Description of the *Nicotiana benthamiana*–*Cercospora nicotianae* Pathosystem

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Accepted for publication 26 August 2017.

ABSTRACT

Nicotiana benthamiana is a valuable model organism in plant biology research. This report describes its extended applicability in the field of molecular plant pathology by introducing a nonbiotrophic fungal pathogen *Cercospora nicotianae* that can be conveniently used under laboratory conditions, consistently induces a necrotic leaf spot disease on *Nicotiana benthamiana*, and is specialized on solanaceous plants. Our inoculation studies showed that *C. nicotianae* more effectively colonizes *N. benthamiana* than its conventional host, *N. tabacum*. The functions of two

critical regulators of host immunity, coronatine-insensitive 1 (COI1) and ethylene-insensitive 2 (EIN2), were studied in *N. benthamiana* using *Tobacco rattle virus*-based virus-induced gene silencing (VIGS). Perturbation of jasmonic acid or ethylene signaling by VIGS of either *COI1* or *EIN2*, respectively, resulted in markedly increased *Cercospora* leaf spot symptoms on *N. benthamiana* plants. These results suggest that the *N. benthamiana*–*C. nicotianae* host–pathogen interaction is a prospective but hitherto unutilized pathosystem for studying gene functions in diseased plants.

Nicotiana benthamiana Domin, a species of tobacco endemic to Australia (Goodspeed 1954), is one of the most widely used model organisms in plant virology (Chakrabarty et al. 2007; Fan et al. 2014; Obrepalska-Stepłowska et al. 2013; Senthil-Kumar and Mysore 2015). Its stunning susceptibility to plant viruses, even in comparison with other *Nicotiana* species, has been linked to a mutation in a gene encoding a crucial RNA-dependent RNA polymerase that results in compromised gene silencing ability and reduced degradation of viral RNAs in the plant (Yang et al. 2004). *N. benthamiana* is an established model organism for experimental plant biology for a number of reasons: (i) it is suitable for heterologous expression of proteins derived from a wide range of organisms by using recombinant plant viral expression systems (Klimyuk et al. 2014; Moon et al. 2014); (ii) virus-induced gene silencing (VIGS) is highly efficient in *N. benthamiana* and serves as a convenient tool for studying gene function (Bubici et al. 2015; Senthil-Kumar and Mysore 2015); and (iii) *N. benthamiana* is also a suitable host for *Agrobacterium*-mediated transient expression for mass production, functional analysis, or intracellular localization of recombinant proteins (Ding et al. 2014; Goodin et al. 2008).

In contrast to a broad spectrum of plant viruses that would be readily available for inoculation studies in *N. benthamiana*, there are only a limited number of phytopathogenic fungi available for similar experimental purposes. Filamentous pathogens (plant pathogenic fungi and oomycetes) of *N. benthamiana* include the biotrophic *Golovinomyces cichoracearum* (Xiao et al. 2003) and *Peronospora hyoscyami* f. sp. *tabacina* (Hall 1989) as well as some hemibiotrophic or necrotrophic pathogens such as *Colletotrichum* spp. (Dean et al. 2002; Shen et al. 2001), *Phytophthora* spp. (Becktell et al. 2006; Rajput et al. 2014), *Sclerotinia sclerotiorum* (Veluchamy et al. 2012), and *Botrytis cinerea* (Asai and Yoshioka 2009). Given that some of these plant pathogenic microbes have extremely wide host ranges, a pathogenic fungus specialized on

tobacco could potentially offer a more suited system for virulence studies in comparison with less specified, polyphagous parasitic microorganisms.

Cercospora is a large genus of the family Mycosphaerellaceae with over 3,000 named species (Pollack 1987). Most of these species are highly successful plant pathogens, causing leaf spot and blight diseases on several hosts including many economically important crops such as corn, soybean, sugar beet, coffee, peanut, rice, banana, and tobacco (Goodwin et al. 2001). *Cercospora nicotianae* Ellis & Everhart is the causal agent of the ‘frog-eye’ leaf spot disease of tobacco (*Nicotiana tabacum* L.). Typically, it has not been a disease of commercial importance in temperate climates, but often responsible for serious losses in tropical and subtropical regions (Alasoadura and Fajola 1970; Holliday 1980; Jahagirdar and Hundekar 2010; Shew and Lucas 1991). The disease is favored by high humidity and warm weather conditions that allow frog-eye leaf spot to increase to damaging levels (Staveland and Nimmo 1969), while also leading to a reduction in the leaf quality of tobacco (Staveland and Chaplin 1972). *C. nicotianae* is an anamorphic ascomycete with no known sexual stage, but a small number of *Cercospora* species have been connected to the teleomorph genus *Mycosphaerella* (Sivanesan 1984; von Arx 1983). It is a hemibiotrophic pathogen that exhibits a symptomless, biotrophic growth for the first few days of infection and later triggers the death of host cells when switches to a necrotrophic phase (Daub et al. 2013). The typical disease symptoms of *C. nicotianae* on tobacco leaves have been associated with secretion of the nonhost specific toxin cercosporin (Upchurch et al. 1991). Cercosporin produced by many *Cercospora* species converts to an electronically excited triplet state when exposed to light and then reacts with oxygen to generate singlet oxygen (Daub and Ehrenschaft 2000). Singlet oxygen (and other ensuing reactive oxygen intermediates) compromise the integrity of host cell membranes that later provides nutrients for fungal hyphae growing in the intercellular spaces of host tissues. In recent years, *C. nicotianae* has become an important model for molecular analysis of the cercosporin biosynthetic gene cluster through targeted gene disruption (Chen et al. 2007; Choquer et al. 2005; Chung et al. 2003; Dekkers et al. 2007; You et al. 2009). Development of a reliable genetic transformation system for *C. nicotianae* represented a major step for cercosporin research

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and helps establish the species as a potential experimental model (Chung et al. 2003). Availability of mutant *C. nicotianae* lines with defects in cercosporin production or resistance holds considerable promise for the elucidation of the pathways leading to the production and regulation of cercosporin toxin (Amnuaykanjanasin and Daub 2009; Chung et al. 1999; Jenns et al. 1995). Today, near-complete genome sequences are available for seven *Cercospora* species, namely *C. arachidicola*, *C. beticola*, *C. canescens*, *C. cf. flagellaris*, *C. sojina*, *C. zea-maydis*, and *C. zeina* (Chand et al. 2015; Muller et al. 2016; Orner et al. 2015; Vaghefi et al. 2017; Zeng et al. 2017). The availability of these genome sequences may pave the way for identification and functional characterization of novel genes in *C. nicotianae*.

To our knowledge, there has been only one account of an interaction between *N. benthamiana* and *C. nicotianae* in the literature of molecular phytopathology (Nielsen et al. 1993). In their work, the fungus was used to test responses of transgenic *N. benthamiana* ectopically expressing a sugarbeet chitinase. Because the presence of the transgene apparently did not affect susceptibility of *N. benthamiana* to *C. nicotianae*, description of the observed symptoms and other details of the host–microbe interaction were not presented.

Consequently, characterization of the *N. benthamiana*–*C. nicotianae* host–pathogen interaction is lacking and it has not been exploited so far in plant sciences.

Here we present results on the interaction between *N. benthamiana* and *C. nicotianae* and demonstrate that *N. benthamiana* is an excellent host for *C. nicotianae*, showing even higher susceptibility to the fungus than *N. tabacum*. The intensity of frog-eye leaf spots and fungal abundance in leaf tissues can be confidently quantified by disease assessments and PCR-based assays, respectively. The potential of this model system is tested by the perturbation of two crucial regulators of host immunity, coronatine-insensitive 1 (COI1) and ethylene-insensitive 2 (EIN2) by RNA interference-based VIGS technology.

MATERIALS AND METHODS

Plant material and growth conditions. *N. benthamiana* and *N. tabacum* ‘Xanthi-nc’ plants were grown in a 1:1 mixture of potting soil (Agroland) and peat (Pindstrup Plus Orange, Pindstrup, Mosenburg) in a greenhouse until inoculation with *C. nicotianae*, and provided with supplemental light when it was necessary to ensure a 16-h photoperiod.

Cultivation of the fungus and inoculation. *C. nicotianae* isolate ATCC 18366 was used throughout this work (Ehrenshaft and Daub 1994). The fungus was maintained on potato dextrose agar (PDA) plates at room temperature and inocula were prepared according to Beckman and Payne (1983). For long-term storage, cultures were maintained on PDA slants covered with sterile paraffin oil and stored at 20°C. To obtain conidia for plant inoculations, mycelia from fresh cultures were harvested, disrupted in sterile distilled water with glass shards using a FastPrep-24 machine (MP Biomedicals), transferred to Petri dishes filled with V8 juice agar medium, and incubated under 16 h light/8 h dark conditions at 18 to 20°C for 7 days. V8 juice medium was prepared by thoroughly mixing 300 ml of V8 juice with 4.5 g of calcium carbonate. The mixture was centrifuged (3,000 rpm, 10 min) and the supernatant was fivefold diluted with distilled water, mixed with 1.5% agar and autoclaved for 15 min. The conidia produced were gently washed off with sterile 0.2% gelatin solution using a paint brush. The resulting suspension of spores and mycelial fragments were filtered through three layers of cheesecloth. The concentration was adjusted to 5×10^4 conidia per milliliter with a hemocytometer and sprayed onto *N. benthamiana* or *N. tabacum* plants until runoff. At the time of inoculation, *N. benthamiana* and *N. tabacum* plants were 9 and 12 weeks old, respectively. Inoculated plants were covered with transparent plastic bags to provide high humidity for the fungal infection to become established and they were incubated in a growth chamber for 4 days at 27°C and 16 h illumination ($160 \mu\text{mol m}^{-2} \text{s}^{-1}$)

per day. After incubation, the plastic bags were removed and the plants were transferred to the greenhouse.

A detached leaf inoculation assay was also developed for the evaluation of *C. nicotianae* symptoms on VIGS-treated *N. benthamiana* leaves. Leaves in middle positions were removed from plants and were placed on wet filter papers in Petri dishes (28 cm in diameter) with their abaxial sides facing up. Ten-microliter drops of *C. nicotianae* conidial suspension (5×10^4 conidia per milliliter) were distributed onto the (abaxial) surface of leaves and the Petri dishes were incubated in a growth chamber for 4 days at 27°C and 16 h illumination ($160 \mu\text{mol m}^{-2} \text{s}^{-1}$) per day. Then, temperature was reduced to 23°C and after 2 days at this temperature the diameters of *C. nicotianae*-induced lesions were measured.

When *C. nicotianae* isolate ATCC 18366 was first received from the ATCC repository we reisolated *C. nicotianae* from infected leaves showing frog-eye symptoms to restore full virulence of the isolate. This was accomplished by placing diseased leaf sections on top of wet pieces of filter paper in Petri dishes, which were incubated in climate chambers programmed for a 16-h-light/8-h-dark cycle at 20°C for 3 days. Conidia developing on leaf surfaces were removed gently by sterile forceps under a stereomicroscope and were transferred to sterile Petri dishes containing PDA medium.

Detection of fungal biomass by real-time PCR. In order to determine the biomass of *C. nicotianae* in infected *N. benthamiana* and *N. tabacum* plants, four fully developed leaves (in positions four through seven) were collected. Samples of two independent experiments were analyzed, both yielding similar results. Leaves were ground with a mortar and pestle in liquid nitrogen and stored at –70°C. Genomic DNA extraction was performed by a Nucleon Phytopure DNA extraction kit (GE Healthcare) following instructions provided by the manufacturer. DNA extracts were adjusted to a concentration of $10 \text{ ng } \mu\text{l}^{-1}$ with nuclease-free water by using NanoDrop-1000 (Thermo Fisher Scientific). The relative fungal biomass within infected leaves was assessed by quantitative real-time PCR (qPCR) on a C1000 Touch Thermal Cycler equipped with a CFX96 Real-Time PCR System (Bio-Rad) using a KAPA SYBR Fast qPCR Kit (KAPA Biosystems). Fungal DNA was quantified using primers designed for amplification of a 140 bp fragment of *C. nicotianae* actin (GenBank JX143144.1): forward 5'-CAGGA AGGAGGAGCTGACAT-3'; reverse 5'-AGTCCTTCTGGCCCAT ACC-3'. Host plant (*Nicotiana* spp.) DNA sequences were quantified with a primer pair specific for an *N. tabacum* actin gene (GenBank X69885.1): forward 5'-CGGAATCCACGAGACTA CATAC-3'; reverse 5'-GGGAAGCCAAGATAGAGC-3', which amplify a 230-bp PCR product. Quantitative PCR was performed in a total volume of 15 μl containing 7.5 μl of 2 $\times$ KAPA SYBR FAST qPCR Master Mix, 1.5 μl of forward and reverse primers (10 μM), 2.5 μl of template, and 2 μl of PCR-grade water. Cycling conditions used were 95°C for 3 min and then 40 cycles of 95°C for 20 s followed by 60°C for 60 s. Finally, a melt curve analysis was performed to determine amplicon specificity with temperature increases from 65 to 95°C in steps of 0.5°C. Fungal biomass content and relative gene expression for the validation of VIGS efficiency were calculated using the comparative $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001) and host actin as a reference gene.

Data collected from one experiment were presented. Each experiment included four biological replicates and each replicate was a pool comprising leaves of three plants (altogether 12 leaves per sample were analyzed).

Vector constructs for virus-induced gene silencing. *Tobacco rattle virus* (TRV), a bipartite virus-based silencing system was used for VIGS experiments. TRV1 contains the viral replicase, the RNA-dependent RNA polymerase and the movement protein while TRV2 contains the coat protein and a multiple cloning site to incorporate host plant derived fragment(s) of target sequences (Hayward et al. 2011). pTRV1 (STOCK: CD3-1039) and pTRV2 in pCAM-BIA3301 (STOCK: CD3-1043) *Agrobacterium* plasmids were obtained from the Arabidopsis Biological Resource Center.

In order to employ VIGS approach to knock down transcript levels of selected host genes, a marker GFP silencing construct (TRV2-GFP) was first created that enabled us to observe the occurrence of silencing events. For this purpose, a 256-bp mGFP5 fragment (Siemering et al. 1996) was amplified from pEarlyGate 103 (Earley et al. 2006) with a GFP-specific primer pair (forward: 5'-CGCTCTAGAAATGCCTGAGGGATACGTGCAG-3', reverse: 5'-CGCTCTAGATTCGATGTTGTGGCGGGTCTT-3') and cloned into a pGEM-T Easy intermediate vector (Promega). The GFP insert containing pGEM-T Easy was digested with *EcoRI* restriction endonuclease (Thermo Scientific) and the gel-purified GFP fragment was ligated into the *EcoRI*-digested pTRV2 vector.

Two sets of primers were used for creating TRV2-GFP-*COI1* and TRV2-GFP-*EIN2* VIGS vectors. The original sequences were adopted from an article published by Shibata et al. (2010) and the restriction site sequences were customized to make them compatible with the restriction endonucleases (*Bam*HI and *Nco*I) we were going to use. Target sequence specificity of the silencing and avoidance of off-target gene silencing were controlled and confirmed by the VIGS tool of the Sol Genomics Network website (Fernandez-Pozo et al. 2015) searching its *Nicotiana benthamiana* v1.0.1 database. The *EIN2* VIGS primer pair (forward: 5'-TAGGATCCGCCCCCCCTCCAATTTCAA-3', reverse: 5'-TCCCATGGATTACTTTGCGCGGTCC-3') amplified a 268-bp fragment from *N. benthamiana* *EIN2* cDNA (Niben101Scf04548g00001.1), whereas the *COI1* VIGS primer pair (forward: 5'-TAGGATCCGCCACTTGATAATGGTGT-3', reverse: 5'-AGGGATCCAGGCCTTCATCGGATTCC-3') amplified a 174-bp fragment from *N. benthamiana* Niben101Scf02280g08005.1 *COI1* cDNA. Using the VIGS tool of the Sol Genomics Network website our *COI1* silencing construct was predicted to knock down transcript levels of host *Niben101Scf02280g08005.1*, *Niben101Scf01227g01004.1*, and *Niben101Scf03500g01002.1 coronatine insensitive 1* genes, whereas the *EIN2* construct targeted transcripts for *Niben101Scf04548g00001.1* and *Niben101Scf23355g00004.1 ethylene-insensitive 2* genes. By using proper restriction enzymes, the amplified cDNA fragments were ligated into the *Bam*HI and *Nco*I restriction sites of the TRV2-GFP vector and the complete product was transferred into *Escherichia coli* DH5 α by the heat shock method (Tu et al. 2005). The identity of inserts in PCR positive clones were confirmed by Sanger DNA sequencing. VIGS plasmids were finally purified from *E. coli* using NucleoSpin Plasmid kit (Macherey-Nagel) and introduced into *Agrobacterium tumefaciens* MOG301 (Hood et al. 1993) by electroporation (Mahmood et al. 2008).

VIGS. *Agrobacterium* strains containing pTRV1, pTRV2, or P14 silencing suppressor constructs (Mérat et al. 2005) were grown overnight at 28°C on Luria Bertani medium supplemented with the appropriate antibiotics (kanamycin sulfate at 30 μ g ml⁻¹, rifampicin at 50 μ g ml⁻¹). For inoculum preparation, bacterial cells were suspended in *Agrobacterium* incubation buffer (1.95 g of MES, 2 g of MgCl₂·6H₂O in 1 liter of distilled water, pH 5.6) and supplemented with acetosyringone (final concentration of 150 mM). Bacterial cell densities were adjusted with a spectrophotometer to OD₆₀₀ = 0.4 for TRV1 and TRV2 and OD₆₀₀ = 0.2 for P14. After a 3-h incubation at

room temperature, the bacterial suspensions mixed in a ratio of 1:1:1 (vol/vol) were infiltrated into two lower leaves of 3- to 4-week-old GFP-expressing transgenic *N. benthamiana*. At least 15 plants were used for each VIGS construct. Fourteen days after inoculation, gene silencing was tested by visual inspection under UV light (for observing the suppression of GFP fluorescence) and by real-time reverse transcription PCR (RT-qPCR).

Evaluation of gene silencing efficiency. For RT-qPCR analyses, four leaves at the middle positions (leaves four through seven) were collected from three plants in each treatment and pooled. Harvested leaves were frozen in liquid nitrogen, ground with a mortar and pestle, and 100 mg of material was used for total RNA extraction by using a Viogene Total RNA Extraction Miniprep Kit according to the manufacturer's instructions. The RNA content and purity were analyzed in a NanoDrop spectrophotometer. Before cDNA synthesis all samples were DNase-treated (Invitrogen, DNase-free DNA Removal Kit). First-strand complementary DNA was synthesized using the Thermo Scientific First Strand cDNA Synthesis Kit according to the manufacturer's instructions. Complementary DNA was used as template for real-time PCR in tenfold dilution. *COI1* and *EIN2* mRNA levels in *COI1*- and *EIN2*-silenced plants were assayed using the same platform and conditions as for real-time PCR detection of fungal biomass and quantified as described above. The primers were the following: *COI1* forward 5'-CTGCAAATCTTACGCTTGA-3' and reverse 5'-ATTCAGCCCTTTGTTCTATGA-3' (amplifying a 162-bp fragment), *EIN2* forward 5'-CGGTGATTACCACTTTTAGTC-3' and reverse 5'-CCTCGTTACAACCTTCTTATCC-3' (amplifying a 179-bp fragment). *COI1* forward and reverse primers were used to monitor transcript abundance for *Niben101Scf02280g08005.1* gene and *EIN2* primer pair was utilized to amplify a corresponding sequence of *Niben101Scf04548g00001.1* transcript. *COI1* and *EIN2* transcript levels were determined in healthy and *C. nicotianae*-infected leaves of GFP-silenced control, and *COI1*- and *EIN2*-silenced plants. Leaf samples analyzed in this assay were collected from plants 3 days after inoculation with *C. nicotianae* using the same method as described above. Total RNA was extracted from leaves in positions four through seven (12 leaves per sample were analyzed). Two independent experiments were conducted with similar results.

Relative gene expressions presented correspond to results provided by one representative experiment and they show means of three biological and three technical replicates (each biological sample was composed of a pool of three plants).

Assessment of disease severity. Necrotic symptoms caused by *C. nicotianae* on VIGS-treated plants were evaluated on nine consecutive leaf levels 7 days after inoculation using the histogram tool of an open-source graphics software (Gimp 2.0) and presented as percentages of necrotic leaf areas. Displayed results represent means calculated for two independent experiments each including four *N. benthamiana* plants spray-inoculated with *C. nicotianae*. In detached-leaf assays, the diameters of *C. nicotianae*-induced necroses following drop-inoculation of detached *N. benthamiana* leaves were determined 6 days after inoculation. Two independent experiments, each containing 10 detached leaves, were evaluated for all the three silencing constructs.

Fig. 1. Symptoms of *Cercospora nicotianae* on *Nicotiana benthamiana* at A, 8 and B, 10 days after inoculation (DAI). C, Conidiophores and conidia of *C. nicotianae* from hyphal strands developing on the surface of an *N. benthamiana* leaf at 11 DAI (including an incubation of the detached leaf in a moist chamber for 3 days at 20°C). Plants were inoculated with a suspension of *C. nicotianae* conidia in a concentration of 5×10^4 spores per milliliter.

Fig. 2. Development of macroscopic symptoms caused by *Cercospora nicotianae* on *Nicotiana benthamiana* (2, 5, 8, and 11 days after inoculation). Plants were inoculated with a suspension of *C. nicotianae* conidia in a concentration of 5×10^4 spores per milliliter.

Data analysis. Experimental data were statistically analyzed by Student's *t* tests or by one-way ANOVA and subsequent Tukey's honestly significant difference test for pairwise comparisons.

RESULTS

N. benthamiana is a host of *C. nicotianae*. Frogeye symptoms caused by *C. nicotianae* typically arose 3 to 4 days after inoculation on lower leaves of 9-week-old *N. benthamiana* plants as small necrotic spots that gradually enlarged and merged into large necrotic areas. Sometimes larger (approximately 5 to 7 mm in diameter) necrotic lesions emerged rapidly on older leaves. Intensity of frogeye symptoms dynamically increased during the following 4 to 5 days in the form of new necroses developing also on the younger, upper leaves, while the first necrotic spots grew larger and completely decayed the lower leaves (Figs. 1 and 2). When leaves showing frogeye leaf spot were detached and incubated for 3 days in a moist chamber, a pronounced growth of gray mycelium was observed on both adaxial and abaxial surfaces of symptomatic leaves. Transferring of this fungal material to Petri dishes containing PDA or V8 media produced cultures that were characteristic of *C. nicotianae*.

When suspensions of conidia harvested from these V8 plates were sprayed onto leaves of healthy *N. benthamiana* plants this inoculation resulted in typical frogeye leaf spot symptoms following incubation at 27°C and in high humidity for 4 days.

N. benthamiana is markedly more susceptible to *Cercospora* leaf spot than *N. tabacum*. One purpose of this study was to compare the susceptibility of *N. benthamiana* and *N. tabacum* to *C. nicotianae*, since *N. tabacum* is the most important host of the fungus.

Nine-week-old *N. benthamiana* and 12-week-old *N. tabacum* plants were inoculated with *C. nicotianae* because they produce similar numbers of leaf levels by this time (the development of *N. tabacum* is somewhat slower). Macroscopic symptoms of frogeye leaf spot first appeared 3 to 4 days after inoculation in *N. benthamiana*, whereas *N. tabacum* plants showed first visible symptoms only 7 to 12 days after inoculation. Necrotic symptoms of the fungal infection were also more pronounced on leaves of *N. benthamiana* in comparison with *N. tabacum* (Fig. 3).

Quantification of fungal DNA in the leaves indicated that the biomass of *C. nicotianae* was consistently higher in *N. benthamiana* than in *N. tabacum* (Fig. 3). This observation together with the

Fig. 3. Symptoms of *Cercospora* leaf spot on 9-week-old *Nicotiana benthamiana* plants (three plants on the left) and 12-week-old *N. tabacum* plants (two plants on the right) 9 days after inoculation with the fungus. Plants were inoculated with a suspension of *Cercospora nicotianae* conidia in a concentration of 5×10^4 spores per milliliter. Inset: Relative amount of *C. nicotianae* to *N. tabacum* and *N. benthamiana* DNA in leaves 9 days after inoculation with the fungus. DNA levels were estimated by quantitative polymerase chain reaction specific for actin genes of *C. nicotianae*, *N. tabacum*, and *N. benthamiana*. Bars represent mean $\pm$ standard error of four biological and three technical replicates (each biological sample was composed as a pool of three plants). Total genomic DNA was extracted from four leaves (in positions four through seven) of plants for each species. Asterisk indicates statistically significant difference in relative amount of *C. nicotianae* DNA between *N. benthamiana* and *N. tabacum* plants calculated by Student's *t* test ($P \leq 0.01$).

Fig. 4. Effect of virus-induced gene silencing on coronatine-insensitive 1 (*COI1*) and ethylene-insensitive 2 (*EIN2*) mRNA transcript levels in *Nicotiana benthamiana*. Gene expression levels were assayed by real-time reverse transcription PCR. The GFP-silencing construct (TRV-GFP) was used as a control. Bars represent mean $\pm$ standard error of three biological and three technical replicates (each biological sample was composed as a pool of three plants). Total RNA was extracted from silenced healthy and *C. nicotianae*-infected *N. benthamiana* leaves (in positions four through seven) 3 days after inoculation. Means with different letters are significantly different at $P \leq 0.01$ calculated by Tukey's post hoc test.

observed symptoms of the fungus on the two host species led us to the conclusion that *N. benthamiana* is significantly more susceptible to *C. nicotianae* than *N. tabacum*.

Perturbation of jasmonic acid or ethylene signaling by VIGS technology further increases the susceptibility of *N. benthamiana* to *C. nicotianae*. TRV-based silencing constructs were designed for functional analysis of *EIN2* and *COI1* genes in *N. benthamiana*. The effects of the two TRV-VIGS treatments on *N. benthamiana* transcripts were evaluated by observing the suppression of GFP fluorescence (plants ectopically expressing the green fluorescence protein were used throughout this study) and by RT-qPCR analyses of *COI1* and *EIN2* transcript levels. After 2 weeks of VIGS treatments, RT-qPCR assays showed that *COI1* and *EIN2* mRNA levels were consistently decreased in healthy as well as in *C. nicotianae*-infected *N. benthamiana* plants (Fig. 4).

Responses of VIGS-treated plants to inoculation with *C. nicotianae* were tested in whole-plant and in detached-leaf assays. Reduction of *COI1* and also *EIN2* transcript levels apparently promoted the development of necrotic symptoms caused by *C. nicotianae* on *N. benthamiana* plants (Figs. 5 and 6). Consistent with these results, silencing of either *COI1* or *EIN2* resulted in more severe necrosis on inoculated detached leaves compared with the GFP-silenced control (Fig. 7).

DISCUSSION

Results in this work establish a new, previously unutilized pathosystem for the model plant *N. benthamiana*. *C. nicotianae*, a

conidial fungus specialized on species of the *Solanaceae* family is presented as a reliable microbial pathogen for the analysis of host responses in *N. benthamiana*. It was critical to test the suitability of the *C. nicotianae*–*N. benthamiana* system for gene silencing studies. Reliable pathogen responses to VIGS treatments in *N. benthamiana* would demonstrate the usefulness of this fungus in studying functions of plant genes in plant–pathogen interactions.

Suppression of host *COI1* and *EIN2* gene activity by triggering the plants' posttranscriptional gene silencing machinery resulted in consistently higher degree of *Cercospora* leaf spot disease severity. These results suggest that frog-eye symptoms caused by *C. nicotianae* may be inhibited by jasmonic acid- or ethylene-mediated responses in *N. benthamiana* plants. Our findings are in agreement with earlier investigations showing increased host susceptibility to various phytopathogens including *B. cinerea* and *S. sclerotiorum* by disruption of *COI1* and *EIN2* gene functions (Chen et al. 2009; Guo and Stotz 2007; Ho et al. 2009; Liu et al. 2004; Thomma et al. 1999). Ethylene-insensitive transgenic *N. tabacum* plants expressing the mutant *etr1-1* gene from *Arabidopsis thaliana* also showed enhanced susceptibility to *C. nicotianae* (Geraats et al. 2003).

N. benthamiana appeared far more susceptible to *C. nicotianae* than the conventional host of the fungus *N. tabacum*. This seems to be in accord with the pronounced susceptibility of *N. benthamiana* to virus infections as a result of a loss-of-function mutation in a gene encoding an RNA-dependent RNA polymerase (Yang et al. 2004). RNA interference mechanisms are not only crucial components of cellular defense responses to viruses but also to bacteria, fungal, and oomycete pathogens (Ellendorff et al. 2009; Lopez et al. 2011; Staiger et al. 2013; Yang and Huang 2014).

Symptoms of *C. nicotianae* on *N. benthamiana* become intense within a rapid and predictable time frame. Growth of the fungus can be assessed by a qPCR method described here. Our findings, therefore, suggest *C. nicotianae* as a prospective fungal pathogen of *N. benthamiana* for research purposes. This pathosystem might be also beneficial for studying fungal factors of pathogenicity by host-induced gene silencing (Andrade et al. 2016; Koch and Kogel 2014) and for examining biology of the reactive oxygen intermediate

Fig. 5. Suppression of *COI1* and *EIN2* transcript levels enhance symptoms caused by *Cercospora nicotianae* on *Nicotiana benthamiana* plants (four plants on the left and right, respectively). The GFP-silencing construct (TRV-GFP) was used as a control (four plants in the middle). Plants were inoculated with a suspension of *C. nicotianae* conidia in a concentration of 5×10^4 spores per milliliter. Picture was taken 7 days after inoculation.

Fig. 6. Development of leaf spot disease symptoms caused by *Cercospora nicotianae* on leaves of control (TRV-GFP construct), *COI1*-, and *EIN2*-silenced *Nicotiana benthamiana* plants. Percentages of necrotic leaf area were calculated for nine consecutive leaves numbered in ascending order 7 days after inoculation with a suspension of *C. nicotianae* conidia in a concentration of 5×10^4 spores per milliliter. Data points represent mean $\pm$ standard error of two independent experiments ($n = 4$ in each experiment).

Fig. 7. Detached leaves of control (TRV-GFP construct), *COI1*-, and *EIN2*-silenced *Nicotiana benthamiana* plants showing *Cercospora* leaf spot symptoms 6 days after drop-inoculation with 10- μ l droplets of a suspension of *Cercospora nicotianae* conidia in a concentration of 5×10^4 spores per milliliter. Mean $\pm$ standard error of two independent experiments are shown ($n = 10$ in each experiment). Values with different letters are significantly different at $P \leq 0.001$ calculated by Tukey's post hoc test. Virus-induced gene silencing treatments that knock down *COI1* and *EIN2* transcript levels increase mean diameter of necrosis caused by the fungus.

singlet oxygen, since cercosporin toxin that is synthesized by the fungus is known to generate singlet oxygen in infected host tissues (Daub and Ehrenshaft 2000; Leisman and Daub 1992).

ACKNOWLEDGMENTS

This research was supported by grants of the Hungarian Scientific Research Fund (OTKA K 104730 and OTKA K 124131). M. Pogány gratefully acknowledges sponsorship provided by the Bolyai Scholarship (BO/00609/12).

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