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1 **Description of the *Nicotiana benthamiana-Cercospora nicotianae* pathosystem**

2

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10

11 **ABSTRACT**

12 *Nicotiana benthamiana* is a valuable model organism in plant biology research. This report
13 describes its extended applicability in the field of molecular plant pathology by introducing a
14 non-biotrophic fungal pathogen *Cercospora nicotianae* that can be conveniently used under
15 laboratory conditions, consistently induces a necrotic leaf spot disease on *Nicotiana*
16 *benthamiana*, and is specialized on solanaceous plants. Our inoculation studies showed that a
17 filamentous fungal pathogen, *Cercospora nicotianae* more effectively colonizes *N.*
18 *benthamiana* than its conventional host, *N. tabacum*. The functions of two critical regulators
19 of host immunity, coronatine-insensitive 1 (COI1) and ethylene-insensitive 2 (EIN2), were
20 studied in *N. benthamiana* using tobacco rattle virus-based virus-induced gene silencing
21 (VIGS). Perturbation of jasmonic acid or ethylene signaling by VIGS of either *COI1* or *EIN2*,
22 respectively, resulted in markedly increased Cercospora leaf spot symptoms on *N.*
23 *benthamiana* plants. These results suggest that the *N. benthamiana – C. nicotianae* host-
24 pathogen interaction is a prospective but hitherto unutilized pathosystem for studying gene
25 functions in diseased plants.

26

27 **Keywords:** *Cercospora nicotianae*, *Nicotiana benthamiana*, pathosystem, gene silencing,
28 *COI1*, *EIN2*

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1
2 *Nicotiana benthamiana* Domin, a species of tobacco endemic to Australia (Goodspeed 1954)
3 is one of the most widely used model organisms in plant virology (Chakrabarty et al. 2007;
4 Obrepalska-Steplowska et al. 2013; Fan et al. 2014; Senthil-Kumar and Mysore 2015). Its
5 stunning susceptibility to plant viruses, even in comparison with other *Nicotiana* species, has
6 been linked to a mutation in a gene encoding a crucial RNA-dependent RNA polymerase that
7 results in compromised gene silencing ability and reduced degradation of viral RNAs in the
8 plant (Yang et al. 2004). *N. benthamiana* is an established model organism for experimental
9 plant biology for a number of reasons: i) it is suitable for heterologous expression of proteins
10 derived from a wide range of organisms by using recombinant plant viral expression systems
11 (Klimyuk et al. 2014; Moon et al. 2014); ii) virus-induced gene silencing (VIGS) is highly
12 efficient in *N. benthamiana* and serves as a convenient tool for studying gene function (Bubici
13 et al. 2015; Senthil-Kumar and Mysore 2015); and iii) *N. benthamiana* is also a suitable host
14 for *Agrobacterium*-mediated transient expression for mass production, functional analysis, or
15 intracellular localization of recombinant proteins (Goodin et al. 2008; Ding et al. 2014).
16 In contrast to a broad spectrum of plant viruses that would be readily available for
17 inoculation studies in *N. benthamiana*, there are only a limited number of phytopathogenic
18 fungi available for similar experimental purposes. Filamentous pathogens (plant pathogenic
19 fungi and oomycetes) of *N. benthamiana* include the biotrophic *Golovinomyces*
20 *cichoracearum* (Xiao et al. 2003) and *Peronospora hyoscyami* f.sp. *tabacina* (Hall 1989) as
21 well as some hemibiotrophic or necrotrophic pathogens such as *Colletotrichum* spp. (Shen et
22 al. 2001; Dean et al. 2002), *Phytophthora* spp. (Becktell et al. 2006; Rajput et al. 2014),
23 *Sclerotinia sclerotiorum* (Veluchamy et al. 2012) and *Botrytis cinerea* (Asai and Yoshioka
24 2009). Given that some of these plant pathogenic microbes have extremely wide host ranges,
25 a pathogenic fungus specialized on tobacco could potentially offer a more suited system for
26 virulence studies in comparison with less specified, polyphagous parasitic microorganisms.
27 *Cercospora* is a large genus of the family Mycosphaerellaceae with over 3000 named
28 species (Pollack, 1987). Most of these species are highly successful plant pathogens, causing
29 leaf spot and blight diseases on several hosts including many economically important crops
30 such as corn, soybean, sugar beet, coffee, peanut, rice, banana and tobacco (Goodwin et al.,
31 2001). *Cercospora nicotianae* Ellis & Everhart is the causal agent of the ’frogeye’ leaf spot
32 disease of tobacco (*Nicotiana tabacum* L.). Typically, it has not been a disease of commercial
33 importance in temperate climates, but often responsible for serious losses in tropical and
34 subtropical regions (Alasoadura and Fajola 1970; Holliday, 1980, Shew and Lucas 1991;

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1 Jahagirdar and Hundekar, 2010). The disease is favored by high humidity and warm weather
2 conditions that allow frogeye leaf spot to increase to damaging levels (Stavely and Nimmo,
3 1969), while also leading to a reduction in the leaf quality of tobacco (Stavely and Chaplin,
4 1972). *C. nicotianae* is an anamorphic ascomycete with no known sexual stage, but a small
5 number of *Cercospora* species have been connected to the teleomorph genus *Mycosphaerella*
6 (von Arx, 1983; Sivanesan 1984). It is a hemibiotrophic pathogen that exhibits a
7 symptomless, biotrophic growth for the first few days of infection and later triggers the death
8 of host cells when switches to a necrotrophic phase (Daub et al. 2013). The typical disease
9 symptoms of *C. nicotianae* on tobacco leaves have been associated with secretion of the non-
10 host specific toxin cercosporin (Upchurch et al. 1991). Cercosporin produced by many
11 *Cercospora* species converts to an electronically excited triplet state when exposed to light
12 and then reacts with oxygen to generate singlet oxygen (Daub and Ehrenshaft 2000). Singlet
13 oxygen (and other ensuing reactive oxygen intermediates) compromise the integrity of host
14 cell membranes that later provides nutrients for fungal hyphae growing in the intercellular
15 spaces of host tissues. In recent years, *C. nicotianae* has become an important model for
16 molecular analysis of the cercosporin biosynthetic gene cluster through targeted gene
17 disruption (Chung et al., 2003; Choquer et al., 2005; Chen et al., 2007; Dekkers et al., 2007;
18 You et al., 2009). Development of a reliable genetic transformation system for *C. nicotianae*
19 represented a major step for cercosporin research and helps establish the species as a potential
20 experimental model (Chung et al., 2003). Availability of mutant *C. nicotianae* lines with
21 defects in cercosporin production or resistance holds considerable promise for the elucidation
22 of the pathways leading to the production and regulation of cercosporin toxin (Jenns et al.,
23 1995; Chung et al., 1999; Amnuaykanjanasin and Daub, 2009; Beseli et al., 2015a, 2015b).
24 Today, near-complete genome sequences are available for seven *Cercospora* species, namely
25 *C. arachidicola*, *C. beticola*, *C. canescens*, *C.* cf. *flagellaris*, *C. sojina*, *C. zeae-maydis* and *C.*
26 *zeina* (Chand et al., 2015; Orner et al., 2015; Muller et al., 2016; Vaghefi et al., 2017 and
27 references therein, Zeng et al., 2017). The availability of these genome sequences may pave
28 the way for identification and functional characterization of novel genes in *C. nicotianae*.
29 To our knowledge, there has been only one account of an interaction between *N. benthamiana*
30 and *C. nicotianae* in the literature of molecular phytopathology (Nielsen et al. 1993). In their
31 work, the fungus was used to test responses of transgenic *N. benthamiana* ectopically
32 expressing a sugarbeet chitinase. Because the presence of the transgene apparently did not
33 affect susceptibility of *N. benthamiana* to *C. nicotianae*, description of the observed
34 symptoms and other details of the host-microbe interaction were not presented.

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1 Consequently, characterization of the *N. benthamiana* – *C. nicotianae* host-pathogen
2 interaction is lacking and it has not been exploited so far in plant sciences.
3 Here we present results on the interaction between *N. benthamiana* and *C. nicotianae* and
4 demonstrate that *N. benthamiana* is an excellent host for *C. nicotianae*, showing even higher
5 susceptibility to the fungus than *N. tabacum*. The intensity of frogeye leaf spots and fungal
6 abundance in leaf tissues can be confidently quantified by disease assessments and PCR-
7 based assays, respectively. The potential of this model system is tested by the perturbation of
8 two crucial regulators of host immunity, coronatine-insensitive 1 (COI1) and ethylene-
9 insensitive 2 (EIN2) by RNA interference-based VIGS technology.
10

11 MATERIALS AND METHODS

12 Plant material and growth conditions

13 *N. benthamiana* and *N. tabacum* cv Xanthi-nc plants were grown in a 1:1 mixture of potting
14 soil (Agroland) and peat (Pindstrup Plus Orange, Pindstrup Mosenburg) in a greenhouse until
15 inoculation with *C. nicotianae*, and provided with supplemental light when it was necessary to
16 ensure a 16-h photoperiod.
17

18 Cultivation of the fungus and inoculation

19 *C. nicotianae* isolate ATCC 18366 was used throughout this work (Ehrenshaft and Daub
20 1994). The fungus was maintained on potato dextrose agar (PDA) plates at room temperature
21 and inocula were prepared according to Beckman and Payne (1983). For long-term storage,
22 cultures were maintained on PDA slants covered with sterile paraffin oil and stored at 20 °C.
23 To obtain conidia for plant inoculations, mycelia from fresh cultures were harvested,
24 disrupted in sterile distilled water with glass shards using a FastPrep-24 machine (MP
25 Biomedicals), transferred to Petri dishes filled with V8 juice agar medium and incubated
26 under 16 h light/8 h dark conditions at 18−20 °C for 7 days. V8 juice medium was prepared
27 by thoroughly mixing 300 ml of V8 juice with 4.5 g of calcium carbonate. The mixture was
28 centrifuged (3000 rpm, 10 min) and the supernatant was 5-fold diluted with distilled water,
29 mixed with 1.5% agar and autoclaved for 15 minutes. The conidia produced were gently
30 washed off with sterile 0.2% gelatine solution using a paint brush. The resulting suspension of
31 spores and mycelial fragments were filtered through 3 layers of cheesecloth, the concentration
32 was adjusted to 5×10^4 conidia per milliliter with a hemocytometer and sprayed onto *N.*
33 *benthamiana* or *N. tabacum* plants until runoff. At the time of inoculation, *N. benthamiana*
34 and *N. tabacum* plants were 9 and 12 weeks old, respectively. Inoculated plants were covered

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1 with transparent plastic bags to provide high humidity for the fungal infection to become
2 established and they were incubated in a growth chamber for 4 days at 27 °C and 16 h
3 illumination (160 µmol m^{-2} s^{-1}) per day. After incubation, the plastic bags were removed and
4 the plants were transferred to the greenhouse.
5 A detached leaf inoculation assay was also developed for the evaluation of *C. nicotianae*
6 symptoms on VIGS-treated *N. benthamiana* leaves. Leaves in middle positions were removed
7 from plants and were placed on wet filter papers in Petri dishes (28 cm in diameter) with their
8 abaxial sides facing up. Ten microliter drops of *C. nicotianae* conidial suspension (5×10^4
9 conidia per milliliter) were distributed onto the (abaxial) surface of leaves and the Petri dishes
10 were incubated in a growth chamber for 4 days at 27 °C and 16 h illumination (160 µmol m^{-2}
11 s^{-1}) per day. Then temperature was reduced to 23 °C and and after 2 days at this temperature
12 the diameters *of C. nicotianae*-induced lesions were measured.
13 When *C. nicotianae* isolate ATCC 18366 was first received from the ATCC repository we
14 reisolated *C. nicotianae* from infected leaves showing frogeye symptoms to restore full
15 virulence of the isolate. This was accomplished by placing diseased leaf sections on top of
16 wet pieces of filter paper in Petri dishes, which were incubated in climate chambers
17 programmed for a 16-h-light/8-h-dark cycle at 20 °C for 3 days. Conidia developing on leaf
18 surfaces were removed gently by sterile forceps under a stereomicroscope and were
19 transferred to sterile Petri dishes containing PDA medium.
20
21 **Detection of fungal biomass by real-time PCR**
22 In order to determine the biomass of *C. nicotianae* in infected *N. benthamiana* and *N.*
23 *tabacum* plants, four fully developed leaves (in positions 4 through 7) were collected.
24 Samples of two independent experiments were analyzed, both yielding similar results. Leaves
25 were ground with a mortar and pestle in liquid nitrogen and stored at -70 °C. Genomic DNA
26 extraction was performed by a Nucleon Phytopure DNA extraction kit (GE Healthcare)
27 following instructions provided by the manufacturer. DNA extracts were adjusted to a
28 concentration of 10 ng μl^{-1} with nuclease-free water by using NanoDrop-1000 (Thermo Fisher
29 Scientific). The relative fungal biomass within infected leaves was assessed by quantitative
30 real-time PCR (qPCR) on a C1000 Touch Thermal Cycler equipped with a CFX96 Real-Time
31 PCR System (Bio-Rad) using a KAPA SYBR Fast qPCR Kit (KAPA Biosystems). Fungal
32 DNA was quantified using primers designed for amplification of a 140 bp fragment of *C.*
33 *nicotianae* actin (GenBank: JX143144.1): forward 5′-CAGGAAGGAGGAGCTGACAT-3′;

reverse 5′-AGTCCTTCTGGCCCATACC-3′. Host plant (*Nicotiana* spp.) DNA sequences were quantified with a primer pair specific for a *N. tabacum* actin gene (GenBank: X69885.1): forward 5′-CGGAATCCACGAGACTACATAC-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 2X KAPA SYBR FAST qPCR Master Mix, 1.5-1.5 µl forward and reverse primers (10 µM), 2.5 µl template and 2 µl PCR-grade water. Cycling conditions used were 95°C for 3 min 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°C 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 $2^{-\Delta\Delta CT}$ 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 pCAMBIA3301 (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′-CGCTCTAGAATGCCTGAGGGATACGTGCAG-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 *Eco*RI restriction endonuclease (Thermo Scientific) and the gel-purified GFP fragment was ligated into the *Eco*RI-digested pTRV2 vector.

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1 Two sets of primers were used for creating *TRV2-GFP-COI1* and *TRV2-GFP-EIN2* VIGS
2 vectors. The original sequences were adopted from an article published by Shibata et al.
3 (2010) and the restriction site sequences were customized to make them compatible with the
4 restriction endonucleases (*Bam*HI and *Nco*I) we were going to use. Target sequence
5 specificity of the silencing and avoidance of off-target gene silencing were controlled and
6 confirmed by the VIGS tool of the Sol Genomics Network website (Fernandez-Pozo et al.
7 2015) searching its *Nicotiana benthamiana* v1.0.1 database. The *EIN2* VIGS primer pair
8 (forward: 5′-TAGGATCCGCCCCCCTCCAATTTCAA-3′ , reverse: 5′-
9 TCCCATGGATTACTTTGCGCGGTCC-3′) amplified a 268 bp fragment from *N.*
10 *benthamiana EIN2* cDNA (Niben101Scf04548g00001.1), whereas the *COI1* VIGS primer
11 pair (forward: 5′- TAGGATCCGCCACTTGATAATGGTGT -3′, reverse: 5′-
12 AGGGATCCAGGCCTTCATCGGATTCC -3′) amplified a 174 bp fragment from *N.*
13 *benthamiana* Niben101Scf02280g08005.1 *COI1* cDNA. Using the VIGS tool of the Sol
14 Genomics Network website our *COI1* silencing construct was predicted to knock down
15 transcript levels of host *Niben101Scf02280g08005.1*, *Niben101Scf01227g01004.1* and
16 *Niben101Scf03500g01002.1 coronatine insensitive 1* genes, whereas the *EIN2* construct
17 targeted transcripts for *Niben101Scf04548g00001.1* and *Niben101Scf23355g00004.1*
18 *ethylene-insensitive 2 genes.* By using proper restriction enzymes the amplified cDNA
19 fragments were ligated into the *Bam*HI and *Nco*I restriction sites of the TRV2-GFP vector and
20 the complete product was transferred into *Escherichia coli* DH5α by heat shock method (Tu et
21 al. 2005). The identity of inserts in PCR positive clones were confirmed by Sanger DNA
22 sequencing. VIGS plasmids were finally purified from *E. coli* using NucleoSpin Plasmid kit
23 (Macherey-Nagel) and introduced into *Agrobacterium tumefaciens* MOG301 (Hood et al.
24 1993) by electroporation (Mahmood et al. 2008).
25
26 **Virus-induced gene silencing**
27 *Agrobacterium* strains containing pTRV1, pTRV2 or P14 silencing suppressor constructs
28 (Mérai et al. 2005) were grown overnight at 28 °C on LB medium supplemented with the
29 appropriate antibiotics (kanamycin sulfate: 30 µg ml^{-1} , rifampicin: 50 µg ml^{-1}). For inoculum
30 preparation, bacterial cells were suspended in *Agrobacterium* incubation buffer (1.95 g MES,
31 2 g $MgCl_2 \cdot 6H_2O$ in 1 l distilled water, pH 5.6) and supplemented with acetosyringone (final
32 concentration of 150 mM). Bacterial cell densities were adjusted with a spectrophotometer to
33 OD_{600}=0.4 for TRV1 and TRV2 and OD_{600}=0.2 for P14. After a 3-hour incubation at room
34 temperature, the bacterial suspensions mixed in a ratio of 1:1:1 (v/v) were infiltrated into two

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lower leaves of 3−4-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, 4 leaves at the middle positions (leaves 4 through 7) were collected from 3 plants in each treatment and pooled. Harvested leaves were frozen in liquid nitrogen, ground with a mortar and pestle, and 100 mg 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, DNA-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′- CCTCGTTACAACTTCTTATCC -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 4 through 7 (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

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1 Necrotic symptoms caused by *C. nicotianae* on VIGS-treated plants were evaluated on
2 9 consecutive leaf levels 7 days after inoculation using the histogram tool of an open-source
3 graphics software (Gimp 2.0) and presented as percentages of necrotic leaf areas. Displayed
4 results represent means calculated for two independent experiments each including four *N.*
5 *benthamiana* plants spray-inoculated with *C. nicotianae*. In detached-leaf assays, the
6 diameters of *C. nicotianae*-induced necroses following drop-inoculation of detached *N.*
7 *benthamiana* leaves were determined 6 days after inoculation. Two independent experiments.,
8 each containing ten detached leaves were evaluated for all the three silencing constructs.
9
10 **Data analysis**
11 Experimental data were statistically analyzed by Student's t-tests or by one-way ANOVA
12 and subsequent Tukey's honestly significant difference test for pairwise comparisons.
13
14 **RESULTS**
15 ***N. benthamiana* is a host of *C. nicotianae***
16 Frogeye symptoms caused by *C. nicotianae* typically arose 3−4 days after inoculation on
17 lower leaves of 9-week-old *N. benthamiana* plants as small necrotic spots that gradually
18 enlarged and merged into large necrotic areas. Sometimes larger (approximately 5−7 mm in
19 diameter) necrotic lesions emerged rapidly on older leaves. Intensity of frogeye symptoms
20 dynamically increased during the following 4−5 days in the form of new necroses developing
21 also on the younger, upper leaves, while the first necrotic spots grew larger and completely
22 decayed the lower leaves (Figs. 1 and 2). When leaves showing frogeye leaf spot were
23 detached and incubated for 3 days in a moist chamber, a pronounced growth of grey
24 mycelium was observed on both adaxial and abaxial surfaces of symptomatic leaves.
25 Transferring of this fungal material to Petri dishes containing PDA or V8 media produced
26 cultures that were characteristic of *C. nicotianae*. When suspensions of conidia harvested
27 from these V8 plates were sprayed onto leaves of healthy *N. benthamiana* plants this
28 inoculation resulted in typical frogeye leaf spot symptoms following incubation at 27 °C and
29 in high humidity for 4 days.
30
31 ***N. benthamiana* is markedly more susceptible to Cercospora leaf spot than *N. tabacum***
32 One purpose of this study was to compare the susceptibility of *N. benthamiana* and *N.*
33 *tabacum* to *C. nicotianae*, since *N. tabacum* is the most important host of the fungus.

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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 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 two 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 as compared to 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*

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1 system for gene silencing studies. Reliable pathogen responses to VIGS treatments in *N.*
2 *benthamiana* would demonstrate the usefulness of this fungus in studying functions of plant
3 genes in plant-pathogen interactions.
4 Suppression of host *COI1* and *EIN2* gene activity by triggering the plants' post transcriptional
5 gene silencing machinery resulted in consistently higher degree of Cercospora leaf spot
6 disease severity. These results suggest that frogeye symptoms caused by *C. nicotianae* may be
7 inhibited by jasmonic acid- or ethylene-mediated responses in *N. benthamiana* plants. Our
8 findings are in agreement with earlier investigations showing increased host susceptibility to
9 various phytopathogens including *B. cinerea* and *S. sclerotiorum* by disruption of *COI1* and
10 *EIN2* gene functions (Thomma et al. 1999; Liu et al. 2004; Guo and Stotz 2007; Chen et al.
11 2009; Ho et al. 2009). Ethylene-insensitive transgenic *N. tabacum* plants expressing the
12 mutant *etr1-1* gene from *Arabidopsis thaliana* also showed enhanced susceptibility to *C.*
13 *nicotianae* (Geraats et al. 2003).
14 *N. benthamiana* appeared far more susceptible to *C. nicotianae* than the conventional host of
15 the fungus *N. tabacum*. This seems to be in accord with the pronounced susceptibility of *N.*
16 *benthamiana* to virus infections as a result of a loss-of-function mutation in a gene encoding
17 an RNA-dependent RNA polymerase (Yang et al. 2004). RNA interference mechanisms are
18 not only crucial components of cellular defense responses to viruses but also to bacteria,
19 fungal, and oomycete pathogens (Ellendorf et al. 2009; Lopez et al. 2011; Staiger et al. 2013;
20 Yang and Huang 2014).
21 Symptoms of *C. nicotianae* on *N. benthamiana* become intense within a rapid and predictable
22 time frame. Growth of the fungus can be assessed by a qPCR method described here. Our
23 findings, therefore suggest *C. nicotianae* as a prospective fungal pathogen of *N. benthamiana*
24 for research purposes. This pathosystem might be also beneficial for studying fungal factors
25 of pathogenicity by host-induced gene silencing (Koch and Kogel 2014; Andrade et al. 2016)
26 and for examining biology of the reactive oxygen intermediate singlet oxygen, since
27 cercosporin toxin that is synthesized by the fungus is known to generate singlet oxygen in
28 infected host tissues (Leisman and Daub 1992; Daub and Ehrenshaft 2000).
29
30
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FIGURE LEGENDS

Fig. 1. A and B, Symptoms of *C. nicotianae* on *N. benthamiana* at 8 (A) and 10 (B) days after inoculation (DAI). C, Conidiophores and conidia of *C. nicotianae* from hyphal strands developing on the surface of a *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 *C. nicotianae* on *N. benthamiana* (2, 5, 8 and 11 DAI). Plants were inoculated with a suspension of *C. nicotianae* conidia in a concentration of 5×10^4 spores per milliliter.

Fig. 3. Symptoms of Cercospora leaf spot on 9-week-old *N. benthamiana* plants (3 plants on the left) and 12-week-old *N. tabacum* plants (2 plants on the right) 9 days after inoculation

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1 with the fungus. Plants were inoculated with a suspension of *C. nicotianae* conidia in a
2 concentration of 5 x 10^4 spores per milliliter.
3 **Inset**: Relative amount of *C. nicotianae* to *N. tabacum* and *N. benthamiana* DNA in leaves 9
4 days after inoculation with the fungus. DNA levels were estimated by quantitative polymerase
5 chain reaction specific for actin genes of *C. nicotianae*, *N. tabacum* and *N. benthamiana*. Bars
6 represent mean ± standard error of four biological and three technical replicates (each
7 biological sample was composed as a pool of three plants). Total genomic DNA was extracted
8 from 4 leaves (in positions 4 through 7) of plants for each species. Asterisk indicates
9 statistically significant difference in relative amount of *C. nicotianae* DNA between *N.*
10 *benthamiana* and *N. tabacum* plants calculated by Student's t-test ($P \leq 0.01$).
11
12
13 **Fig. 4.** Effect of VIGS on *coronatine-insensitive 1* (*COI1*) and *ethylene-insensitive 2* (*EIN2*)
14 mRNA transcript levels in *N. benthamiana*. Gene expression levels were assayed by real-time
15 reverse transcription PCR. The GFP-silencing construct (TRV–GFP) was used as a control.
16 Bars represent mean ± standard error of three biological and three technical replicates (each
17 biological sample was composed as a pool of three plants). Total RNA was extracted from
18 silenced healthy and *C. nicotianae*-infected *N. benthamiana* leaves (in positions 4 through 7)
19 3 days after inoculation. Means with different letters are significantly different at $P \leq 0.01$
20 calculated by Tukey's post hoc test.
21
22
23 **Fig. 5.** Suppression of *COI1* and *EIN2* transcript levels enhance symptoms caused by *C.*
24 *nicotianae* on *N. benthamiana* plants (4 plants on the left and right, respectively). The GFP-
25 silencing construct (TRV–GFP) was used as a control (4 plants in the middle). Plants were
26 inoculated with a suspension of *C. nicotianae* conidia in a concentration of 5 x 10^4 spores per
27 milliliter. Picture was taken 7 days after inoculation.
28
29
30 **Fig. 6.** Development of leaf spot disease symptoms caused by *C. nicotianae* on leaves of
31 control (TRV–GFP construct), *COI1*- and *EIN2*-silenced *N. benthamiana* plants. Percentages
32 of necrotic leaf area were calculated for nine consecutive leaves numbered in ascending order
33 7 days after inoculation with a suspension of *C. nicotianae* conidia in a concentration of 5 x

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1 10^4 spores per milliliter. Data points represent mean ± standard error of two independent
2 experiments (n = 4 in each experiment).
3
4
5 **Fig. 7.** Detached leaves of control (TRV-GFP construct), *COI1*- and *EIN2*- silenced *N.*
6 *benthamiana* plants showing Cercospora leaf spot symptoms 6 days after drop-inoculation
7 with 10-µl droplets of a suspension of *C. nicotianae* conidia in a concentration of 5 x 10^4
8 spores per milliliter. Mean ± standard error of two independent experiments are shown (n =
9 10 in each experiment). Values with different letters are significantly different at $P \leq 0.001$
10 calculated by Tukey's post hoc test. VIGS treatments that knock down *COI1* and *EIN2*
11 transcript levels increase mean diameter of necroses caused by the fungus.
12
13

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Fig. 1. A and B, Symptoms of *C. nicotianae* on *N. benthamiana* at 8 (A) and 10 (B) days after inoculation (DAI). **C,** Conidiophores and conidia of *C. nicotianae* from hyphal strands developing on the surface of a *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 x 10^4 spores per milliliter.

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

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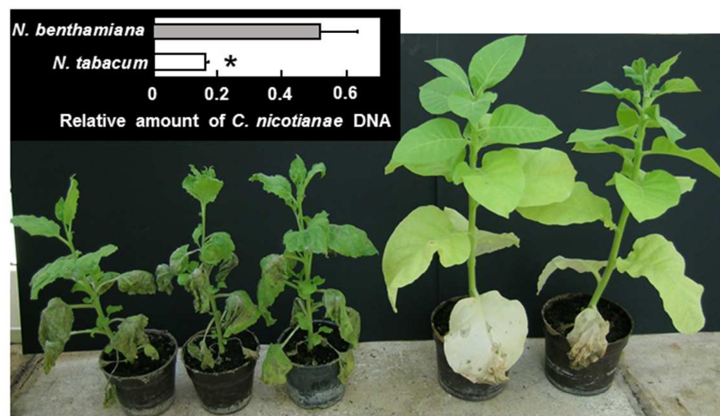

Fig. 3. Symptoms of Cercospora leaf spot on 9-week-old *N. benthamiana* plants (3 plants on the left) and 12-week-old *N. tabacum* plants (2 plants on the right) 9 days after inoculation with the fungus. Plants were inoculated with a suspension of *C. nicotianae* conidia in a concentration of 5 x 10^4 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 ± 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 4 leaves (in positions 4 through 7) 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 ($P \leq 0.01$).

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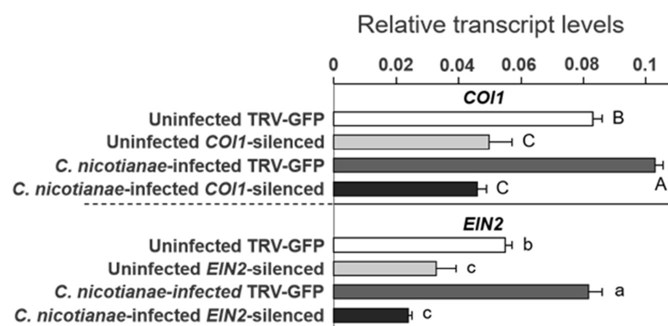

Fig. 4. Effect of VIGS on *coronatine-insensitive 1* (*COI1*) and *ethylene-insensitive 2* (*EIN2*) mRNA transcript levels in *N. benthamiana*. Gene expression levels were assayed by real-time reverse transcription PCR. The GFP-silencing construct (TRV–GFP) was used as a control. Bars represent mean ± 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 4 through 7) 3 days after inoculation. Means with different letters are significantly different at P ≤ 0.01 calculated by Tukey's post hoc test.

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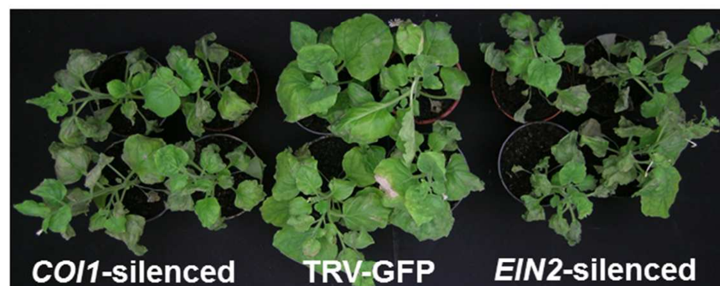

Fig. 5 Suppression of *COI1* and *EIN2* transcript levels enhance symptoms caused by *C. nicotianae* on *N. benthamiana* plants (4 plants on the left and right, respectively). The GFP-silencing construct (TRV–GFP) was used as a control (4 plants in the middle). Plants were inoculated with a suspension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

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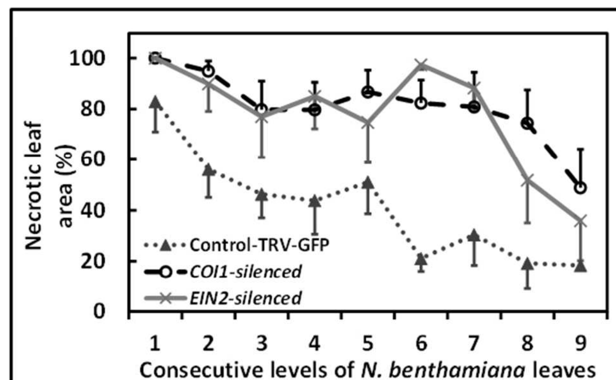

Fig. 6. Development of leaf spot disease symptoms caused by *C. nicotianae* on leaves of control (TRV–GFP construct), *COI1*- and *EIN2*-silenced *N. benthamiana* plants. Percentages of necrotic leaf area were calculated for nine consecutive leaves numbered in ascending order 7 days after inoculation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

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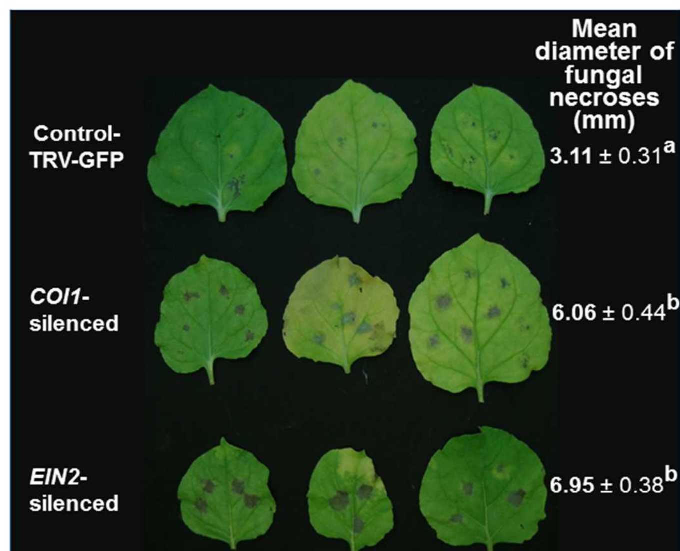

Fig. 7. Detached leaves of control (TRV-GFP construct), *COI1*- and *EIN2*- silenced *N. benthamiana* plants showing Cercospora leaf spot symptoms 6 days after drop-inoculation with 10-µl droplets of a suspension of *C. nicotianae* conidia in a concentration of 5 x 10^4 spores per milliliter. Mean ±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

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