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Cladosporium fulvum CfHNNI1 induces hypersensitive necrosis, defence gene expression and disease resistance in both host and nonhost plants

Xin-Zhong Cai · Xin Zhou · You-Ping Xu · Matthieu H. A. J. Joosten · Pierre J. G. M. de Wit

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Abstract Nonhost resistance as a durable and broad-spectrum defence strategy is of great potential for agricultural applications. We have previously isolated a cDNA showing homology with genes encoding bZIP transcription factors from tomato leaf mould pathogen *Cladosporium fulvum*. Upon expression, the cDNA results in necrosis in *C. fulvum* host tomato and nonhost tobacco plants and is thus named *CfHNNI1* (for *C. fulvum* host and nonhost plant necrosis inducer 1). In the present study we report the induction of necrosis in a variety of nonhost plant species belonging to three families by the transient *in planta* expression of *CfHNNI1* using virus-based vectors. Additionally, transient expression of *CfHNNI1* also induced expression of the HR marker gene *LeHSR203* and greatly reduced the accumulation of recombinant *Potato virus X*. Stable *CfHNNI1* transgenic tobacco plants were generated in which the expression of *CfHNNI1* is under the control of the pathogen-inducible hsr203J promoter. When infected with the oomycetes pathogen *Phytophthora parasitica* var. *nicotianae*, these transgenic plants manifested enhanced expression of *CfHNNI1* and subsequent accumulation of CfHNNI1 protein, resulting in high expression of the *HSR203J* and *PR* genes, and strong resistance to the pathogen. The *CfHNNI1* transgenic plants also exhibited induced resistance to *Pseudomonas syringae* pv. *tabaci* and *Tobacco mosaic virus*. Furthermore, *CfHNNI1* was highly expressed and the protein was translocated into plant cells during the incompatible interactions between *C. fulvum* and host and nonhost plants. Our results demonstrate that CfHNNI1 is a potential general elicitor of hypersensitive response and nonhost resistance.

Keywords CfHNNI1 · *Cladosporium fulvum* · Elicitor · Hypersensitive response (HR) · Nonhost resistance · Transgenic tobacco

Abbreviations

CfHNNI1	*Cladosporium fulvum* host and nonhost plant necrosis inducer 1
dpi	Days post inoculation
HR	Hypersensitive response
PVX	*Potato virus X*
TRV	*Tobacco rattle virus*
TMV	*Tobacco mosaic virus*

X.-Z. Cai (✉) · X. Zhou
Institute of Biotechnology, and Department of Plant Protection, Zhejiang University, 268 Kai Xuan Road, Hangzhou 310029, P.R. China
e-mail: xzhcai@zju.edu.cn

Y.-P. Xu
Centre of Analysis and Measurement, Zhejiang University, 268 Kai Xuan Road, Hangzhou 310029, P.R. China

M. H. A. J. Joosten · P. J. G. M. de Wit
Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, 6709 PD Wageningen, The Netherlands

Introduction

Nonhost resistance is a type of resistance shown by an entire plant species against all isolates of a microbial species, which enables plants to protect themselves against the majority of potential pathogens (Heath 2000). It is durable and broad-spectrum,

and therefore is promising for application in plant protection.

The mechanism of nonhost resistance is still not well understood, although some important progress has been made in this regard (reviewed in Thordal-Christensen 2003; Holub and Cooper 2004; Mysore and Ryu 2004; Nurnberger and Lipka 2005). It can be generally classified into preformed and induced defence mechanisms. Preformed defence mechanisms include formation of plant cytoskeleton and barriers against pathogenic invasion (Kobayashi et al. 1997; Yun et al. 2003), and constitutive accumulation of a variety of secondary metabolites, especially those with antimicrobial activity (Osbourn 1996). Induced defence mechanisms comprise of accumulation of components involved in preformed defence mechanisms, activation of plant defence signalling, accumulation of reactive oxygen species and initiation of hypersensitive response (HR) in some cases (Kamoun et al. 1999; Heath 2000; Lauge et al. 2000; Mellersh et al. 2002; Mellersh and Heath 2003; Christopher-Kozjan and Heath 2003; Shimizu et al. 2003; Mysore and Ryu 2004). Some essential nonhost resistance genes have recently been cloned from *Arabidopsis*. Among these are a glycerol kinase gene *NHO1* (Kang et al. 2003), a syntaxin gene *PEN1* (Collins et al. 2003), a glycosyl hydrolase gene *PEN2* (Lipka et al. 2005), and an ATP binding cassette transporter gene *PEN3/PDR8* (Stein et al. 2006). Accumulated evidence reveals that host and nonhost resistance shares some defence mechanisms. First, some nonhost resistance genes also play important roles in host resistance (Collins et al. 2003; Kang et al. 2003). Secondly, some well-known defence signalling pathways and components involved in host resistance, such as salicylic acid- and ethylene-dependent signalling pathways, protein kinase cascade, and pivotal host resistance signalling genes such as *EDS1*, *SGT1*, *HSP90*, are crucial components of nonhost resistance as well (Mysore and Ryu 2004). Thirdly, gene-for-gene interactions leading to host resistance also operate in nonhost resistance in some cases such as nonhost resistance to *Phytophthora* (Kamoun et al. 1999; Kamoun 2001) and *Xanthomonas oryzae* pv. *oryzicola* (Zhao et al. 2004, 2005) and nonhost resistance resulting from recognition of some general elicitors such as flagellin (Asai et al. 2002; Nurnberger and Brunner 2002; Jones and Takemoto 2004; Nurnberger and Lipka 2005).

Relatively less attention has been paid to the identification of nonhost resistance determinants from the pathogen as compared to those from the plant. Nonhost resistance determinants of pathogens known to date include: (1) specific elicitors, i.e. nonhost avirulence gene (*Avr*) products such as INF1 (Kamoun et al. 1999; Kamoun 2001), avrPphD (Arnold et al. 2001) and avrRxo1 (Zhao et al. 2004, 2005), (2) general elicitors or PAMPs (pathogen-associated molecular patterns), including nonhost pathogen-derived structural molecules such as fungal cell wall constituents (e.g. chitin, glucan, protein and glycoprotein), bacterial flagellin and lipopolysaccharide (LPS), and non-structural components, such as harpin of *Pseudomonas syringae* (Nurnberger and Brunner 2002; Nurnberger and Lipka 2005), and (3) cell wall degrading enzymes that produce elicitors, e.g. endopolygalacturonase (Boudart et al. 2003; Poinssot et al. 2003) and xylanase (Rotblat et al. 2002).

Tomato is the only host of *Cladosporium fulvum*, the pathogen of tomato leaf mould disease. The interaction between tomato and *C. fulvum* is a model pathosystem to study gene-for-gene hypothesis (Joosten and De Wit 1999; Cai and Zheng 1999; Cai et al. 2001; Wang et al. 2005, 2006b). However, nonhost resistance induced by *C. fulvum* is much less studied. During infection the pathogen secrets proteins into plant extracellular space. Upon expression, the gene encoding one of the secreted proteins, *ECP2*, induces HR and resistance to recombinant *Potato virus X* in nonhost plant *Nicotiana paniculata* (Lauge et al. 2000), probably through recognition by *Cf-ECP2* (Lauge et al. 1998). Therefore ECP2 might be a specific Avr elicitor of *C. fulvum* nonhost resistance.

Previously, in an attempt to isolate HR inducers, we have cloned a cDNA of *C. fulvum* race 5. Upon functional expression in tomato and tobacco plants it induces a fast and severe HR, and is later named *CfHNNI1* (for *C. fulvum* host and nonhost plant necrosis inducer 1) (Takken et al. 2000; Cai 2004). This clone has homology with genes encoding bZIP transcription factors (Takken et al. 2000). Mutational analysis demonstrates that some amino acids conserved in bZIP transcription factors are required for necrosis-inducing function of CfHNNI1 (Cai 2004). In this study, we investigated the nonhost plant spectrum of *CfHNNI1* in a set of 18 species belonging to five families. Furthermore, through transient expression analysis and stable transgenic plants analysis, the role of CfHNNI1 in defence response and resistance to viral, bacterial and oomycetes pathogens was examined. Finally, localisation of the CfHNNI1 protein in plant cells during *C. fulvum* and plant interactions was analysed by immunocytological approach to provide evidence on its biological role.

Materials and methods

Plant materials and growth condition

The following species were used in this study: Solanaceous *Nicotiana* species (*N. benthamiana*, *N. clevelandii*, *N. cordifolia*, *N. glutinosa*, *N. langsdorfii*, *N. paniculata*, *N. rustica*, *N. solanifolia*, and *N. tabacum* cv. *Samsun* and *Xanthi*), *Lycopersicon esculentum* (tomato cv. Moneymaker near isogenic lines with different *Cf* gene background), *Capsicum annuum* (pepper), and *Solanum melongena* (eggplant); Cruciferous species *Brassica chinensis* and *Brassica pekinensis*; Cucurbitaceous species *Cucumis sativus* (cucumber); Leguminous species *Pisum sativum* (pea) and *Vicia faba* (broad bean); and Chenopodiaceous species *Spinacia oleracea* (spinach). All plants were grown in the green house at 23–25°C during daytime and 19–21°C during night with a light/dark regime of 16 h/8 h.

Constructs for *CfHNNI1* transient expression in plant species

The PVX-based construct pSfinx::*CfHNNI1* and negative control pSfinx::*Avr4* was made previously (the clone 43-7G in the study of Takken et al. 2000), and was used for transient expression study in *Nicotiana* species. To facilitate analysis of *CfHNNI1* transient expression in plant species other than Solanaceous *Nicotiana* species, a TRV-based construct, containing the *CfHNNI1* open reading frame (ORF) and a His-tag sequence fused at the 5 prime of *CfHNNI1* ORF, was made. The forward primer cxz141a (5′-cg gaattc ATG CAC CAC CAC CAC CAC CAC ATG GCC GGG GGA TAC TTC AC-3′) in which an *Eco*RI restriction site (underlined) and a tag sequence encoding six consecutive histidine residues (underlined and capitalized) were introduced at the 5′, and the reverse primer cxz142a (5′-gc ggatcc CTA CTT CCC AAC CAA CCC TCG-3′) in which a *Bam*HI restriction site (underlined) was introduced at the 5 prime, were used to amplified the chimeric sequence carrying the His-tag and the *CfHNNI1* ORF (*His-CfHNNI1*). This sequence was then inserted through *Eco*RI and *Bam*HI restriction sites into the TRV vector pYL156, frequently used for virus-induced gene silencing analysis (Liu et al. 2002), to release the expression construct pYL156::*His-CfHNNI1*. A control expression construct pYL156::*His-Avr4*, which carries the ORF of *C. fulvum* avirulence gene *Avr4*, was made in the same way with the primers cxz52a (5′-cg ggatcc ATG CAC CAC CAC CAC CAC CAC ATG CAC TAC ACA ACC CTC CT-3′) and cxz53a (5′-gc ggatcc TCA ATA GCC AGG ATG TCC AAC-3′). The expression constructs obtained were confirmed by sequencing (MegaBACE 1000 DNA Analysis System, conducted at Centre of Analysis and Measurement, Zhejiang University, China).

Transient expression of *CfHNNI1* in plant species

Two approaches were employed to express *CfHNNI1* in different plant species. The pSfinx::*CfHNNI1*-mediated expression in *Nicotiana* species was achieved by the so-called toothpick-colony inoculation method. Leaves of plants at 3–4 leaf stage were inoculated by pricking with sterilized toothpicks that were stuck on colonies of *Agrobacterium* transformed with pSfinx::*CfHNNI1*, pSfinx::*CfHNNI1*Δ100, which contains the loss-of-function 100 bp deletion mutant of *CfHNNI1* (Xu et al. unpublished data), and control construct pSfinx::*Avr4*. The pYL156::*His-CfHNNI1*-mediated expression analysis in other species was executed by agro-infiltration (Cai 2004). Leaf sectors of cucumber plants at 2–3 leaf stage and other species at 5–6 leaf stage were infiltrated using needleless syringes with 1:1 ratio mixed liquid culture of *Agrobacterium* carrying pTRV1 and pYL156::*His-CfHNNI1*, respectively (Cai et al. 2006). The liquid culture of *Agrobacterium* was prepared as described (Wang et al. 2006a).

Generation of stable *His-CfHNNI1* transgenic tobacco plants

The forward primer hsr203-F (5′-GC GAA TTC GGA TCT TAA TGT TAG TTT ATC-3′) including an *Eco*RI restriction site (underlined) and the reverse primer hsr203-R (5′-GC GAG CTC TTG GCA AAG TTT GAA GTG TT-3′) including a *Sac*I restriction site (underlined) were used to amplify a 1411-nucleotide promoter sequence of the tobacco *HSR203J* gene (X77136, Keller et al. 1999), which was inserted into the binary vector pCHF3 by using *Eco*RI and *Sac*I restriction sites to replace the original CaMV 35S promoter in pCHF3 to release pCHF3-hsr. The chimeric sequence *His-CfHNNI1* was subcloned through *Bam*HI and *Sal*I sites into pCHF3-hsr to produce pCHF3-hsr::*His-CfHNNI1* for tobacco transformation.

The construct pCHF3-hsr::*His-CfHNNI1* was introduced into tobacco by *Agrobacterium*-mediated leaf disc method. It was electroporated into *Agrobacterium tumefaciens* strain EHA105 with a Gene Pulser II system (Bio-Rad, USA). The bacterium cells were collected from liquid culture with an OD_{600} of 0.6–0.8, washed three times with MS medium, and diluted

10–20 times. Tobacco leaf discs were prepared from sterile plant leaves, cultured for about 5 days on MS plates supplied with 6-benzyl aminopurine (6-BA), and then dipped into the diluted *Agrobacterium* suspensions for 10–25 min. After removal of the remaining suspension with sterile filter papers, the leaf discs were incubated on MS plate at 25°C in the dark for 3 days, then transferred to MS plate containing 6-BA, 100 μg/ml kanamycin and 500 μg/ml cefotaxime for selective culture at 25°C with 35 mmol/m^2/s illumination from cool white fluorescent lamps under a 16 h photoperiod. After grown to over 1 cm long, the induced shoots were excised from the primary explants and subcultured in MS medium containing 100 μg/ml kanamycin and 300 μg/ml cefotaxime for shoot proliferation. Kanamycin resistant shoots were selected and transferred to the half-strength MS medium containing 100 μg/ml kanamycin and 250 μg/ml cefotaxime for rooting. The rooted plantlets were exposed to air for 3 days and then transferred to soil for pot-growing in greenhouse. The T_0 progeny of lines containing single copy *His-CfHNNI1* gene as identified with Southern Blot analysis were selected for screening for T_2 homozygous progeny using standard kanamycin selection approach.

Pathogen inoculation and detection

Phytophthora parasitica var. *nicotianae*, causing tobacco black shank disease, was grown on PDA medium. The *His-CfHNNI1* transgenic tobacco plants and controls were inoculated by putting two mycelium discs with a diameter of 4 mm per leaf with one per half side onto the surface of leaves. The inoculated plants were grown at 25°C. High humidity was maintained for 24 h by covering the tray with a transparent polyethylene sheet (Wang et al. 2003).

Cladosporium fulvum, races 4 and 4.5, causing tomato leaf mould disease, was grown on PDA medium. Conidiospores were harvested and prepared into suspensions to a concentration of 10^5/ml, and spray-inoculated 5-6-leaf stage tomato (cv. MM-Cf4, MM-Cf5), tobacco (*N. tabacum* cv. Samsun nn) and cucumber (cv. Jinyiu No. 1) plants as described (Cai and Zheng 1999; De Wit and Flach 1979).

Pseudomonas syringae pv. *tabaci*, the bacterial pathogen of tobacco wild fire disease, was grown on King's B (KB) agar plates at 28°C. Two days later, bacterial cells were suspended with sterile water, washed twice, and resuspended in 10 mM $MgSO_4$. Cell density was determined by optical density at OD_{600}. Bacterial suspensions (10^8 cfu/ml) were infiltrated into tobacco leaves using a needleless syringe for symptom observation. To investigate bacterial growth, suspensions (10^6 cfu/ml) were used, and the bacterial populations in the leaves were measured daily within 5 days post infiltration. Leaf disks (1 cm diameter) were punched out from inoculated areas and homogenized in 10 mM $MgSO_4$, serial dilutions of the homogenate were plated on KB agar plates. After 48 h of incubation at 28°C, colonies were counted. The final result was indicated as the mean value and the standard deviation of triple experiments. The data of in planta bacterium growth study were analysed and illustrated with the software Origin 6.0 (Microcal Software Inc.)

Tobacco mosaic virus (TMV) inoculation was performed in 4-week-old tobacco plants by gently rubbing the sap of *N. clevelandii* containing TMV particles on to carborundum-dusted leaves. Equal amount of viral sap (200 μl per leaf) was used in parallel for the leaves at a same age of both *CfHNNI1* transgenic plants and nontransgenic controls. To detect accumulation of the virus in leaves, the inoculation leaves were sampled for RT-PCR analysis (Cai et al. 2006) to amplify the viral coat protein gene. Total RNA was extracted from plant leaves with TRIzol reagents (Invitrogen, USA). The first-strand cDNA was synthesized from 0.1 mg of total RNA with oligo (dT) primer and superscript reverse transcriptase (Promega, USA). A 319 bp-fragment of the viral coat protein gene was amplified using primers 5′-TCA GCG AGG TGT GGA AAC CT-3′ and 5′-GAG GTC CAG ACC AAC CCA GA-3′. The ubiquitin gene served as a plant internal constitutively expressed control. The primers specific for a 234 bp-fragment of a tobacco ubiquitin gene were 5′-ATG CAG ATC TTC GTG AAG AC-3′ and 5′-CTA GAA ACC ACC ACG GAG A-3′. For a negative control, RT reaction mix without reverse transcriptase was used in the reaction.

Southern and Northern Blot analyses

Genomic DNA was isolated from tobacco leaves following the standard procedure (Sambrook et al. 1989) and digested with *Eco*RI. Total RNA was extracted from plant leaves with TRIzol reagents (Invitrogen, USA). The digested genomic DNA and total RNA were separated on agarose gels and blotted on to Hybond N^+ membranes according to the product manual (Amersham Biosciences, UK). Blot hybridisation with ^{32}P dCTP-labelled probes in Ultrasensitive Hybridization Buffer (Ambion, USA) and subsequent washing with SSC/0.1% SDS were conducted as described (Wang et al. 2005). Kodak XAR 5 films were exposed to the blots at –80°C. The probes used for hybridisation include the full-length cDNA of *CfHNNI1*, *LeHSR203*

(Pontier et al. 1998), *ChiA*, which encode an acidic chitinase (Danhash et al. 1993), and *GluA*, which encode an acidic glucanase (Van Kan et al. 1992), and 1.5 kb *Cla*I/*Sst*I fragment of the coat protein gene of PVX (Takken et al. 2000).

Western blot analysis and immunolocalisation assay

Leaf tissues (about 0.5 g) were harvested and ground into fine powder in liquid nitrogen. Ground tissues were resuspended in 3 ml of extraction buffer (50 mM Tris–HCl, pH 7.0, 5 mM EDTA, 5 mM DTT, 50 mM NaCl, 20 µl protease inhibitor cocktail for plant cell and tissue extracts (Sigma-Aldrich, USA) and centrifuged at 15,000×*g* for 15 min at 4°C. The supernatant was subjected to protein concentration determination using the Bradford method (Bradford 1976). In the CfHNNI1 immunolocalisation analysis, intercellular and intracellular parts were fractionated. Ten days after inoculation with *C. fulvum*, the inoculation leaves were collected, and infiltrated with distilled water *in vacuo* as described previously (De Wit and Flach 1982). After removal of water on surface, the leaves were put into centrifuge tubes and centrifuged for 10 min at 3,000×*g* to obtain intercellular fluids. The leaves were then subjected to intracellular protein extraction as described above. The protein samples (50 µg) was boiled for 5 min in 5× SDS sample buffer, separated by 12% SDS-PAGE and then transferred to PVDF membrane by electroblotting using a TE77 Semi-dry Transfer Unit (Amersham Biosciences, USA) according to standard protocols. The membranes were probed with anti-His-tag monoclonal antibody to detect His-epitope-tagged CfHNNI1 protein as recommended by the manufacturer (Novagen, EMD Biosciences, Germany). In the CfHNNI1 immunolocalisation analysis, CfHNNI1 protein was detected with specific antibody raised against this protein. Antibody directed against the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Agri-Sera, Sweden), was used as a control for intracellular localisation.

Results

Transient expression of *CfHNNI1* induces necrosis in a variety of plant species belonging to three families

To investigate whether expression of *CfHNNI1* induces necrosis in plant species other than tomato cv. Moneymaker lines Cf4 and common tobacco that we reported previously (Takken et al. 2000), a screen of 18 species belonging to five families was undertaken. These species are selected because they are the hosts of the viruses from which the expression vectors derived, easily infiltrated, and some of them are economically important. Necrosis-inducing ability of *CfHNNI1* was first examined by toothpick inoculation of *Agrobacterium* containing pSfinx::*CfHNNI1*, a binary vector modified from *Potato virus X* (PVX), to express foreign cDNA in plants (Takken et al. 2000), in near-isogenic tomato lines Cf0, Cf2, Cf4, Cf5, Cf9, Cf-veda and Cf18, and nine *Nicotiana* species; *N. benthamiana*, *N. clevelandii*, *N. cordifolia*, *N. glutinosa*, *N. langsdorfii*, *N. paniculata*, *N. rustica*, *N. solanifolia*, and *N. tabacum*. All these species developed a *CfHNNI1*-specific necrosis, since mock inoculation with *Agrobacterium* expressing *Avr4*, an *Avr* gene of *C. fulvum*, did not produce any necrosis in the same plants except tomato line Cf4 (Fig. 1A). The development and severity of necrotic symptoms varied in these species. Necrosis in inoculated leaves appeared 7 days post inoculation (dpi) in *N. benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. cordifolia* and *N. rustica*; 9 dpi in tomato; and 10 dpi in *N. langsdorfii*, *N. tabacum*, *N. paniculata* and *N. solanifolia*. In the four species of *Nicotiana*; *N. rustica*, *N. tabacum*, *N. paniculata* and *N. solanifolia*, necrosis only occurred around the inoculation holes, while in others, necrosis developed in both the inoculated and noninoculated systemically infected leaves (Fig. 1A).

In order to examine whether transient expression of *CfHNNI1* could induce necrosis in other species including those belong to families other than Solanaceae, *CfHNNI1* was cloned into the expression vector pYL156 modified from *Tobacco rattle virus* (TRV), which can infect a wide range of plant species. In the expression construct a His-tag-encoding six consecutive histidine residues was fused to the *CfHNNI1* ORF so that the CfHNNI1 protein can be easily detected by antibody raised against the His-tag epitope. The resulting expression construct pYL156::*His-CfHNNI1* was delivered into plant leaves by agro-infiltration method. Necrosis first occurred 3 dpi and developed strongly in the Solanaceous species *Capsicum annuum* (pepper); the Cruciferous species *Brassica chinensis*; and the Cucurbitaceous species *Cucumis sativus* (cucumber); while it first appeared 5 dpi, developed weakly and only in some of the infiltrated leaf sectors in the Cruciferous species *B. pekinensis*. However, necrosis never developed in the Solanaceous species *Solanum melongena* (eggplant); the Leguminous species *Pisum sativum* (pea) and *Vicia faba* (broad bean); and the Chenopodiaceous species *Spinacia oleracea* (spinach). Mock

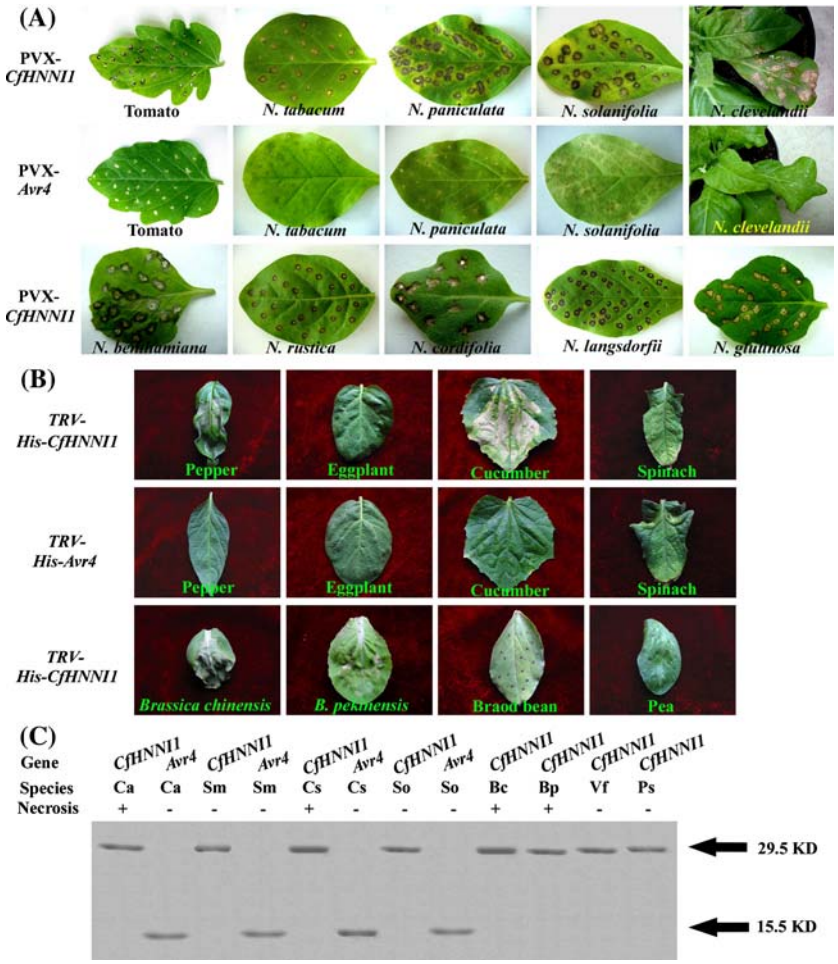

Fig. 1 Transient expression of CfHNNI1 induces necrosis in a variety of plant species belonging to several families. (**A**) *CfHNNI1* was expressed using a PVX-based vector pSfinx in tomato and *Nicotiana* species by toothpick-colony inoculation approach. Leaves of these plant species were inoculated by pricking with sterilized toothpicks that were stuck on colonies of *Agrobacterium* carrying pSfinx::*CfHNNI1* or pSfinx::*Avr4* as a negative control. Necrosis in inoculated leaves, and upper noninoculated leaves in case of *N. clevelandii*, is shown. Photographs were taken 12 days post inoculation (dpi). (**B**) *CfHNNI1* was expressed using a TRV-based vector pYL156 in other plant species by agro-infiltration approach. Leaves of eight plant species belonging to five families were infiltrated with suspensions of *Agrobacterium* carrying pYL156::*His-CfHNNI1* or pYL156::*His-Avr4* as a negative control. Photographs were taken 6 dpi. (**C**) Western Blot analysis for detection of the His-tagged CfHNNI1 protein or control Avr4 protein using anti-His-tag monoclonal antibody in infiltrated leaves of the eight tested plant species shown in (**B**). The molecular weight of the His-tagged CfHNNI1 and Avr4 proteins is 29.5 KD and 15.5 KD, respectively. The abbreviations for plant species are: Ca: *Capsicum annuum* (pepper); Sm: *Solanum melongena* (eggplant); Cs: *Cucumis sativus* (cucumber); So: *Spinacia oleracea* (spinach); Bc: *Brassica chinensis*; Bp: *Brassica pekinensis*; Vf: *Vicia faba* (broad bean); Ps: *Pisum sativum* (pea)

agro-infiltration expressing *Avr4* did not induce any necrosis in any tested plant species (Fig. 1B).

To ascertain the expression of *CfHNNI1* in the inoculated plants that did not show necrosis, Western Blot analysis using anti-His-tag monoclonal antibody was conducted for the agro-infiltrated leaves. Accumulation of the His-tagged CfHNNI1 protein or control Avr4 protein was detected in infiltrated leaves of all tested plant species (Fig. 1C), confirming that the absence of necrosis development in agro-infiltrated eggplant, pea, broad bean and spinach is not due to lack of the CfHNNI1 protein in these species.

Expression of *CfHNNI1* induces HR marker gene expression and resistance against recombinant PVX

To establish whether the *CfHNNI1*-induced necrosis is HR-like, expression of the HR marker gene *LeHSR203* (Pontier et al. 1998) was analysed. The expression level of *LeHSR203* correlated well with the severity of necrosis developed in *CfHNNI1*-expressed tomato plants (Fig. 2). *LeHSR203* was highly expressed in all tested plants that were inoculated with pSfinx::*CfHNNI1*-transformed *Agrobacterium* and in MM-Cf4 plants, harbouring the *Cf-4* gene, inoculated with pSfinx::*Avr4*-transformed

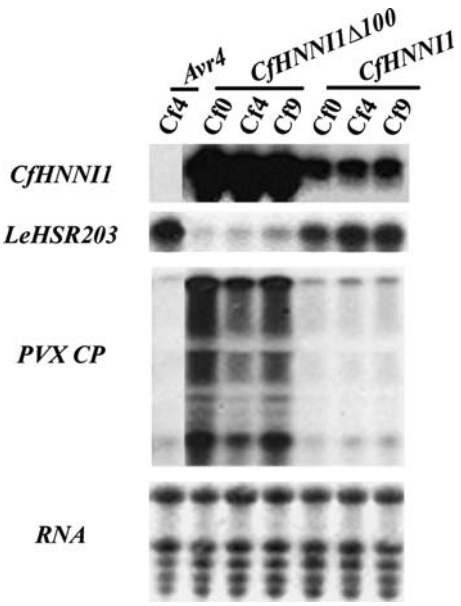

Fig. 2 Transcript accumulation of genes encoding CfHNNI1, LeHSR203 and PVX coat protein in plants expressing *CfHNNI1*. Total RNA was isolated from leaves of MM-Cf4 tomato plants inoculated by toothpicks that were stuck on colonies of *Agrobacterium* expressing pSfinx::*Avr4*, which served as a HR-expressing positive control, and from leaves of MM-Cf0, MM-Cf4 and MM-Cf9 plants inoculated in the same way with *Agrobacterium* carrying pSfinx::*CfHNNI1* and the loss-of-function 100 bp-deletion mutant construct pSfinx::*CfHNNI1*Δ100, which served as a necrosis-not-expressing negative control. Probes used for hybridisation analysis included the full-length cDNA of *CfHNNI1*, *LeHSR203* and the 1.5 kb *Cla*I/*Sst*I fragment of the coat protein gene of PVX

Agrobacterium, which resulted in strong and widespread necrosis. However the gene was only very weakly expressed in the plants inoculated with *Agrobacterium* carrying pSfinx::*CfHNNI1*Δ100, which contains a deletion mutant of *CfHNNI1* with a deletion of the first 100 bp, resulting in frame shift and thus losing the ability to induce necrosis in plants (Xu et al. unpublished data). This result indicates that the *CfHNNI1*-induced necrosis in the plants may be a kind of an HR mimicking response of plants to infection by necrotising pathogens.

To further investigate the relationship between the *CfHNNI1*-induced necrosis and disease resistance, the accumulation of PVX coat protein (CP) gene transcript was determined to quantify the amount of virus present in the plants. The transcripts of the PVX *CP* accumulated at a significantly lower level in all necrosis-developing plants including those were inoculated with pSfinx::*CfHNNI1*-transformed *Agrobacterium* and MM-Cf4 plants that were inoculated with pSfinx::*Avr4*-transformed *Agrobacterium*. However it accumulated at a much higher level in all plants inoculated with pSfinx::*CfHNNI1*Δ100-transformed *Agrobacterium*, which did not show necrosis, thus allowing a much higher multiplication of the virus (Fig. 2). This result fits the observation that transcript abundance of *CfHNNI1*Δ100 is higher than that of *CfHNNI1* in correspondingly inoculated plants (Fig. 2). These data indicate that the HR-like symptoms induced by *CfHNNI1* might result in resistance against PVX. Additionally, the *CfHNNI1*-induced resistance appears to be less strong than *Avr4*-induced resistance in MM-Cf4 plants, as indicated by the lower amount of PVX CP transcripts and higher expression level of HR marker *LeHSR203* in the latter case (Fig. 2).

Generation of stable *CfHNNI1* transgenic tobacco

For the sake of further examining the role of *CfHNNI1* in disease resistance, efforts were made to generate stable *CfHNNI1* transgenic plants. Considering high expression of *CfHNNI1* induces hypersensitive necrosis, a pathogen-inducible expression strategy was employed for generation of *CfHNNI1* transgenic plants in which expression of *CfHNNI1* is under the control of the pathogen-inducible hsr203J promoter (Keller et al. 1999). Through confirmation by PCR and Southern Blot analysis, a total of 15 independent *CfHNNI1* transgenic tobacco lines (CTL) were obtained using the *Agrobacterium*-mediated leaf disc method. Among these, five transgenic lines, named CTL3, 6, 8, 11 and 13, carried a single copy of the *CfHNNI1* gene (Fig. 3A). Lines CTL3, 8 and 11 were selected for further production of plants homozygous for the *CfHNNI1* gene. The T_2 plants homozygous for the *CfHNNI1* gene were subjected to further molecular assays and disease resistance investigation. These CTL plants did not develop necrosis and grew normally as non-transgenic control plants (data not shown). Neither *CfHNNI1* nor *HSR203J* was significantly expressed in these CTL plants according to the Northern blot analysis (Fig. 3B). Western Blot analysis revealed no detectable accumulation of the CfHNNI1 protein in these CTL plants (Fig. 3C).

Pathogen infection induces expression of *CfHNNI1*, *HSR203J* and *PR* genes, accumulation of CfHNNI1 protein, and resistance to *P. parasitica* var. *nicotianae* in transgenic plants

To gain more insight into the role of CfHNNI1 in plant defence and resistance, the T_2 tobacco plants of CTL3, 8 and 11 were inoculated by putting two mycelium discs onto the leaf surface. In the non-transgenic control plants, necrosis developed 2 dpi and was scattered rapidly around the mycelium discs, rendering the whole leaf completely necrotic 5–6 dpi. However, in CTL plants, necrosis was strongly limited within only

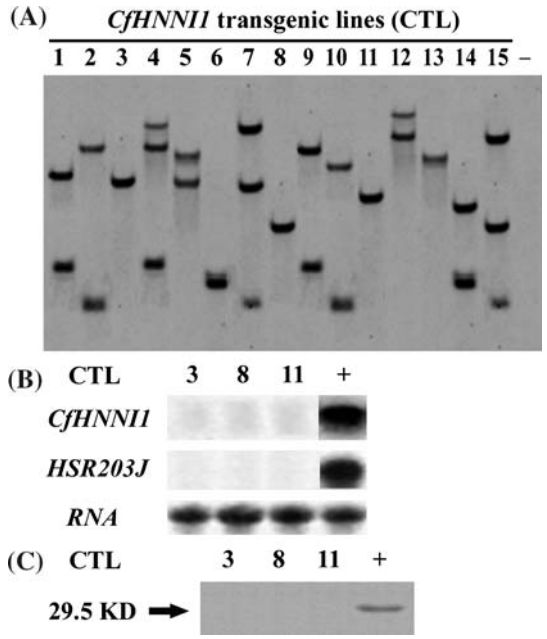

Fig. 3 Molecular analysis of the *CfHNNI1* transgenic tobacco lines. (**A**) Southern Blot analysis of the *CfHNNI1* transgenic tobacco lines. Genomic DNA of T_0 plants of 15 independent candidate *CfHNNI1* transgenic tobacco lines was isolated and digested with *Eco*RI. The blot was probed with the full-length cDNA of *CfHNNI1*. The "–" indicates a non-transgenic negative control. (**B**) Northern Blot analysis of *CfHNNI1* and *HSR203J* in T_2 homozygous plants of the *CfHNNI1* transgenic tobacco lines (CTL) 3, 8 and 11. Total RNA was isolated from leaves of the CTL plants and the positive control (+) non-transgenic tobacco plants infiltrated with *Agrobacterium* expressing TRV::*His-CfHNNI1*. The blot was probed with the full-length cDNA of *CfHNNI1* and *HSR203J*. (**C**) Western Blot detection of the His-tagged CfHNNI1 protein in T_2 plants of CTL 3, 8 and 11. Total protein was extracted from leaves of the CTL plants and the positive control (+) as described in B. The blot was probed with the anti-His-tag monoclonal antibody

0.1, 0.2, and 0.3 cm-wide circular areas around the mycelium discs at 5 dpi in CTL3, 8 and 11 plants, respectively (Fig. 4A). Daily time-course expression of *CfHNNI1*, *HSR203J* and *PR* genes in CTL plants was examined. Transcript accumulation of *CfHNNI1* appeared at a low level at 1 dpi, increasing steadily, peaked at 4 dpi, and maintained at 5 dpi. Transcript of *HSR203J* and *PR* genes encoding acidic glucanase and chitinase accumulated to a low level at 1 dpi, and was elevated continuously thereafter to 5 dpi. In non-transgenic control plants, however, *HSR203J* and *PR* genes were expressed with 1–2 days lag at a significantly lower level (Fig. 4B). Daily time-course expression of the CfHNNI1 protein in these CTL plants was investigated by Western Blot analysis. The His-tagged CfHNNI1 protein was detected at a low level at 1 dpi. The abundance of the protein consistently increased henceforward, reaching the highest level at 4 dpi, and retained thereafter (Fig. 4C). Taken together, these data demonstrate that pathogen infection induced expression of *CfHNNI1*, resulted in expression of *HSR203J* and *PR* genes, and induced resistance to *P. parasitica* var. *nicotianae*.

CfHNNI1 transgenic plants exhibit induced resistance to *Pseudomonas syringae* pv. *tabaci* and TMV

In order to investigate the role of CfHNNI1 in resistance to bacterial and viral pathogens, the transgenic tobacco plants of line CTL3 were used to evaluate the CfHNNI1-induced resistance to *P. syringae* pv. *tabaci* (Pst) and TMV. In Pst-infiltrated leaves of nontransgenic tobacco plants, disease symptoms gradually developed, initially in infiltrated areas, where the tissues turned into firstly chlorotic lately necrotic. Six to seven days later, necrosis was extended over the veins into the neighbouring non-infiltrated sectors, finally resulting in large necrotic areas with a brown centre surrounded by chlorosis (Fig. 5A). However, in Pst-infiltrated leaves of *CfHNNI1* transgenic plants, necrosis developed rapidly in infiltrated areas and turned into grey 2–3 dpi. It was strictly limited within the infiltrated areas (Fig. 5B). Bacterial growth analysis revealed that Pst rapidly propagated within 2–3 dpi and maintained the high population thereafter in non-transgenic tobacco plants, while the growth was significantly restricted in transgenic plants, finally causing about 40 times less than in nontransgenic plants (Fig. 5C). This result indicates that *CfHNNI1* transgenic plants display induced resistance to Pst.

In TMV-inoculated nontransgenic plants, numerous lesions appeared in inoculation leaves 5–6 dpi, gradually enlarged and coalesced, resulting in large tissue yellowing and necrosis (Fig. 5D). While in TMV-inoculated transgenic plants, smaller and significantly less necrotic spots exhibited in inoculation leaves (Fig. 5E). Much lower abundance of viral coat protein gene transcript was detected by RT-PCR in inoculation leaves of transgenic plants than in that of nontransgenic plants (Fig. 5F), indicating that *CfHNNI1* transgenic plants exhibit induced resistance to TMV.

Expression of *CfHNNI1* is induced by *C. fulvum* infection, and CfHNNI1 protein is translocated into plant cell during incompatible interactions

To investigate the possible biological role of *CfHNNI1*, expression of the *CfHNNI1* gene in tomato

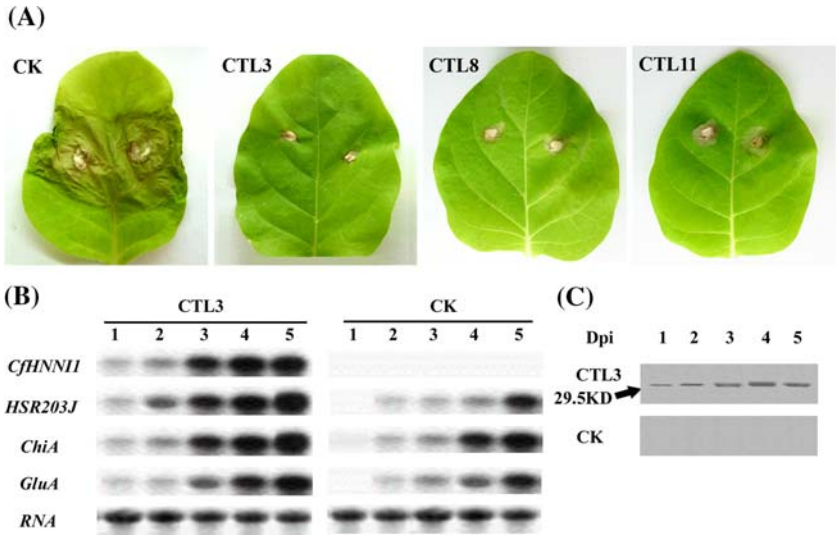

Fig. 4 Molecular analysis of the *CfHNNI1* transgenic tobacco plants after inoculation with the oomycetes pathogen *Phytophthora parasitica* var. *nicotianae*. (**A**) Symptom of the T_2 homozygous CTL plants and non-transgenic control plants (CK). The plants were inoculated by putting two mycelium discs per leaf with one per half side onto surface of leaves. High humidity was maintained for 24 h. Photographs were taken 6 dpi. (**B**) Northern Blot analysis of *CfHNNI1*, *HSR203J* and *PR* genes encoding acidic chitinase (ChiA) and glucanase (GluA) in the T_2 CTL3 plants post inoculation. Total RNA was isolated from 1 cm circular area around the necrotic zone in inoculated leaves of CTL3 plants and non-transgenic plants (CK) daily 1–5 dpi. The blot was probed with the full-length cDNA of *CfHNNI1*, *HSR203J*, *ChiA* and *GluA*. (**C**) Western Blot detection of the His-tagged CfHNNI1 protein in T_2 CTL3 plants post inoculation. Total protein was extracted from leaves sampled as described in (**B**). The blot was probed with the anti-His-tag monoclonal antibody

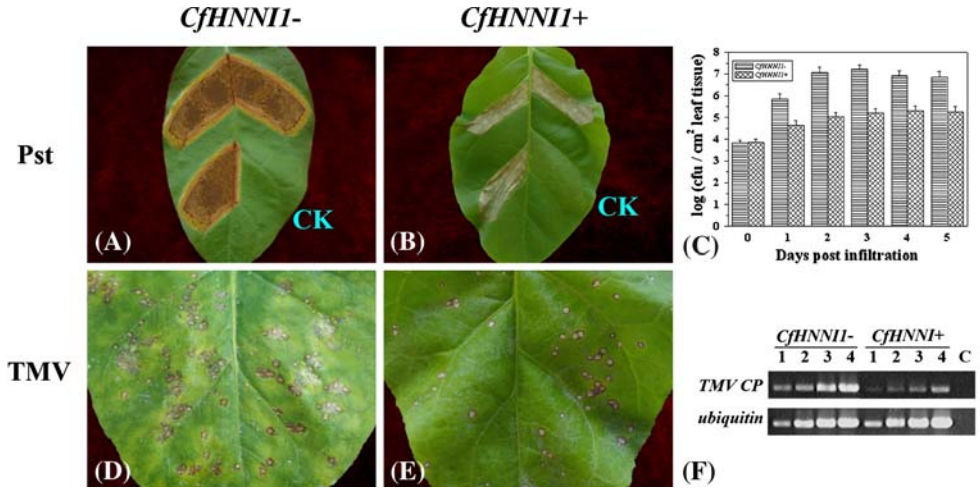

Fig. 5 CfHNNI1 transgenic tobacco plants exhibit induced resistance to *Pseudomonas syringae* pv. *tabaci* and TMV. (**A**–**C**) Resistance test for *P. syringae* pv. *tabaci* (Pst). (**A**, **B**) Symptom observation. Bacterium suspension (10^8 cfu/ml) or 10 mM $MgSO_4$ (CK) was infiltrated into leaf sectors of *CfHNNI1* transgenic tobacco plants of line CTL3 (CfHNNI1+) and nontransgenic plants (CfHNNI1–) using a needleless syringe for symptom observation. Photographs were taken 8 dpi. (**C**) Bacterium growth analysis. Bacterium suspension (10^6 cfu/ml) was infiltrated into leaf sectors. The bacterium populations in the leaves were measured daily 0–5 dpi. (**D**–**F**) Resistance test for TMV. (**D**, **E**) Symptom observation. Four-week-old tobacco plants were inoculated by gently rubbing the sap of *Nicotiana clevelandii* containing TMV particles on to carborundum-dusted leaves. Equal amount of viral sap (200 μl per leaf) was used in parallel for the leaves at a same age of both *CfHNNI1* transgenic plants and nontransgenic controls. Photographs were taken 9 dpi. (**F**) Virus detection. The inoculation leaves were sampled for RT-PCR analysis to amplify a 319 bp-fragment of the viral coat protein (CP) gene. The ubiquitin gene served as a plant internal constitutively expressed control. The primers specific for a 234 bp-fragment of a tobacco ubiquitin gene were used for amplification analysis. Lanes 1–4 correspond to products from PCR of cycles 23, 27, 31 and 35, respectively. Lane C represents the negative control, in which the RT reaction mix without reverse transcriptase was used in the reaction

host plants during pathogen infection was examined by Northern analysis. *CfHNNI1* was expressed in *C. fulvum*-inoculated plants, but not in mock inoculated plants. Transcript of *CfHNNI1* accumulated to a much higher level in Cf5 plants inoculated with the incompatible race 4, compared to Cf4 and Cf5 plants inoculated with the compatible race 4.5 (Fig. 6A), indicating the potential involvement of *CfHNNI1* in disease resistance.

In order to further examine the biological role of *CfHNNI1*, localisation of CfHNNI1 protein in host and nonhost plant cells during *C. fulvum*–plant interactions was analysed by immunocytological approach. Intercellular and intracellular parts were fractionated from tomato and nonhost plants, including tobacco and cucumber, 10 days after *C. fulvum* inoculation. CfHNNI1 protein in the fractions was detected by specific antibody directed against this protein. The immunocytological study revealed that CfHNNI1 protein accumulated in plants during all *C. fulvum* and plant interactions, however, the protein localisation was different during compatible and incompatible interactions. CfHNNI1 protein was detected in intercellular fraction of tomato Cf4 plants inoculated with *C. fulvum* race 4 (compatible), while it was detected in intracellular fraction of tomato Cf5 plants inoculated with the same race (incompatible). In nonhost tobacco and cucumber plants inoculated with *C. fulvum*, CfHNNI1 protein was detected in intracellular fraction as well (Fig. 6B). This result demonstrates that CfHNNI1 protein is translocated into plant cells during *C. fulvum*–plant incompatible interactions, and further supports the involvement of CfHNNI1 in host and nonhost resistance.

Discussions

Cladosporium fulvum CfHNNI1 might be a general elicitor of HR and nonhost disease resistance

Components that induce plant defence responses are called elicitors, a term originally used to refer to molecules and other stimuli that induce the synthesis and accumulation of phytoalexins in plant cells (Ebel and Cosio 1994). Among these, those that induce defence responses in a variety of plant species or even genera are called general elicitors. Here we report that the expression of a single gene *CfHNNI1*, of the tomato leaf mould pathogen *C. fulvum*, is sufficient to induce hypersensitive necrosis in a variety of nonhost plant species belonging to three families; Solanaceae; Cruciferaceae; and Cucurbitaceae (Fig. 1). Additionally, CfHNNI1 induces the expression of HR marker gene *LeHSR203* and the *PR* genes, and resistance to a variety of pathogens including the viruses PVX and TMV, the bacterium *P. syringae* pv. *tabaci*, and the oomycetes *P. parasitica* var. *nicotianae* (Fig. 2, 4, 5). Furthermore, *CfHNNI1* was expressed in *C. fulvum*-inoculated plants. Transcript of *CfHNNI1* accumulated to a much higher level during an incompatible tomato–*C. fulvum* interaction, compared to a compatible one (Fig. 6A). CfHNNI1 can therefore be regarded as a potential general inducer of HR and host and nonhost disease resistance. However, whether CfHNNI1 is a real general elicitor of HR and disease resistance requires further genetic and biochemical identification.

CfHNNI1, in molecular feature, is quite different from the reported general elicitors. Most of the general elicitors of plant defence responses reported to date are pathogen surface-derived structure molecules,

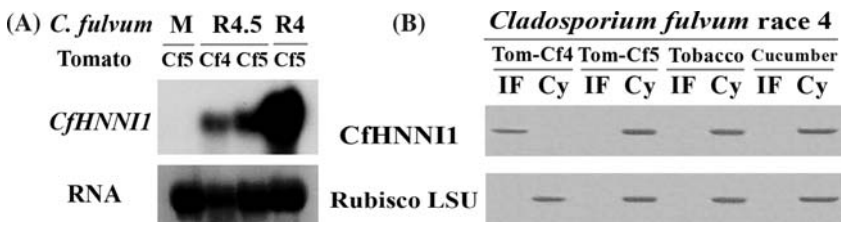

Fig. 6 *CfHNNI1* gene expression and protein accumulation in plants during compatible and incompatible *C. fulvum*–plant interactions. (**A**) *CfHNNI1* gene expression analysis. Total RNA was isolated from leaves of tomato plants line Cf5 inoculated with either sterilised water (M) or spore suspensions of compatible *C. fulvum* race 4.5, and incompatible race 4, as well as line Cf4 inoculated with compatible race 4.5 10 dpi. The blot was probed with the full-length cDNA of *CfHNNI1*. RNA loading is shown in the lower panel. (**B**) CfHNNI1 protein localisation assay. Tomato plants of lines Cf4 (Tom-Cf4) and Cf5 (Tom-Cf5), and nonhost tobacco and cucumber plants were spray-inoculated with spore suspensions of *C. fulvum* race 4. Ten days later, the inoculation leaves were sampled for protein extraction. Intercellular (IF) and intracellular (Cy) parts were fractionated from the sampled leaves, and were then subjected to Western blot analysis. CfHNNI1 protein was detected with specific antibody raised against this protein. Antibody directed against the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco LSU) was used as a control for intracellular localisation

including fungal cell wall constituents such as chitin, glucan, protein and glycoprotein, and bacterial flagellin and liposaccharide (Nurnberger and Brunner 2002; Nurnberger and Lipka 2005), which are similar to animal PAMPs. In addition, a few well-known general elicitors are cell wall degrading enzymes such as endopolygalacturonase (Boudart et al. 2003; Poinssot et al. 2003) and xylanase (Rotblat et al. 2002) that produce effective elicitors. Little is reported for other types of non-structural molecules that act as general elicitors of plant defence responses. CfHNNI1, however, is a non-structural molecule of fungal pathogen *C. fulvum*. This protein contains domains with homology to DNA-binding and leucine zipper domains of bZIP transcription factors. Previous results of mutation assays reveal that amino acids conserved in these domains of bZIP transcription factors are essential for the full function of CfHNNI1 (Cai 2004). It will be intriguing to know whether CfHNNI1 is indeed a member of the bZIP transcriptional factors since no report has yet demonstrated that this kind of transcription factor functions as a general elicitor of plant defence responses.

Possible mechanism of CfHNNI1-dependent HR and nonhost resistance

How is *CfHNNI1*-dependent HR induced? *CfHNNI1* may function as a cytoplasmic bZIP transcription factor, either directly activating HR and defence signalling through the regulation of downstream plant gene expression, or activating the expression of an elicitor gene of plant origin thereby resulting in the accumulation of active plant elicitor, which then initiates downstream HR signalling. In this context it is noteworthy that H52, a tomato transcription factor containing a leucine-zipper domain, is involved in regulation of programmed cell death (Mayda et al. 1999), inferring that the bZIP transcription factor may play a direct role in the regulation of HR and resistance. Additionally, we previously found that amino acids conserved in these domains of bZIP transcription factors are essential for the full function of CfHNNI1 (Cai 2004).

Alternatively, *CfHNNI1* may not act as a bZIP transcription factor, but instead upon entering the plant cell it is recognized through directly or indirectly binding with a receptor, resulting in activation of HR and defence signalling, as reported for other plant general elicitors such as bacterial flagellin (Asai et al. 2002) and animal PAMPs (Jones and Takemoto 2004). It is well known that bacterial effectors including avirulence elicitors are delivered into plant cell. During fungus-plant interactions, some fungal effectors such as avirulence elicitors are also supposed to function intracellularly (reviewed in O'Connell and Panstruga 2006). However, the mechanism by which they are delivered into plant cell is not known until recently a potential host-targeting signal is identified. A conserved amino acid sequence motif, RXLR (R, arginine; L, leucine; X, any amino acid), that is present in close spatial proximity to the predicted signal peptide sequence in all intracellular oomycete effectors characterized to date, might be involved in transporting proteins into the host cell (reviewed in Birch et al. 2006; Ellis et al. 2006). Current results indicate that effectors of *C. fulvum* were secreted into intercellular space during host infection. Interestingly, in this study, we find that CfHNNI1 protein is translocated into plant cells during incompatible interactions between *C. fulvum* and host and nonhost plants, which supports the hypothesis that CfHNNI1 functions inside the plant cell to induce host and nonhost resistance, although the mechanism by which CfHNNI1 is translocated into plant cells is not clear. After entering the cells, *CfHNNI1* could bind to two or more plant proteins in a complex, one of which is the plant target of *C. fulvum* functioning through *CfHNNI1*, another might be the plant protein functioning to recognize *CfHNNI1* and protecting the pathogen target, as suggested by 'guard hypothesis' in the explanation of gene-for-gene host resistance (Hammond-Kosack and Parker 2003). If this is the case, it can be predicted that *CfHNNI1* will elicit HR potentially in all plant species containing these two components. Further studies on the identification of transcription factor activity and isolation of the possible plant targets of *CfHNNI1* will elucidate the mechanism by which *CfHNNI1* functions.

Potential application of *CfHNNI1* in plant protection against diseases

One strategy for engineering resistance is to construct transgenic crop plants that carry a gene encoding a highly active protein elicitor whose expression is under the control of a promoter that is specifically inducible by a virulent pathogen (De Wit 1992). A pathogen-inducible promoter is essential to fulfil this strategy, as they should eliminate any detrimental effects on growth and development owing to unwanted transgene expression in disease-free conditions (Gurr and Rushton 2005). One promising such kind of promoter is the one of the tobacco gene *hsr203J* (Pontier et al. 1994). The hsr203J promoter has been used to drive the elicitors cryptogein and popA in tobacco plants. The transgenic plants expressing cryptogein displayed enhanced resistance to multiple fungal pathogens, such as *P. parasitica* var. *nicotianae*,

Thielaviopsis basicola, *Erysiphe cichoracearum*, and *Botrytis cinerea* (Keller et al. 1999). The transgenic plants expressing *popA* also showed enhanced resistance to *P. parasitica* var. *nicotianae*, but not to some viral pathogens (Belbahri et al. 2001). In this study we obtained stable *CfHNNI1* transgenic tobacco plants in which the *CfHNNI1* gene expression is under the control of the hsr203J promoter. When infected with tobacco black shank pathogen *P. parasitica* var. *nicotianae*, these transgenic plants not only show induced expression of HR marker gene *HSR203J* and *PR* genes *ChiA* and *GluA*, and high resistance to the oomycets (Fig. 4), but also exhibit induced resistance to the bacterial pathogen *P. syringae* pv. *tabaci* and viral pathogen TMV (Fig. 5). Taken together, these data indicate that the hsr203J promoter is a promising candidate promoter and *CfHNNI1* is a good candidate gene to be applied in plant protection against diseases through the strategy of genetic engineering. In this regard, it is interesting that *CfHNNI1* can induce hypersensitive necrosis in a variety of nonhost plant species belonging to at least three families, including Solanaceae, Cruciferaceae and Cucurbitaceae (Fig. 1). It is worthy to examine whether stable transgenic plants of these species can also manifest pathogen-inducible resistance as observed in tobacco.

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References

Arnold DL, Gibbon MJ, Jackson RW, Wood JR, Brown J, Mansfield JW, Taylor JD, Vivian A (2001) Molecular characterization of *avrPphD*, a widely distributed gene from *Pseudomonas syringae* pv. *phaseolicola* involved in non-host recognition by pea (*Pisum sativum*). Physiol Mol Plant Pathol 58:55–62

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in *Arabidopsis* innate immunity. Nature 415:977–983

Belbahri L, Boucher C, Candresse T, Nicole M, Ricci P, Keller H (2001) A local accumulation of the *Ralstonia solanacearum* PopA protein in transgenic tobacco renders a compatible plant–pathogen interaction incompatible. Plant J 28:419–430

Birch PRJ, Rehmany AP, Pritchard L, Kamoun S, Beynon JL (2006) Trafficking arms: oomycete effectors enter host plant cells. Trend Microbiol 14:8–11

Boudart G, Charpentier M, Lafitte C, Martinez Y, Jauneau A, Gaulin E, Esquerre-Tugaye M-T, Dumas B (2003) Elicitor activity of a fungal endopolygalacturonase in tobacco required a functional catalytic site and cell wall localization. Plant Physiol 131:93–101

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254

Cai X (2004) Identification of amino acids of *Cladosporium fulvum* CfHNNI1 required for function of necrosis induction in plants. Acta Bot Sin 46:738–743

Cai X, Takken FLW, Joosten MHAJ, De Wit PJGM (2001) Specific recognition of AVR4 and AVR9 results in distinct patterns of hypersensitive cell death in tomato, but similar patterns of defence-related gene expression. Mol Plant Pathol 2:77–86

Cai X, Xu Q, Wang C, Zheng Z (2006) Development of a virus-induced gene-silencing system for functional analysis of the *RPS2*-dependent resistance signalling pathways in *Arabidopsis*. Plant Mol Biol 62:223–232

Cai X, Zheng Z (1999) Induction of systemic resistance in tomato by an incompatible race of *Cladosporium fulvum* and the accumulation dynamics of salicylic acid in tomato plants. Acta Phytopathol Sin 29:261–264

Christopher-Kozjan R, Heath MC (2003) Cytological and pharmacological evidence that biotrophic fungi trigger different cell death execution processes in host and nonhost cells during the hypersensitive response. Physiol Mol Plant Pathol 62:265–275

Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, Qiu J-L, Hückelhoven R, Stein M, Freialdenhoven A, Somerville SC, Schulze-Lefert P (2003) SNARE-protein-mediated disease resistance at the plant cell wall. Nature 425:973–977

Danhash N, Wagemakers CAM, Van Kan JAL, De Wit PJGM (1993) Molecular characterization of four cDNAs obtained from *Cladosporium fulvum*-infected tomato. Plant Mol Biol 22:1017–1029

De Wit PJGM (1992) Molecular characterisation of gene-for-gene systems in plant–fungus interactions and the application of avirulence genes in control of plant pathogens. Annu Rev Phytopathol 30:391–418

De Wit PJGM, Flach W (1979) Differential accumulation of phytoalexins in tomato leaves, but not in fruits after inoculation with virulent and avirulent races of *Cladosporium fulvum*. Physiol Plant Pathol 15:257–267

De Wit PJGM, Flach W (1982) Evidence for the occurrence of race and cultivar-specific elicitors of necrosis in intercellular fluids of compatible interactions of *Cladosporium fulvum* and tomato. Physiol Plant Pathol 21:1–11

Ebel J, Cosio EG (1994) Elicitors of plant defence responses. Int Rev Cytol 148:1–36

Ellis J, Catanzariti AM, Dodds P (2006) The problem of how fungal and oomycete avirulence proteins enter plant cells. Trend Plant Sci 11:61–63

Gurr SJ, Rushton PJ (2005) Engineering plants with increased disease resistance: how are we going to express it? Trend Biotech 23:283–290

Hammond-Kosack KE, Parker JE (2003) Deciphering plant-pathogen communication: fresh perspectives for molecular resistance breeding. Curr Opin Biotech 14:177–193

Heath MC (2000) Nonhost resistance and nonspecific plant responses. Curr Opin Plant Biol 3:15–319

Holub EB, Cooper A (2004) Matrix, reinvention in plants: how genetics is unveiling secrets of non-host disease resistance. Trends Plant Sci 9:211–214

Jones DA, Takemoto D (2004) Plant innate immunity – direct and indirect recognition of general and specific pathogen-associated molecules. Curr Opin Immunol 16:48–62

Joosten MHAJ, De Wit PJGM (1999) The tomato-*Cladosporium fulvum* interaction: a versatile experimental system to study plant-pathogen interactions. Annu Rev Phytopathol 37:335–367

Kamoun S (2001) Nonhost resistance to *Phytophthora*: novel prospects for a classical problem. Curr Opin Plant Biol 4:295–300

Kamoun S, Huitema E, Vleeshouwers V (1999) Resistance to oomycetes: a general role for the hypersensitive response? Trends Plant Sci 4:196–200

Kang L, Li J, Zhao T, Xiao F, Tang X, Thilmony R, He SY, Zhou J-M (2003) Interplay of the *Arabidopsis* nonhost resistance gene *NHO1* with bacterial virulence. Proc Natl Acad Sci USA 100:3519–3524

Keller H, Pamboukdjian N, Ponchet M, Poupet A, Delon R, Verrier J-L, Roby D, Ricci P (1999) Pathogen-induced elicitin production in transgenic tobacco generates a hypersensitive response and non-specific disease resistance. Plant Cell 11:223–235

Kobayashi Y, Yamada M, Kobayashi I, Kunoh H (1997) Actin microfilaments are required for the expression of nonhost resistance in higher plants. Plant Cell Physiol 38:725–733

Lauge R, Goodwin PH, De Wit PJGM, Joosten MHAJ (2000) Specific HR-associated recognition of secreted proteins from *Cladosporium fulvum* occurs in both host and non-host plants. Plant J 23:735–745

Lauge R, Joosten MHAJ, Haanstra JPW, Goodwin PH, Lindhout P, De Wit PJGM (1998) Successful search for a resistance gene in tomato targeted against a virulence factor of a fungal pathogen. Proc Natl Acad Sci USA 95:9014–9018

Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W, Rosahl S, Scheel D, Llorente F, Molina A, Parker J, Somerville S, Schulze-Lefert P (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*. Science 310:1180–1183

Liu YL, Schiff M, Dinesh-Kumar SP (2002) Virus-induced gene silencing in tomato. Plant J 31:777–786

Mayda E, Tornero P, Conejero V, Vera P (1999) A tomato homeobox gene (HD-zip) is involved in limiting the spread of programmed cell death. Plant J 20:591–600

Mellersh DG, Foulds IV, Higgins VJ, Heath MC (2002) H_2O_2 plays different roles in determining penetration failure in three diverse plant–fungal interactions. Plant J 29:257–268

Mellersh DG, Heath MC (2003) An investigation into the involvement of defence signaling pathways in components of the nonhost resistance of *Arabidopsis thaliana* to rust fungi also reveals a model system for studying rust fungal compatibility. Mol Plant–Microbe Interact 16:398–404

Mysore KS, Ryu C-M (2004) Nonhost resistance: how much do we know? Trends Plant Sci 9:97–104

Nurnberger T, Brunner F (2002) Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. Curr Opin Plant Biol 5:1–7

Nurnberger T, Lipka V (2005) Non-host resistance in plants: new insights into an old phenomenon. Mol Plant Pathol 6:335–345

O'Connell RJ, Panstruga R (2006) Tête à tête inside a plant cell: establishing compatibility between plants and biotrophic fungi and oomycetes. New Phytol 171:699–718

Osbourn AE (1996) Preformed antimicrobial compounds and plant defence against fungal attack. Plant Cell 8:1821–1831

Poinssot B, Vandelle E, Bentejac M, Adrian M, Levis C, Brygoo Y, Garin J, Sicilia F, Coutos-Thevenot P, Pugin A (2003) The endopolygalacturonase 1 from *Botrytis cinerea* activates grapevine defence reactions unrelated to its enzymatic activity. Mol Plant–Microbe Interact 16:553–564

Pontier D, Godiard L, Marco Y, Roby D (1994) *Hsr203J*, a tobacco gene whose activation is rapid, highly localized and specific for incompatible plant/pathogen interactions. Plant J 5:507–521

Pontier D, Tronchet M, Rogowsky P, Lam E, Roby D (1998) Activation of *hsr203*, a plant gene expressed during incompatible plant–pathogen interactions, is correlated with programmed cell death. Mol Plant–Microbe Interact 11:544–554

Rotblat B, Enshell-Seijffers D, Gershoni JM, Schuster S, Avni A (2002) Identification of an essential component of the elicitation active site of the EIX protein elicitor. Plant J 32:1049–1055

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, vol 2, 2nd edn. Cold Spring Harbour Laboratory Press, New York

Shimizu R, Taguchi F, Marutani M, Mukaihara T, Inagaki Y, Toyoda K, Shiraishi T, Ichinose Y (2003) The Δ*fliD* mutant of *Pseudomonas syringae* pv. *tabaci*, which secretes flagellin monomers, induces a strong hypersensitive reaction (HR) in non-host tomato cells. Mol Genet Genomics 269:21–30

Stein M, Dittgen J, Sanchez-Rodriguez C, Hou BH, Molina A, Schulze-Lefert P, Lipka V, Somerville S (2006) *Arabidopsis* PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell 18:731–746

Takken FLW, Luderer R, Gabriels SHEJ, Westerink N, Lu R, De Wit PJGM, Joosten MHAJ (2000) A functional cloning strategy, based on a binary PVX-expression vector, to isolate HR-inducing cDNAs of plant pathogens. Plant J 24:275–283

Thordal-Christensen H (2003) Fresh insights into processes of nonhost resistance. Curr Opin Plant Biol 6:351–357

Van Kan JAL, Joosten MHAJ, Wagemakers CAM, Van den Berg-Velthuis GCM, De Wit PJGM (1992) Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum*. Plant Mol Biol 20:513–527

Wang C, Cai X, Lin J, Zheng Z (2003) The role of salicylic acid and ethylene in resistance of tobacco to black shank. Acta Phytophyl Sin 30:295–299

Wang C, Cai X, Wang X, Zheng Z (2006a) Optimisation of tobacco rattle virus-induced gene silencing in *Arabidopsis*. Func Plant Biol 33:347–355

Wang C, Cai X, Xu Y (2006b) Molecular mechanism of interaction between tomato and leaf mold pathogen *Cladosporium fulvum*. Acta Phytopathol Sin 36:385–391

Wang C, Cai X, Zheng Z (2005) High humidity represses *Cf-4/Avr4*-and *Cf-9/Avr9*-dependent hypersensitive cell death and defense gene expression. Planta 222:947–956

Yun BW, Atkinson HA, Gaborit C, Greenland A, Read ND, Pallas JA, Loake GJ (2003) Loss of actin cytoskeletal function and EDS1 activity, in combination, severely compromises non-host resistance in *Arabidopsis* against wheat powdery mildew. Plant J 34:768–777

Zhao BY, Ardales EY, Raymundo A, Bai JF, Trick HN, Leach JE, Hulbert SH (2004) The *avrRxo1* gene from the rice pathogen *Xanthomonas oryzae* pv. *oryzicola* confers a nonhost defense reaction on maize with resistance gene *Rxo1*. Mol Plant–Microbe Interact 17:771–779

Zhao BY, Lin XH, Poland J, Trick H, Leach J, Hulbert S (2005) A maize resistance gene functions against bacterial streak disease in rice. Proc Natl Acad Sci USA 102:15383–15388