). The identities of the fungi were also established by cloning and partial sequencing of the internal transcribed spacer (ITS) region from the genomic DNA as described earlier (Shanmugam et al. 2008). The ITS sequences were aligned using the ClustalX program. The highly pathogenic isolate, FOL, was deposited in the Microbial Type Culture Collection (MTCC 10278), Institute of Microbial Technology, IMTECH (Chandigarh, India).

Isolation and characterization of constitutively expressing fungal genes induced *in planta*

The infected and healthy root samples of the pathogenicity studies were carefully uprooted, washed in running tap water and homogenized with liquid nitrogen in a pre-chilled mortar and pestle. Total RNA was isolated from the powdered samples (100 mg) using Tri-reagent (Invitrogen) according to the manufacturer's protocol. For reverse transcriptase-polymerase chain reaction (RT-PCR), 2 mg of RNA was treated with RNase-free DNase (Genetix) for 30 min at 37 °C followed by DNase stop solution for 10 min at 65 °C for DNase inactivation. The treated RNA (2 μg) was added to oligo (dT) 12–18 primer, and the sample (20 μL) was briefly denatured at 65 °C for 10 min and chilled on ice for 2 min. Reverse transcription was carried out following the protocol supplied with

MuMLv (Fermentas) at 42 °C for 1 h. Two μL of an eightfold dilution of the cDNA was used as template for amplification by PCR in a 25-μL reaction mixture consisting of 2.5 μL of 10× PCR buffer, 2.5 μL of 2 mM dNTPS each, 1 μL of each primer (1 μM), 0.5 μL of *Taq* polymerase (Merck Genei, 1 U/μL) and the final volume was adjusted using 15.5 μL of nuclease free water. Eleven hypothetical fungal genes were amplified at 94 °C for 4 min (initial denaturation), 32 cycles of 94 °C for 30 s (denaturation), 55 °C for 40 s (annealing), 72 °C for 1 min (extension), with a final extension at 72 °C for 5 min.

Constitutively expressing fungal genes were isolated and characterized from the highly pathogenic *Fusarium* isolates, FOL and FS. To prepare the fungal mycelium, conidial suspensions (1×10^5 conidia mL^{-1}) of each of the isolates were inoculated in 100 mL of minimal synthetic broth (0.2 g $MgSO_4.7H_20$, 0.9 g K_2HPO_4, 0.2 g KCl, 1.0 g NH_4NO_3, 2 mg $FeSO_4$ $7H_2O$, 2 mg $ZnSO_4$ $7H_2O$, 2 mg $MnCl_2$ $7H_2O$ supplemented with 0.1% glucose; pH 5.6 g/L). After incubation at 28 ± 2 °C for 7 days on a rotary incubator shaker (180 rpm), the mycelium was filtered through pre-weighed filter paper, weighed and fine ground with liquid nitrogen.

Total RNA was isolated from the mycelial samples (100 mg) and cDNA was synthesized as described earlier for plant samples. *Fusarium* actin (5′-CACCGAGGCTCCCA TCAA-3′ and R- 5′-GGCCTGGATGGAGACGTAGA-3′) gene was used as a house-keeping gene to balance the amounts of cDNA in the samples and the expression of the fungal genes were determined by semi-quantitative PCR assays. The PCR products of the highly expressing *FOW2* and *chsV* genes were purified, and cloned in a T-vector following manufacturer's (Promega) protocols and were sequenced (Eurofins). Phylogenetic analyses of the deduced nucleotide sequences were performed using the ClustalX program.

hpRNAi construct preparation

Fungal transformation vector pSilent-1 (Nakayashiki et al. 2005) was used to construct the hairpin RNAi cassette for *FOW2* or *chsV* as described earlier (Zhong et al. 2012). Briefly, partial cDNA fragments (sense) of 370 bp (*FOW2*) and 310 bp (*chsV*) were PCR amplified with the primers, CFRFOW2-SF2 and CFRFOW2-SR2, and CFRCHSV-SF4 and CFRCHSV-SR4 (Table 1). A 25-μL reaction mixture contained 2.5 μL of 10× PCR buffer, 2.5 μL of 2 mM dNTPS each, 1 μL of each primer (1 μM), 0.5 μL of *Taq* polymerase (Merck Genei, 1 U/μL) and 15.5 μL of nuclease-free water. The thermal cycling conditions were as follows: initial denaturation (94 °C, 4 min), followed by 32 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 40 s) and extension (72 °C, 5 min), and then one final cycle of extension (72 °C, 5 min). The amplified products were digested individually with *Xho*I and *Hind*III, and cloned separately into the

Table 1 Primers used to identify the constitutively expressing pathogenicity genes of *Fusarium* spp.

Gene/Accession No./Identifier	Blast annotation	Primer sequence (5′-3′)
FOXG_12281	Uncharacterized protein	s-TCAACTATGCGCCGACCTAC
		as-ATTTTGCCGAACGCTTCACC
FOXG_10206	Uncharacterized protein	s-TGGATAAGTCCACCGACCCT
		as-CAGCACCGTTGGTGTCAAAG
FOXG_01583	Uncharacterized protein	s-CCTGAGTGGCTTAACACGGT
		as-TGGCTGATCCAAAGGACGAC
FOXG_03544	Uncharacterized protein	s-GCACATTTCGATGAGTCGGC
		as-CCTGTTCCGACCGTATCTCG
FOXG_16265	Uncharacterized protein	s-CAAGATGATTCAGCGCCACG
		as-GGGTTGAACGCGCTTCTTTT
FOXG_05506	Uncharacterized protein	s-GGGTATCGAGCAGACTGAGC
		as-GAGTCGGAGTGTGTGAGCAA
FOXG_03031	Uncharacterized protein	s-GCCAACGCCTCAATTTTGGT
		as-TGTGAGGAGGTCTTTGCGAC
FOXG_13729	Actin like protein 2	s-GGTTTCCTCAAGGTCGGATATG
		as-GCACATCTTCTCTCGGTTCTT
FOW1	Mitochondrial carrier protein	s-CTGCATGAAGACGGTCGAGA
		as-ACGCCGTTGGTCTCGTAAAT
FOW2	Zn(II)2Cys6 transcriptional regulator	s-TATCGGCCTTCTGTCCAACG
		as-TACAAACTGAGTTCCCAGGGC
chsV	Chitin synthase	s-ATCGTCGATTTCCCCGGTTT
		as-GCGTTTTTGCCATCGAAACG

*Xho*I-*Hind*III site of the vector, resulting in pSilent FOW2–1 and pSilent chsV-1. The same fragments (antisense) were also amplified using primers CFRFOW2-ASF2 and CFRFOW2-ASR2 (*FOW2*), and CFRCHSV-ASF4 and CFRCHSV-ASR4 (*chsV*). The amplified products were digested individually with *Kpn*I and *Bgl*II, and cloned into the *Bgl*II- *Kpn*I site of the pSilent FOW2–2 and pSilent chsV-2 vectors resulting in pSilentFOW-2 and pSilentCHSV-2, respectively, to express hpRNAi of the target genes.

Transformation, selection and characterization of fungal transformants

To prepare fungal protoplasts, a spore suspension was inoculated in 100 mL of Potato Dextrose Broth to a final concentration of 10^5 spore mL^{-1} and incubated overnight at 28 °C in shaking (200 rpm). The fungal suspension was centrifuged at 8000 rpm for 10 min and the pellet was suspended in 50 mL enzyme mixture containing β-1,3 glucanase (2.85 mg mL^{-1}), driselase (5.70 mg mL^{-1}) and chitinase (0.05 mg mL^{-1}) dissolved in 0.7 M NaCl. The suspension was incubated at 28 °C with gentle shaking (70 rpm) for 3–5 h. The protoplast was collected from the mycelium by centrifugation at 4500 rpm for 5 min. The pellet was washed in 0.7 M NaCl followed by STC (sorbitol 1.2 M, Tris pH 7.5 10 mM and $CaCl_2$ 50 mM) and diluted to 10^8 mL^{-1} after suspending in 200–500 μL of STC.

Fungal transformants were obtained via polyethylene glycol (PEG)-mediated transformation of the protoplasts as described previously (Hohn and Desjardins 1992) with 10 μg of either linearized plasmids, pSilent FOW2–2 or pSilent ChsV-2. Transformation with pSilent-1 alone served as control. The transformed colonies were selected on PDA containing 250 μg mL^{-1} hygromycin B (Merck, USA) as a selection marker. The transformation of the transgenes in the transformants was verified separately by PCR of genomic DNA using fungal gene specific primers (Table 1).

To study the expression of the pathogenicity genes in the fungal tranformants by semi- and quantitative RT-PCR assays, cDNA was synthesized by reverse transcription from the total RNA isolated from the mycelial samples as described earlier. The amounts of cDNA in the samples were balanced employing *Fusarium* actin (5′-CACCGAGGCTCCCA TCAA-3′ and R-5′-GGCCTGGATGGAGACGTAGA-3′) as a house-keeping gene. In qRT-PCR, the optimal number of cycles was determined for each primer pair (Table 1). The 20 μL of reaction volume included 2 μL of the cDNA template, 10 μL of SYBR Green PCR Master Mix (Thermo, USA) and 1 μL each of 100 nM final concentration of fungal gene primers (Table 2). The assays were conducted in a BioRad iCycler with an initial denaturation for 180 s at 95 °C, 40 cycles of 95 °C for 15 s, 55 °C for 20 s and 72 °C for 20 s. Relative gene expression levels were assessed following the procedure of Livak and Schmittgen (2001). Each of the samples along with a no template control was used in triplicate and the experiment was done in duplicate.

Table 2 Primers used for making hpRNA-expressing constructs and phenotypic analyses of hpRNA-induced silencing in *Fusarium* spp.

Primer name	Primer sequence (5′-3′)
CFRCHSV-SF4	AACTCGAGATCGTCGATTTCCCCGGTTT
CFRCHSV-SR4	AAAAAGCTTGCGTTTTTGCCATCGAAACG
CFRCHSV-ASF4	AAAGGTACCATCGTCGATTTCCCCGGTTT
CFRCHSV-ASR4	AAAAGATCTGCGTTTTTGCCATCGAAACG
CFRFOW2-SF2	AACTCGAGTATCGGCCTTCTGTCCAACG
CFRFOW2-SR2	AAAAAGCTTTACAAACTGAGTTCCCAGGGC
CFRFOW2-ASF2	AAAGGTACCTATCGGCCTTCTGTCCAACG
CFRFOW2-ASR2	AAAAGATCTTACAAACTGAGTTCCCAGGGC
RTFOW2-F	GAGGTTATCTCGGTGCTTCTAG
RTFOW2-R	ATGTCTGGCGATGTATCATGG
RTCHSV-F	AGACGTTTCGATGGCAAAAA
RTCHSV-R	CGGTCGAAGAAGTTCTGGAG

Phenotypic characterization of fungal mutants and infection assays

To estimate the aerial mycelium growth rate of the fungal strains, fungal colonies were tested in vitro in triplicate for their abilities to grow on solid PDA at 28 ± 2 °C. Fungal discs of 5 mm diameter were punched separately from 7-day-old fungal colonies and placed in the middle of solid PDA plates. Solid medium inoculated with the wild-type individual isolate was maintained as a control. The inoculated plates were incubated at 28 ± 2 °C and the linear mycelial growth was measured when the respective control plate showed full growth.

To test the isolates for sporulation, 1 mL of conidial suspension (1×10^5 conidia mL^{-1}) prepared from 5-day-old cultures of each of the fungal strains was inoculated onto the centre of three 70-mm PDA plates and the plates incubated at 28 ± 2 °C. Changes in sporulation of the mutants were measured after 10 days and expressed with respect to the control. The infectivity of the fungal strains was assessed on the 3-week-old tomato cultivar, Pusa Rohini as described earlier in a completely randomized design (CRD) in a greenhouse with 15 replicates. The cultivar developed systemic infection within a week when the humidity and temperature were 90–95% and 20–30 °C, respectively. The wilt development on each tomato plant was calculated as the wilt development on each tomato plant was rated as described by Shanmugam et al. (2011): 0 = no symptoms; 1 = <25% of leaves with symptoms; 2 = 26–50% of leaves with symptoms; 3 = 51–75% of leaves with symptoms; 4 = 76–100% of leaves with symptoms. The percent disease index (PDI) was calculated as follows: Disease index = [Σ(rating × number of plants rated)/total number of plants × highest rating] × 100.

Statistical analyses

The experiments were analysed using the package IRRISTAT v.92–1 developed by the International Rice Research Institute Biometrics Unit, the Philippines. Differences between treatment mean values were determined following LSD test at a 0.05 probability level.

Results

Characterization of the *Fusarium* isolates

Vascular wilt of tomato was widely prevalent to the extent of 15–20% in all the surveyed locations (data not shown) of H.P. and Delhi. Isolation from the infected stem vascular tissues of the symptomatic plants on potato dextrose agar revealed two types of fungi (Fig. 1) for H.P. or Delhi but predominated by type 1 in H.P. In the pathogenicity tests, the inoculated plants with either type upon incubation at 28 °C and 85% humidity for 3–4 weeks exhibited the same symptoms as those of the diseased tomato plants. The fungi re-isolated from the diseased tissues of the inoculated plants displayed characteristic colony and conidial morphologies of the respective inoculated fungus. No fungus was isolated from the asymptomatic control plants. Two isolates belonging to types 1 and 2 that exhibited systemic infection within 3 weeks and representing H.P. and Delhi, respectively, were selected as highly pathogenic and used for further studies. On PDA (Fig. 1a), type 1 was fast growing, reaching up to 7.0 cm in 10 days, with white aerial mycelium tinged with purple, delicate, loosely floccose, pigmentation from pale beige, rose to strong purple or dark bluish violet; bluish violet sclerotial bodies occasionally seen, reverse colourless to dark purple. Conidiophores branched and unbranched monophialides which are short in comparison to those of *Fusarium solani* or *F. verticillioides*, microconidia abundant, mostly 1-celled, oval to kidney shaped, produced in false heads, macroconidia abundant, slightly curved, thin walled and delicate, with an attenuated apical cell and a foot shaped basal cell, 4–6 septate measuring 25–56 X 2.5–6.0 μM (Fig. 1a), chlamydospores formed singly or in pairs. The presence of chlamydospores and microconidia borne in false heads on short monophialides distinguishes the fungus from the closely related *Fusarium* species. On these characteristics, the type 1 isolate was putatively identified as *Fusarium oxysporum* (FOL) Mart. Sacc. (Nelson et al. 1983). On PDA (Fig. 1a), the type 2 isolate displayed rapid growth, dense and white aerial mycelium, with orange sporodochia appearing on old cultures. Microconidia were abundant and lemon- to pear-shaped with 0–1 septum. Macroconidia (Fig. 1a) were also abundant and sickle-shaped with their basal cells distinctly foot-shaped or notched; chlamydospores were formed singly or in chains. On these characteristics, the type 2 isolate was

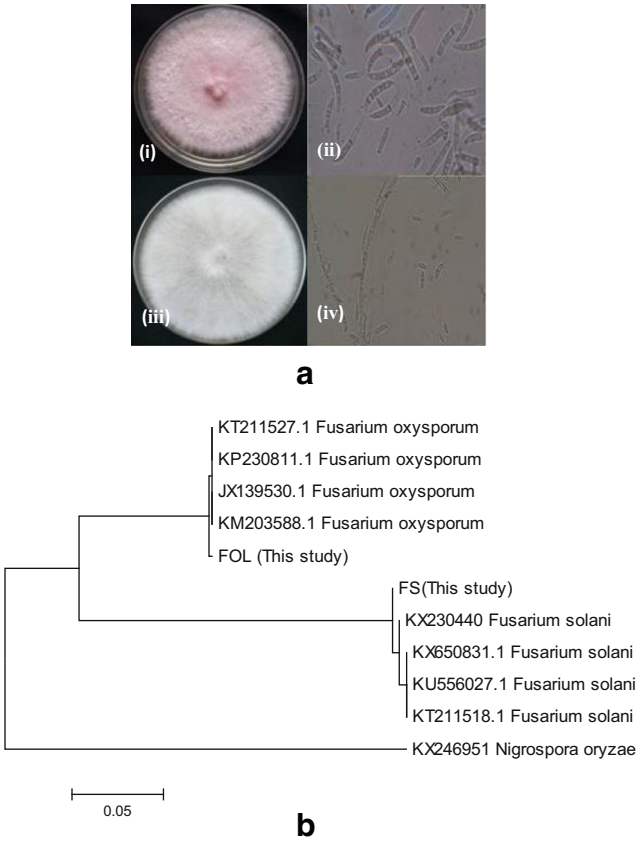

Fig. 1 Characterization of *Fusarium* isolates of tomato. **a** Morphological characteristics of two highly pathogenic isolates: (*i*) growth of FOL on PDA (*ii*) conidia of FOL, *scale bar* 20 μm (*iii*) growth of FS on PDA (*iv*) conidia of FS, *scale bar*= 20 μm. **b** Phylogenetic tree based on ITS sequences, drawn using the neighbour-joining method and showing the relation between selected and other *usarium* isolates. The isolate name is given after the GenBank accession numbers of each species. *Bar* 0.05 substitution per site

putatively identified as *Fusarium solani* (FS) Mart. Sacc. (Nelson et al. 1983). Further characterization to establish the identity of the strains FOL and FS by sequencing of internal transcribed spacer (ITS) region displayed 100 and 99% sequence identities, respectively, to that of the strains, CBPPR0035 of *F. oxysporum* (Accession No KT211527.1) and AL1 of *F. solani* (Accession No. KX650831.1) from the databases (Table 1). In phylogenetic analyses, the ITS sequences of FOL and FS were grouped along with other isolates of *F. oxysporum* and *F. solani*, respectively, as a single cluster (Fig. 1b). Thus, based on colony morphologies, sequence analyses of the ITS region, and by fulfilling Koch's postulates, the highly pathogenic strains FOL and FS were identified as *F. oxysporum* and *F. solani*, respectively.

Isolation and characterization of constitutively expressing fungal genes induced *in planta*

In evaluating the fungal strains, FOL and FS for expression of these genes upon culturing in the minimal medium, among the

11 host induced genes, none of the genes encoding the uncharacterised proteins were inductive. However, both *FOW2* (370 bp) and *chsV* (310 bp) were expressed constitutively (Fig. 2a). In homology analyses of the deduced sequences, the *FOW2* of FOL and FS were closely associated (96 and 99%, respectively) to a putative transcription regulator belonging to the Zn(II)2Cys6 family (Accession No AB266616.1) of *F. oxysporum* from the databases (Table 1). The sequence identities of the *chsV* of FOL and FS were 98 and 99%, repectively, of that of the chitin synthase (Accession No KC 840941.1) of *F. oxysporum*. In phylogenetic analyses, *FOW2* and *chsV* of *F. oxysporum* or *F. solani* were grouped along with respective genes from other isolates of *F. oxysporum* as a single cluster (Fig. 2b).

Hairpin RNA interference (hpRNAi) construct preparation

To down-regulate the expression of the pathogenicity genes *FOW2* or *chsV* in FOL and FS, an RNA silencing vector pSilent-1 containing an inverted repeat (IR) sequence corresponding to the cDNA fragment was constructed. The flow chart for making the RNAi construct is presented in Fig. 3a. The IR sequence is under the control of *A. nidulans gpdA* promoter and *trpC* terminator. The vector carries a hygromycin resistance cassette and a spacer of a cutinase gene intron from the rice blast fungus *Magnaporthe oryzae*. The pSilent-1 construct is predicted to produce a self-complimentary transcript forming a hairpin RNA. The construct was introduced into the FOL and FS genome by PEG-mediated transformation.

Putative transformants for each gene were selected for the PCR assays. To ascertain the transformation of the transgenes in the transformants, genomic DNA of all the putatively transgenic and control fungi was isolated and amplified using forward and reverse primers for sense and antisense arms. A single band of 370 bp for *FOW2* and 310 bp for *chsV* was observed in transgenic fungal lines derived from hairpinRNA-FOW2 and hairpinRNA-chsV constructs, respectively, whereas the same were absent in control clones (Fig. 4b, c). Greater transformation efficiency was observed for FOL (80.0 to 81.3%) than FS (71.4 to 78.6%) (Table 3). To assess the effect

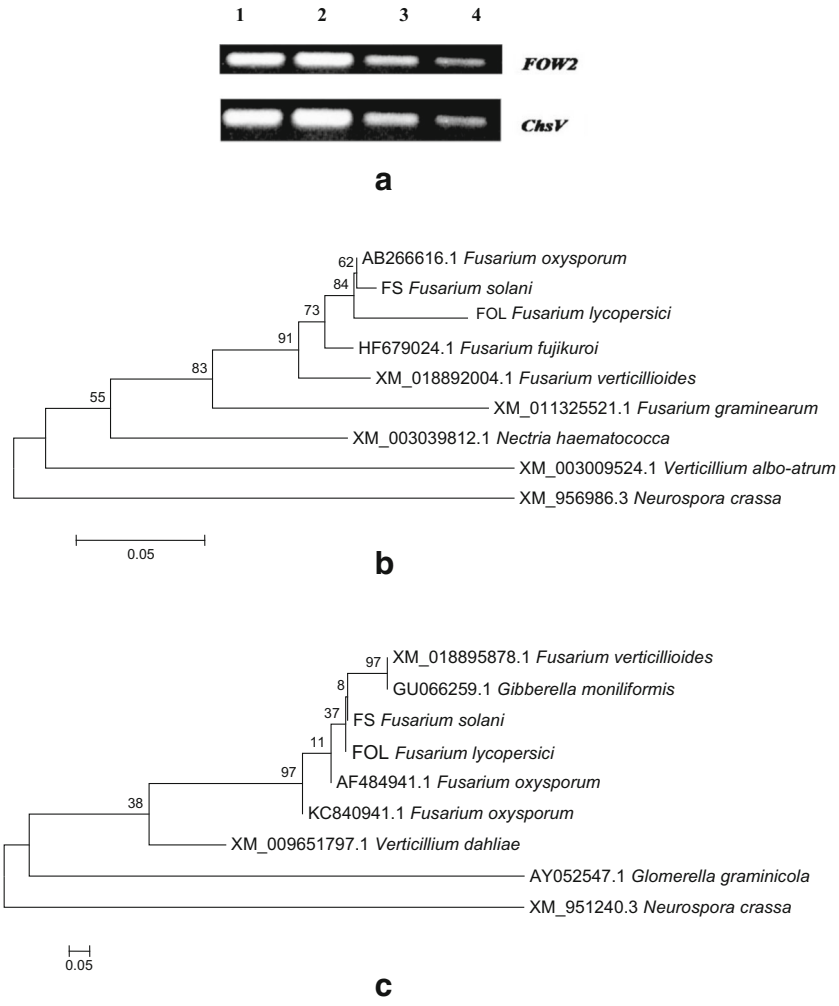

Fig. 2 Selection and characterization of constitutively expressing *Fusarium* genes induced *in planta*. **a** Expression in FOL (*lanes 1 and 2*) and FS (*lanes 3 and 4*) on culturing in a minimal synthetic broth at 28 ± 2 °C. **b**, **c** Phylogenetic analyses of deduced cDNA of *Fusarium* spp.: **b** *FOW2* and **c** *chsV*. *Bar* 0.05 substitution per site

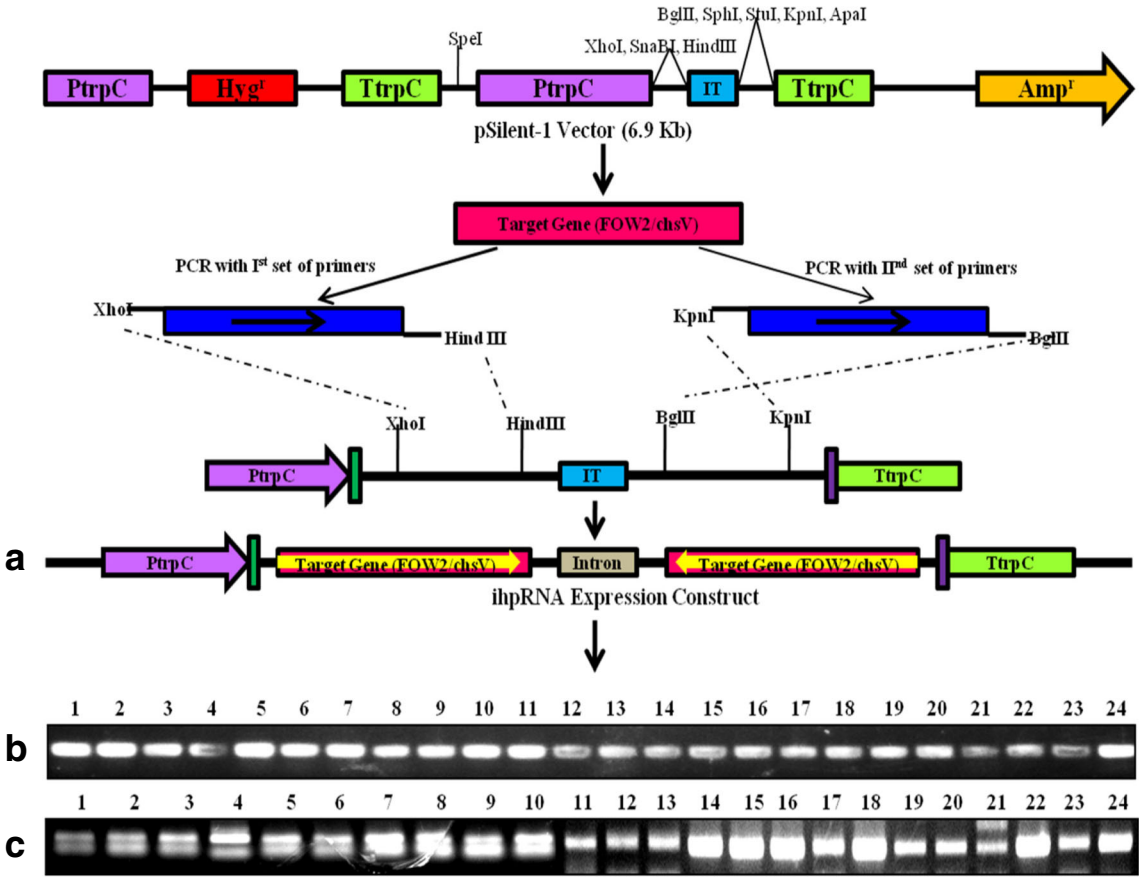

Fig. 3 hpRNAi-expressing constructs. **a** Schematic representation showing the construction of RNAi construct expressing hairpin RNA of the target gene using pSilent-1 vector. **b** Verification of hpRNAi expression construct for *FOW2* by PCR assays: *lanes 1–13* FOL and *14–24* FS. **c** Verification of ihpRNA expression construct for *chsV* by PCR assays: *lanes 1–10* FOL and *11–24* FS

of silencing of the genes on subculturing, monoconidial cultures of the transformants of FOL and FS were obtained through a single spore isolation technique and cultured on PDA medium. The progenies exhibited the same phenotype as the parent transformant suggesting that RNA silencing triggered by the silencing vector was stable during asexual propagation in *F. oxysporum and F. solani* (data not shown).

Expression analysis of fungal transformants

Putative transformants obtained for each of the genes isolated through PDA and hygromycin-containing selective agar plates and tested by PCR were further confirmed by RT-PCR. Analysis of gene expression level of *FOW2* and *chsV* was done in the fungal transformants using gene-specific primers employing fungus actin-encoding gene as endogenous control. In semi-quantitative assays, in comparison to the wild-type strains, FOL and FS, and to the pSilent-1 mutant (control), the expression levels of *FOW2* and *chsV* were significantly decreased (Fig. 4). Likewise, in q-PCR assays, the mean mRNA levels of *FOW2* in the two *FOW2*-silencing transformants of FOL and FS were decreased to 80 and 90%, respectively, in comparison to that of the respective wild-type (Fig. 4). In the two *chsV*-silencing transformants of FOL and FS, the mRNA levels of *chsV* were 90%, indicating effective endogenous gene silencing in either FOL or FS.

Phenotypic characterization of fungal mutants and infection assays

Aerial mycelium growth rate and conidial production assays were performed to determine the effect of *FOW2* or *chsV* silencing on fungal growth. As shown in Fig. 5 and Table 4, the transformants for either of the genes exhibited significantly lesser growth and conidiation over the wild-type strain indicated that silencing of *FOW2* or *chsV* expression adversely affected mycelial growth and propagation. In determining the mycelial growth rate over the wild-type control, among the genes, *FOW2* transformants of FOL exhibited greater reduction in mycelial growth (76.7 and 65.7%) than *chsV* (43.3 and 63.3%). In contrast, *chsV* transformants of FS exhibited no growth, whereas those of *FOW2* showed 82.2 and 67.8% reduction in mycelial growth (Fig. 5). Likewise, the transformants of FOL and FS though exhibited greater reduction in mean sporulation (70.2 and 60.3%) for *FOW2*, while the reduction was 56.7 and 74.1% for the *chsV* transformants.

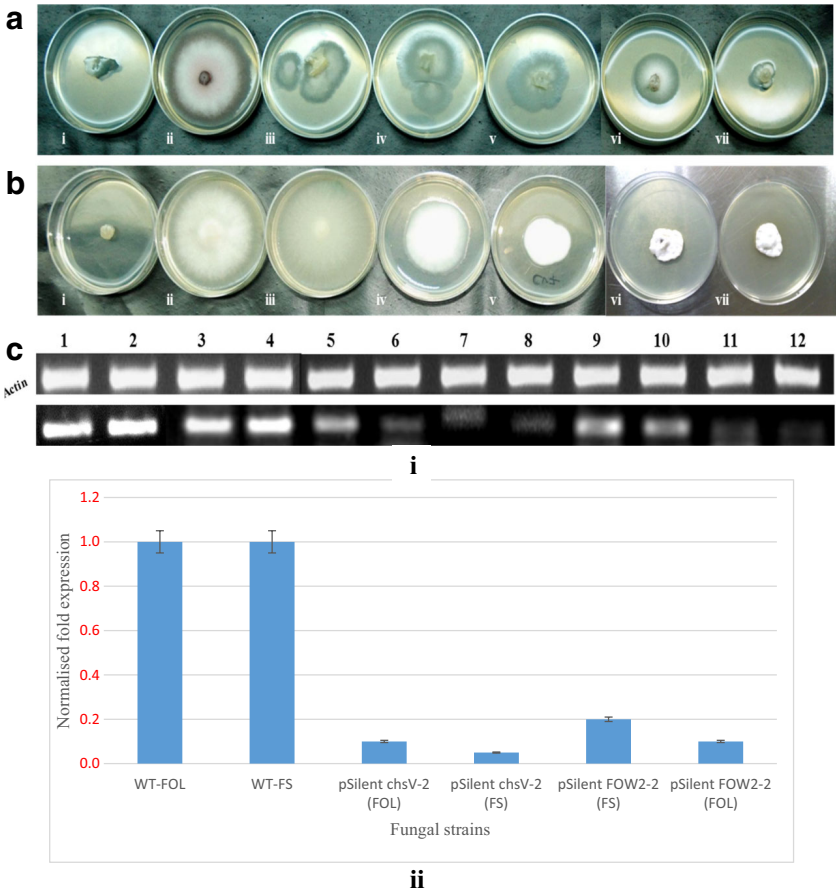

Fig. 4 Phenotypic analyses of hpRNA-induced silencing in *Fusarium* spp. **a**, **b** Colony morphologies of the RNAi-silenced FOL and FS transformants, respectively; *i* WT on PDA with hygromycin *ii* WT on PDA alone; *iii* pSilent-1 *iv, v* pSilent chsV-2, *vi, vii* pSilent FOW2–2 on PDA with hygromycin. **c** *i* RT-PCR detection of gene transcripts *1* WT-FOL, *2* WT-FS, *3* pSilent-1 (FOL), *4* pSilent-1 (FS), *5*, *6* pSilent FOL chsV-2 i and ii, *7*, *8*) pSilent FOL–FOW2–2 i and ii, *9*, *10* pSilent FS-chsV-2 i and ii, *11*, *12* pSilent FS-FOW2–2 i and ii on PDA with hygromycin. **c** *ii* Relative mRNA expression levels in silencing transformants (the amounts of cDNA in the samples were normalized employing *Fusarium* actin as an internal control)

To determine the role of *FOW2* or *chsV* in fungal virulence, infection assays were performed (Fig. 6). Infection with the *FOW2* and *chsV* transformants resulted in reduced PDI, with decreases in the disease indices ranging from 73.3 to 93.3% relative to the wild-type. Among the transformants, silencing of *chsV* in FS resulted in the least mean PDI of 9.2%, whereas that of *FOW2* in FOL recorded 16.7% PDI (Fig. 6).

Discussion

Isolation of *F. solani* isolates other than that of *F. oxysporum* from tomato plants exhibiting characteristic vascular wilt symptoms in both H.P and Delhi regions indicated the significance of the pathogen. Interestingly,the absence of *F. solani* in *F. oxysporum*-associated vascular wilt samples and vice versa revealed lack of complexity in vascular wilt aetiology in the surveyed regions. Recently, based on molecular phylogenetic analysis of *Fusarium* species associated with tomato, Imazaki and Kadota (2015) revealed *F. solani* as a species complex distinct from that of *F. oxysporum*. Hence, *F. solani* is addressed along with *F. oxysporum* to control vascular wilt of tomato.

Table 3 Characterization of fungal transformants

Strains	Putative transformants	Confirmed transformants	Transformation efficiency
pSilent FOW2 (FOL)	16	13	81.3
pSilent FOW2 (FS)	14	11	78.6
pSilent chsV-2 (FOL)	15	12	80.0
pSilent chsV-2 (FS)	14	10	71.4

Because the host-induced genes of fungi that are constitutively expressed may be the potential ones, the 11 fungal genes were assessed for their inductions in a liquid minimal medium. High expressions of *FOW2* and *chsV* over *FOW1* in both FOL and FS established them as molecular targets for downstream applications. Phylogenetic analyses of *FOW2* and *chsV* of both FOL and FS revealed that they are conserved among the species, indicating a common function for pathogenicity in them. Searches in fungal genome databases also revealed that *FOW2* is conserved in other species of *Fusarium*, *F. graminearum*, *F. verticilloides* and *F. fujikuroi*. *Fow2* homologues with significant similarity were also observed in *Verticillium albo-atrum* and *N. crassa*. Likewise, *chsV* homologues were observed in in

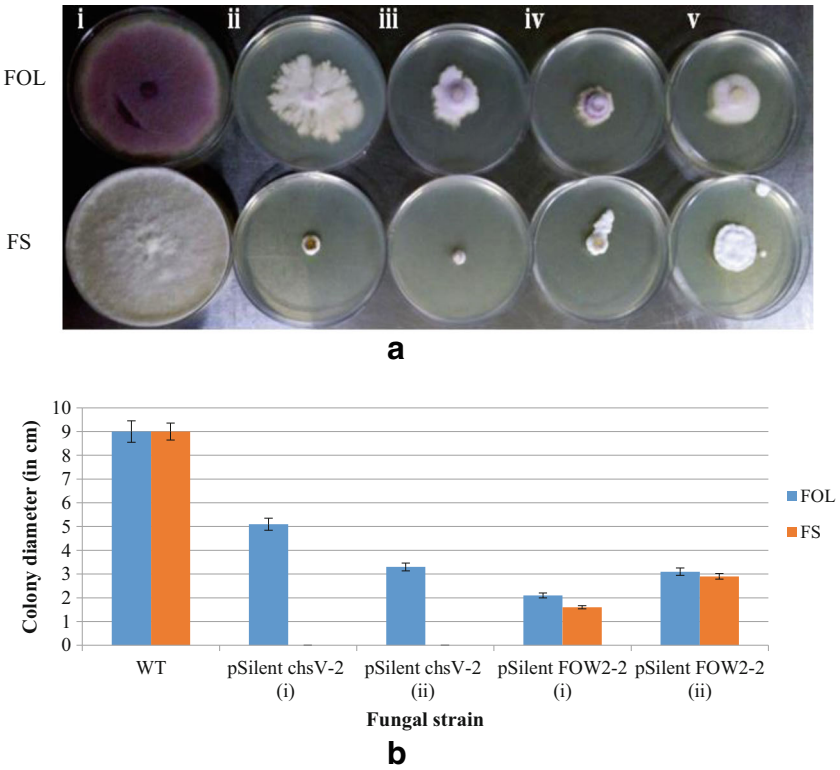

Fig. 5 Aerial mycelium growth rate of the fungal strains. **a** Growth pattern (representative) of the wild-type, *WT* (*lane i*) and hygromycin-resistant transformants, pSilent *chsV-2* (*lanes ii and iii*) and pSilent *FOW2–2* (*lanes iv and v*) on solid potato dextrose agar at 28 ± 2 °C after 4 days of incubation. **b** Mean colony diameter (mean and SD for two transformants of three replications each). *Error bars* SD ($P < 0.05 = 0.4$)

other species of *Fusarium*, *F. verticilloides*, *Verticillium dahliae*, *Colletotrichum graminocola* and *N. crassa*.

The role of *FOW2* and *chsV* in the pathogenesis of *forma specialis* of *F. oxysporum* has earlier been established through development of loss-of-function mutants (Imazaki et al. 2007; Madrid et al. 2003) and HIGS (Hu et al. 2015). However, in some species, transcriptional gene silencing due to expression of hairpin RNA transcripts has been reported (Schumann et al. 2013) suggesting more effective RNA silencing technology for gene function studies. This study successfully demonstrated that the pSilent-1 vector expressing a hairpin RNA possessed the capability to induce efficient gene silencing of either of the genes in *F. solani* and *F. oxysporum*. In comparison to the wild-type strains, the *FOW2*-silenced FOL or FS strains exhibited significant change in mycelium growth rate or spore production. Phenotypic effects of *FOW2* mutants of both FOL and FS are in contrast to an earlier study on *FOW2* of *F. oxysporum* (Madrid et al. 2003). *FOW2*-targeted mutants completely lost pathogenicity and were not impaired in vegetative growth and conidiation in cultures. These differences in the phenotypic effects of FOL might have been accounted by the difference in the method of developing the mutants, as the earlier reported ones were of loss-of-function ones (knock-out). Madrid et al. (2003) speculated the loss of pathogenicity to a failure of expression of a number of pathogenicity genes, as the mutants expressed multiple defects: invasion into roots and colonization in plant tissues.

Table 4 Conidiation of fungal transformants

Strains	Mean conidiation ($\times 10^5$/mL)[a]
WT-FOL	6.7 ± 0.0
WT-FS	5.8 ± 0.0
pSilent FOW2 (FOL)	2.0 ± 0.4
pSilent FOW2 (FS)	2.3 ± 0.4
pSilent chsV-2 (FOL)	2.9 ± 0.4
pSilent chsV-2 (FS)	1.5 ± 0.4
LSD ($P = 0.05$)	0.4

[a] Initial inoculum was 1×10^5 /mL; Conidiation was tested after 8 days; mean and SD for two transformants of three replications each

In *F. oxysporum*, nine CHS genes have been identified (Kong et al. 2012). Due to the presence of multiple genes, in general, the deletion of a single CHS gene may often impart no or only minor effects on hyphal growth. However, in certain cases, some of the multiple CHSs play unique and essential roles in the physiology of the fungal organism (Madrid et al. 2003). Among the four CHS genes that have been characterized, the *CHS1* (III) gene is not important for plant infection but the *chs2* (II) mutant is reduced in virulence on tomato plants. Though deletion of the MMD CHS genes, *CHSV* or *CHSVb* resulted in a significant reduction in virulence, as the *chsV chsVb* double mutant was viable but failed to infect and colonize tomato plants (Kong et al. 2012). Similar to the effect of deletion of *chsV* in *F. oxysporum* (Madrid et al. 2003), in the present study, silencing of the gene in *F. solani* or

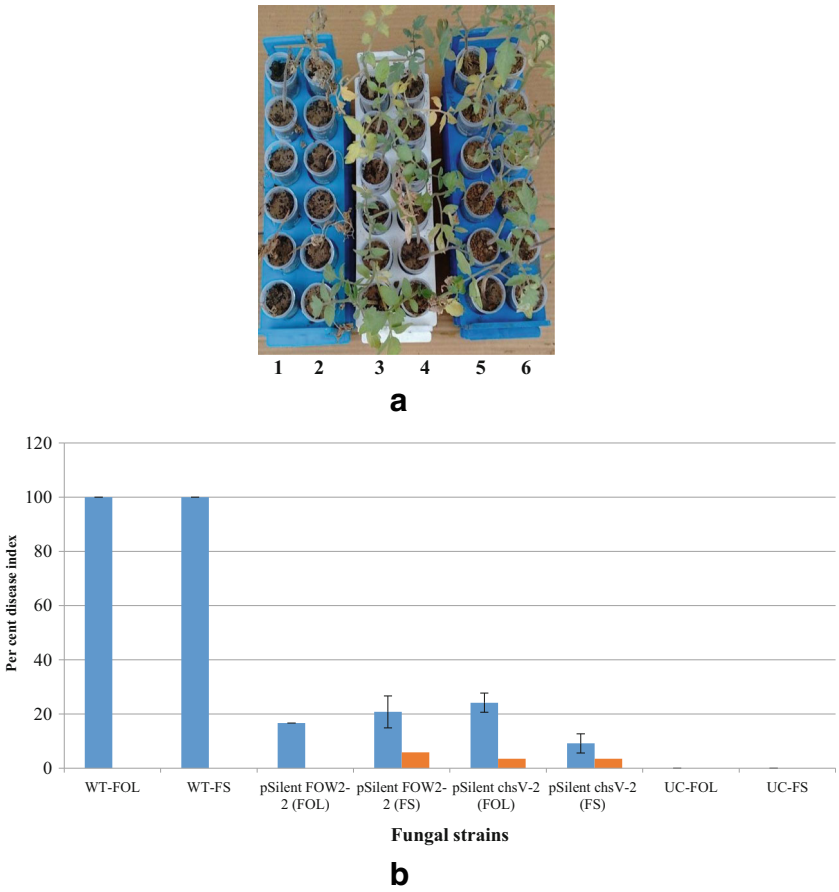

Fig. 6 Infectivity assays of fungal strains on tomato. **a** In vitro assays (representative) for infectivity. *Lane 1* WT-FOL; *2* WT-FS; *3* pSilent FOW2–2 (FOL); *4* pSilent chsV-2 (FS); *5* uninoculated control (UC)-FOL; *6* UC-FS. **b** Per cent disease (wilt) indices on infected tomato seedlings. Values are mean of two transformants of 15 replications each. For rating of wilt development on each tomato plant, see text. The percent disease index was calculated as follows: Disease index = [Σ(rating × number of plants rated)/total number of plants × highest rating] × 100. *Error bars* indicate SD (*$P < 0.05$)

F. oxysporum displayed altered colony growth rate and sporulation besides significant reduction in pathogenic ability in comparison to that of the wild-type. Loss of pathogenicity for host infection was mainly attributed to cell wall integrity offered by *chsV* that prevents the access of antifungal plant compounds to their cellular targets. The possibilities of efficient adhesion during host penetration by ensuring increased cell wall rigidity or through infection-related morphogenesis have been ruled out (Madrid et al. 2003).

Loss of pathogenesis in *Fusarium* species due to RNA silencing indicate that the tool can be a useful means to rapidly screen candidate fungal pathogenicity genes without the need to produce fungal knock-out mutants, and is also likely to be broadly applicable to other *Fusarium* species. Further, *FOW2* and *chsV*, by virtue of their roles in pathogenicity which were proven by efficient gene silencing, could serve as ideal candidates for the development of *Fusarium* wilt-resistant tomato crops through HIGS.

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