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FEMS Microbiology Letters 228 (2003) 241–248

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Quantification of disease progression of several microbial pathogens on *Arabidopsis thaliana* using real-time fluorescence PCR

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Received 25 June 2003; received in revised form 22 August 2003; accepted 1 October 2003

First published online 27 October 2003

Abstract

An accurate monitoring of disease progression is important to evaluate disease susceptibility phenotypes. Over the years, *Arabidopsis thaliana* has become the model species to serve as a host in plant–pathogen interactions. Despite the efforts to study genetic mechanisms of host defense, little efforts are made for a thorough pathogen assessment, often still depending on symptomology. This manuscript describes the use of real-time polymerase chain reaction (PCR) to assess pathogen growth in the host Arabidopsis for a number of frequently studied pathogens. A wide range of correlations between pathogen biomass and fluorescence is demonstrated, demonstrating the theoretical sensitivity of the technique. It is also demonstrated that host DNA does not interfere with the quantification of pathogen DNA over a wide range. Finally, quantification of pathogen biomass in different plant genotypes with a varying degree of resistance shows the capability of this technique to be used for assessment of pathogen development in disease progression.

Keywords: LightCycler; SYBR Green I; *Alternaria brassicicola*; *Arabidopsis thaliana*; *Botrytis cinerea*; *Erwinia carotovora*; *Peronospora parasitica*; *Pseudomonas syringae*

1. Introduction

Detection and quantification of pathogen biomass in planta is a crucial step when monitoring disease resistance. To this purpose, several methods have been developed over the years that depend either on symptom display or on actual pathogen assessment.

By far the easiest way to evaluate disease resistance is to score for the display of visual symptoms. For pathogens that can kill complete plants one can score survival rates to evaluate disease resistance. Alternatively, for pathogens that cause necrosis without killing the host, the degree of necrosis over time can be used as a measure to monitor resistance. Although these techniques are relatively quick and easy to perform, there are disadvantages associated with these techniques. First, they can only be used for those pathogen–host interactions that result in the display of macroscopically visible disease symptoms. Furthermore, these techniques only provide a measure for the extent of disease symptoms and not for the extent of pathogen colonization. To overcome these limitations, techniques have to be developed that are able to actually assess pathogen presence. These techniques can be based on the measurement of components that are present in the pathogen but not in the host, like the fungal constituents ergosterol and chitin [1,2]. However, such techniques are not very sensitive and can only be used to assess colonization by pathogens that possess these constituents. Other techniques for assessing fungal biomass are based on immunological methods like enzyme-linked immunosorbent assay (ELISA) [3,4], or on monitoring *UidA* (GUS) activity in inter-

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0378-1097/03/$22.00
doi:10.1016/S0378-1097(03)00759-6

actions with microorganisms transformed with the bacterial *UidA* gene [5–7]. For these methods, interference of plant factors with the measured parameter constitutes the major drawback [7–9].

Finally, nucleic acid-based techniques like RNA hybridization [10,11] and quantitative polymerase chain reaction (PCR) [12,13] can be used to quantify pathogen ingress. A great step forward for nucleic acid-based techniques is the development of real-time PCR applications in the second half of the 1990s [14]. In a real-time PCR application, the accumulation of the amplicon is monitored by each cycle based on the emission of fluorescence [15]. Amplicons can be detected using different chemistries, which can be divided into either amplicon specific detection methods [16–19] or amplicon non-specific methods [20]. Though the advantage of amplicon specific detection methods is the high specificity of the fluorescent signal that is generated, the disadvantage of these specific chemistries is that detection of different amplicons requires different probes. This disadvantage does not apply to amplicon non-specific detection chemistries that are based on fluorophores that associate with double-stranded DNA. Such fluorophores can also associate with primer-dimers and non-specific amplification products, which can seriously disturb interpretation of the results. However, by melting analysis at the end of the PCR the accuracy of the amplification PCR can be evaluated.

Arabidopsis has become the model organism for the dissection of plant disease responses [21,22]. Remarkably, pathogen susceptibility is often monitored through scoring of disease symptoms. However, several studies have shown that development of disease symptoms not always correlates with actual pathogen colonization [23–25]. Therefore it is of utmost importance to assess pathogen growth rather than disease symptoms. Up till now real-time PCR has mainly been applied for diagnosis rather than quantification of plant diseases [26]. In this manuscript we describe real-time PCR applications to accurately assess pathogen colonization of a number of pathogens that are frequently used in Arabidopsis pathosystems based on SYBR®Green I technology. These pathogens include fungi as well as bacteria and biotrophs as well as necrotrophs.

2. Materials and methods

2.1. Plant inoculations

All inoculations were performed on 4-week-old soil-grown Arabidopsis plants of the Columbia-0 ecotype unless mentioned differently. Inoculation with *Alternaria brassicicola* was performed as described previously [11]. Essentially, one 5-μl drop containing 5×10^5 spores ml^{-1} water was placed on each leaf. For inoculations with *Erwinia carotovora*, one 5-μl drop of a bacterial suspension ($OD_{600}=0.05$) prepared in 10 mM $MgCl_2$ was placed on each leaf. *Botrytis cinerea* was inoculated by spraying a suspension of 5×10^5 spores ml^{-1} in half strength potato dextrose broth (Difco, Detroit, MI, USA) until droplet runoff. For inoculations with *Pseudomonas syringae* pv. *tomato* DC3000, plants were placed at high humidity 1 day before inoculation to promote stomatal opening. At the time of inoculation, a bacterial suspension ($OD_{600}=0.05$) prepared in 10 mM $MgCl_2$ supplemented with 0.01% Silwet L-77 was used to dip-inoculate the plants for 30 s. For all inoculations, plants were kept at 100% relative humidity for the remainder of the experiment in a growth chamber with 14 h light per day at 21°C. *Peronospora parasitica* isolate Waco9 was inoculated on *eds1* mutants of the WS-0 ecotype [27]. A conidial suspension of 5×10^4 spores ml^{-1} in water was sprayed until droplet runoff. After allowing the plants to dry for 1 h, plants were kept at 100% relative humidity in a growth chamber with 9 h light per day at 17°C.

At the appropriate time points, tissue was harvested and subsequently frozen in liquid nitrogen. For *A. brassicicola*, *E. carotovora* and *P. syringae*, samples containing 10 leaf discs cut with a cork borer were taken. For *P. parasitica* and *B. cinerea*, 100 mg tissue was weighed. Samples were stored at −80°C until further handling. All experiments were performed at least twice with comparable results.

2.2. DNA isolation

To the samples, five 2-mm glass beads and approximately 0.2 g 212–300-μm glass beads, 300 μl lysis buffer (2.5 M LiCl, 50 mM Tris–HCl, 62.5 mM Na_2-ethylenediamine tetraacetic acid (EDTA), and 4.0% Triton X-100, pH 8.0) and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) were added. Cells were mechanically disrupted in a Fast Prep system high-speed shaker (Bio 101/Thermo Savant, Holbrook, NY, USA) by reciprocal shaking the samples for 30 s at maximum speed. Especially the small glass beads are required for disruption of microbial tissues. The supernatant was collected after centrifugation (5 min at 10 000 rpm) and the DNA was precipitated by addition of 2 Vol of absolute ethanol followed by incubation for 15 min at −20°C and subsequent centrifugation (5 min at 10 000 rpm). The DNA pellet was washed with 70% ethanol, air-dried, and resuspended in ultrapure water.

For the generation of a standard curve DNA was isolated from in vitro grown pathogen material similar as described above. The amount of DNA was determined spectrophotometrically at 260 nm.

2.3. Real-time PCR

Irrespective of the detection chemistry, for quantification purposes a standard curve is generated by plotting the crossing cycle number for each sample of a standard

series versus the logarithm of the concentration. This crossing cycle number is the point where the amplification is in the exponential phase and the fluorescence rises above the background fluorescence level. Using a regression line through the data points allows for any sample via its crossing cycle number to extrapolate the respective concentration.

Quantitative PCR was performed using SYBR®Green I technology on a LightCycler™ instrument (Roche Diagnostics Corp., Indianapolis, IN, USA) in the presence of 3 mM $MgCl_2$. For each PCR, samples (20 μl) were prepared in sealed LightCycler capillaries containing 2 μl DNA extract, 10 μl Lithos qPCR Mastermix QGS (Eurogentec, Liège, Belgium) and 0.5 μM of each primer. Samples were run for 40 cycles under the following thermal cycling protocol. Samples were preheated at 95°C for 15 s. Then, 40 amplification cycles were run: 0 s at 95°C, 5 s at the annealing temperature as indicated in Table 1, and elongation at 72°C for the time period indicated in Table 1. Fluorescence (520 nm) was detected at the end of the elongation phase for each cycle. Following the final amplification cycle, a melting curve was acquired by heating to 95°C, cooling to 60°C and slowly heating to 95°C at 0.1°C s^{-1} with continuous measurement of fluorescence at 520 nm.

For each Arabidopsis pathogen the performance of one or two primer pairs was tested on a dilution series of genomic DNA of the target organism as well as Arabidopsis DNA. Based on the melting analysis and gel electrophoresis of the generated amplicons it could be determined whether the primer pairs were able to specifically generate the desired amplicon.

3. Results

3.1. *Quantification of* P. parasitica *infections*

One of the most frequently studied Arabidopsis pathogens is the downy mildew fungus *P. parasitica*. Resistance against this biotrophic pathogen is controlled through a gene-for-gene interaction, and pathogen susceptibility is often determined qualitatively by assessing the host for pathogen presence through microscopic observation. Incidentally, pathogen colonization is assessed by determining the amount of sporulation structures. In Fig. 1A a standard curve is shown, generated from analysis of a 10-fold dilution series of genomic DNA starting from 100 ng using the primer pair AFP293–AFP294. The second primer pair (AFP295–AFP296, see Table 1) essentially gave similar results (data not shown). Next, a 10-fold dilution series of genomic DNA starting from 50 ng was added to samples containing 50 ng of Arabidopsis genomic DNA, such that the ratio between pathogen and plant DNA varied from 1:1 (which could possibly reflect a very heavy infestation) to 1:1 000 000 (reflecting early stages of infections). This was done to test whether the presence of a plant-derived DNA extract would interfere with accurate quantification. Again a perfect correlation was shown, using the primer pair AFP293–AFP294, demonstrating the feasibility of this technique for quantification of *P. parasitica* infections (Fig. 1B). Finally, disease progression was assessed on *eds1* mutants that are compromised in *P. parasitica* resistance, 2, 4 and 6 days after inoculation using the primer pair AFP293–AFP294. In Fig. 1C it is shown that on days 4 and 6 an increasing biomass of *P. parasitica* is

Table 1
Sequences and origins of primers for real-time PCR

Organism	Primer[a]	Sequence (5′–3′)	origin	T_{ann}[b]	T_{el}[c]
A. brassicicola	OWB577 (S)	ACAATATGAAAGCGGGCTGG	5.8S rDNA	62°C	16
	OWB578 (A)	AAGACGCCCAACACCAAGCA	5.8S rDNA	62°C	16
	OWB579 (S)	CGTACCACACGACCTGTCCA	cutinase	60°C	24
	OWB580 (A)	CGTTTCAAGCTCGTTTCTGG	cutinase	60°C	24
B. cinerea	AFP24 (S)	CCGTCATGTCCGGTGTTACCAC	tubulin	63°C	10
	AFP25 (A)	CGACCGTTACGGAAATCGGAAG	tubulin	63°C	10
	AFP26 (S)	TGGAGATGAAGCGCAATCCAA	actin	62°C	12
	AFP27 (A)	AAGCGTAAAGGGAGAGGACGGC	actin	62°C	12
E. carotovora	AFP16 (S)	TGTCGTCAGCTCGTGTTGTGAA	16S rDNA	62°C	12
	AFP17 (A)	GAGTTGCAGACTCCAATCCGGA	16S rDNA	62°C	12
	AFP18 (S)	GTTTGCTGTCCACGTTCTGCTG	AF046928[d]	62°C	12
	AFP19 (A)	CCGGTCGTCATCGAGAAACAAT	AF046928[d]	62°C	12
P. parasitica	AFP293 (S)	TTTCCGTAGGTGAACCTGCG	18S rDNA	60°C	12
	AFP294 (A)	GCGAGCCTAGACATCCAC	18S rDNA	60°C	12
	AFP295 (S)	CACGTCCCTTATGTCGAGG	AW737077[d]	58°C	12
	AFP296 (A)	CAAGTTGTCGCAGATCACG	AW737077[d]	58°C	12
P. syringae	OWB575 (S)	AACTGAAAAACACCTTGGGC	*oprf* gene	58°C	15
	OWB576 (A)	CCTGGGTTGTTGAAGTGGTA	*oprf* gene	58°C	15

[a](S) is sense primer, (A) is antisense primer.
[b]Annealing temperatures.
[c]Elongation time (s).
[d]GenBank accession number.

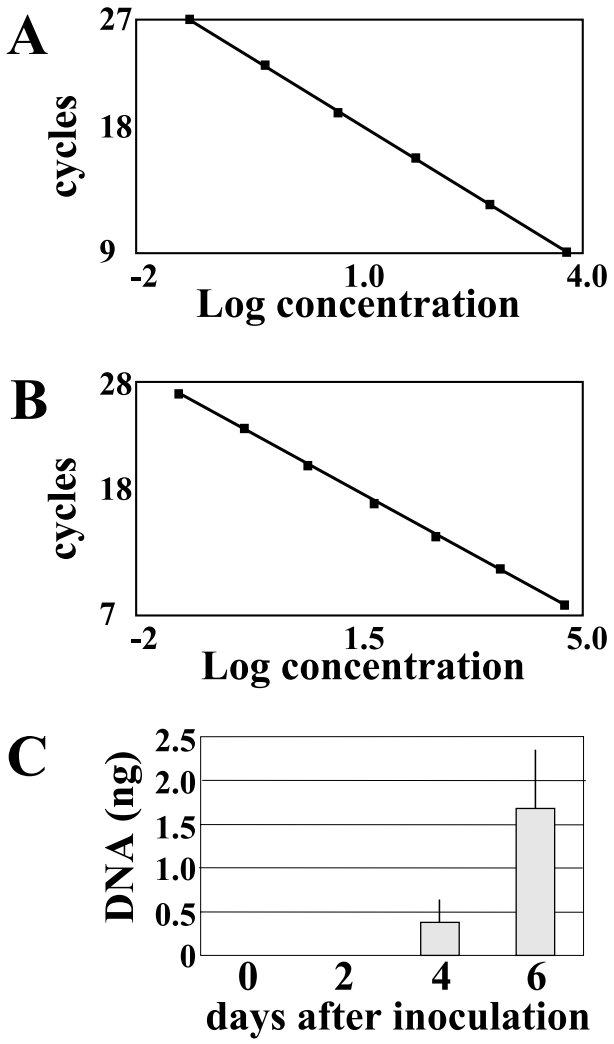

Fig. 1. Detection and quantification of *P. parasitica* in real-time PCR analysis using the primer pair AFP293–AFP294. A: Regression line for real-time PCR analysis of a 10-fold dilution series of *P. parasitica* genomic DNA starting from 100 ng using the primer pair AFP293–AFP294. B: Regression line for real-time PCR analysis of a 10-fold dilution series in 50 ng Arabidopsis genomic DNA, starting from 50 ng of *P. parasitica* genomic DNA using the primer pair AFP293–AFP294. C: Disease progression of *P. parasitica* isolate Waco9 on Arabidopsis *eds1* plants of the WS ecotype at 2, 4 and 6 days after inoculation. Data represent average plus standard deviation from 1 μl extract of three samples.

detected, an observation that was verified microscopically (data not shown).

3.2. *Quantification of infections with the necrotrophic fungi* A. brassicicola *and* B. cinerea

Arabidopsis wild-type plants are susceptible to a number of necrotrophic fungal pathogens [22]. The most frequently studied are *A. brassicicola* and *B. cinerea*. *A. brassicicola* is a leaf-spot pathogen that causes relatively small, necrotic spots on Arabidopsis wild-type plants of the Columbia ecotype [11,28]. On Arabidopsis, *B. cinerea* typically colonizes the inoculated leaves, which subsequently decay [11,25]. Based on the aggressiveness of the fungal strain used, the fungus is able to spread to non-inoculated tissues and the whole plant decays. For detection and quantification of each of these pathogens, two sets of primers were designed (Table 1). In a first assay, genomic DNA of each of these pathogens was serially diluted in several steps and quantified in a real-time PCR. For *A. brassicicola*, in total 25 ng of DNA was five-fold diluted in five steps to 8 pg, and a nearly perfect correlation between the cycle number at which the fluorescence exceeds the background level and the concentration using the primer pair based on a ribosomal DNA sequence (OWB577–OWB578) was found (Fig. 2A). Using the primers designed on the cutinase gene (OWB579–OWB580), an accurate quantification was not possible due to aberrant kinetics of amplicon generation in the PCR (data not shown). Therefore it was decided to use the primers designed on the ribosomal sequence for further experiments.

For *B. cinerea* both primer pairs behaved equally for quantification purposes. In Fig. 2B results are shown for the quantification of a five-step 10-fold dilution series starting with 10 ng using the primer pair designed on the tubulin sequence (AFP24–AFP25). The primer pair designed on the actin sequence essentially gave similar results (data not shown).

In a next assay we wanted to assess whether we were also able to quantify pathogen biomass in a plant extract.

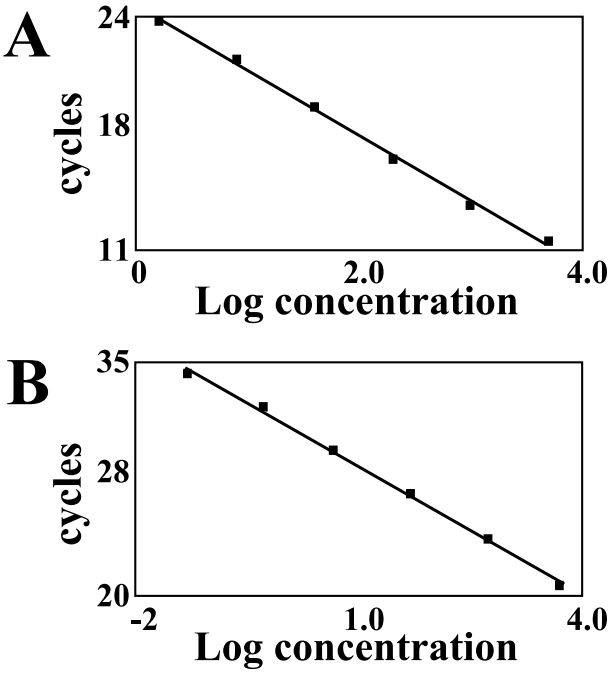

Fig. 2. Quantification of a standard series of genomic DNA isolated from *A. brassicicola* and *B. cinerea*. A: Regression line for real-time PCR analysis of a five-fold dilution series of *A. brassicicola* genomic DNA starting from 25 ng using the primer pair OWB577–OWB578. B: Regression line for real-time PCR analysis of a 10-fold dilution series of *B. cinerea* genomic DNA starting from 10 ng using the primer pair AFP24–AFP25.

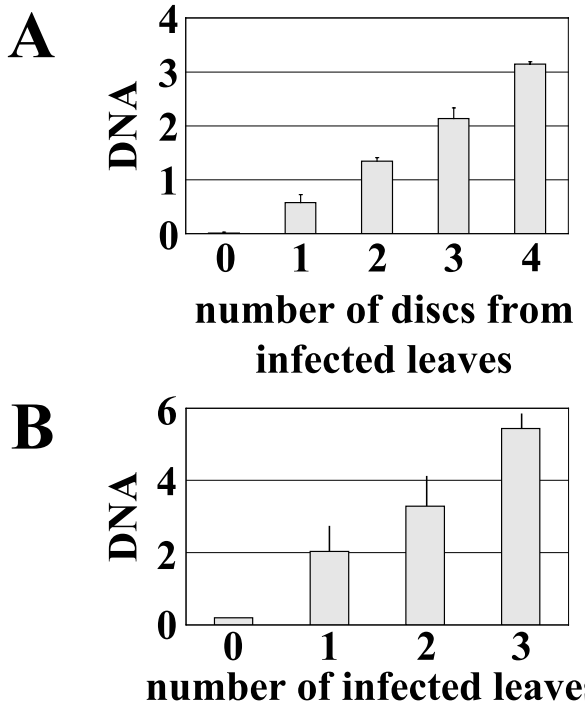

Fig. 3. Detection of *A. brassicicola* and *B. cinerea* in plant extracts. Relative DNA content for samples containing known amounts of pathogen-inoculated plant material. A: For *A. brassicicola*, samples were composed containing 10 leaf discs of which up to four were collected from pathogen-inoculated plants. Data represent average plus standard deviation of three samples. B: For *B. cinerea*, samples were composed containing five leaves of which up to three were collected from pathogen-inoculated plants. Data represent average plus standard deviation of three samples.

Addition of 25 or 10 ng of plant DNA extracted from healthy Arabidopsis leaf material to all samples of the *A. brassicicola* and *B. cinerea* dilution series, respectively, did not influence the quantification of pathogen biomass (results not shown). However, the major difference in the interaction of a host with a biotrophic or a necrotrophic pathogen lies in the extent of tissue decay. The massive tissue necrosis that is generally associated with infections of necrotrophic pathogens leads to severe biochemical changes that can potentially lead to components that influence pathogen quantification based on real-time PCR. We therefore chose not to quantify dilution series of pathogen DNA mixed with plant DNA, but compose samples with mixes of healthy and pathogen-infected plant material for quantification. For *B. cinerea*, wild-type plants were inoculated. However, wild-type plants display a relatively high degree of resistance against *A. brassicicola*. For this pathogen, *pad3-1* mutants that are deficient in production of the antimicrobial phytoalexin camalexin and thus display enhanced susceptibility [28] were inoculated. For *A. brassicicola*, leaf discs cut with a cork borer around a lesion site were collected and mixed with leaf discs of non-inoculated plants such that on a total amount of 10 leaf discs, samples contained from zero to four pathogen-inoculated discs. The relative amount of pathogen DNA detected correlates to the amount of pathogen-infected leaf discs included in the sample (Fig. 3A). For *B. cinerea*, samples were collected containing five Arabidopsis leaves, of which up to three were collected from spray-inoculated wild-type plants 4 days after treatment. Also for this pathogen accurate quantification could be performed using the primer pair designed on the actin gene sequence (Fig. 3B) as well as the pair designed on the tubulin gene sequence (results not shown).

Several Arabidopsis mutants have been identified that display enhanced susceptibility to either of these pathogens. While wild-type plants have a high degree of resis-

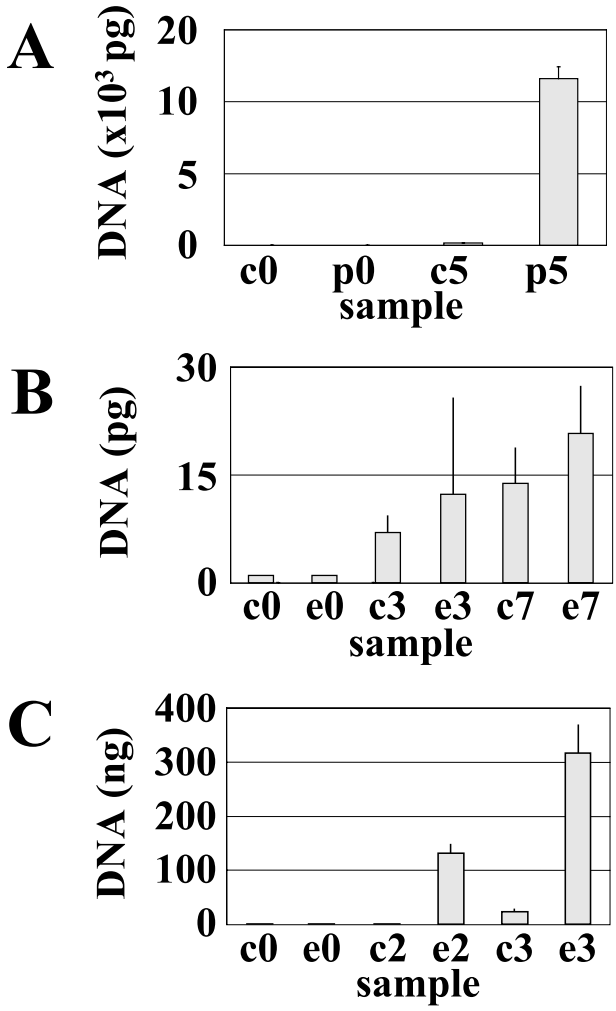

Fig. 4. Determining enhanced susceptibility phenotypes in Arabidopsis towards *A. brassicicola* and *B. cinerea*. A: DNA extracts from samples containing 10 leaf discs collected from *A. brassicicola*-inoculated Col-0 (c) and *pad3-1* (p) plants at 0 and 5 days after inoculation were assessed for the presence of *A. brassicicola*. Data represent average DNA content plus standard deviation from 1 μl extract of two samples. B: DNA extracts from samples containing 10 leaf discs collected from *A. brassicicola*-inoculated Col-0 (c) and *ein2-1* (e) plants at 0, 3 and 7 days after inoculation were assessed for *A. brassicicola* presence. Data represent average plus standard deviation from 1 μl extract of three samples. C: DNA extracts from samples containing 10 leaf discs collected from *B. cinerea*-inoculated Col-0 (c) and *ein2-1* (e) plants at 0, 2 and 3 days after inoculation were assessed for *B. cinerea* presence. Data represent average plus standard deviation from 1 μl extract of three samples.

tance against *A. brassicicola*, *pad3-1* mutants are hypersusceptible [28]. In addition, though *ein2-1* mutants display enhanced symptom development upon inoculation with this pathogen compared to wild-type plants, it was shown that this is not correlated with enhanced pathogen colonization [25]. For each of these genotypes, samples were composed containing 10 leaf discs of pathogen-inoculated plants. Both *A. brassicicola* and *B. cinerea* are necrotrophic pathogens, able to induce extensive host tissue necrosis. Because it can be anticipated that host cell death is correlated with a downregulation of transcriptional activity, we have chosen not to normalize extracts using a host reference DNA standard as this could lead to an actual overestimation of pathogen biomass. To be able to compare different samples we ensured collecting equal amounts of host tissue areas that were treated with an equal amount of inoculum. With an optimized extraction procedure, not giving rise to large variations in sample-to-sample extraction efficiencies, justified comparisons between samples should be ensured. Based on real-time PCR the enhanced susceptibility phenotypes are confirmed as in *A. brassicicola*-inoculated *pad3-1* much more *A. brassicicola* DNA is amplified, while in *ein2-1* it is not, compared to inoculated wild-type plants (Fig. 4A and B). Similarly, the observation that *ein2-1* mutants are hypersusceptible to *B. cinerea* [25] could be verified using real-time PCR (Fig. 4C).

3.3. Quantification of bacterial infections

The results above show that real-time PCR can be successfully implemented for the quantification of Arabidopsis colonization by fungal pathogens. Next we assessed whether this technology could be successfully applied to detect and quantify infections by the pathogenic bacteria *E. carotovora* subsp. *carotovora* and *P. syringae* pv. *tomato* DC3000. Up till now, plate countings are often performed to assess bacterial colonization. However, this procedure is very labor intensive. For *E. carotovora*, two primer pairs were designed (see Table 1). A five-step 10-fold dilution series starting with 10 ng of bacterial DNA could be accurately quantified as demonstrated in Fig. 5A for primer pair AFP16–AFP17. The other primer pair essentially gave the same results (data not shown). Also for this pathogen in planta detection was established by assaying extracts from samples containing in total 10 leaf discs of which up to five were collected from pathogen-inoculated plant material (Fig. 5B).

For *P. syringae*, one primer pair was designed. Also for this primer pair a five-step 10-fold dilution series starting with 10 ng of bacterial DNA could be accurately quantified as demonstrated in Fig. 5C. When DNA extracts of infected plants were mixed with extracts from healthy tissues, relative fluorescence levels correlated well with pathogen concentrations (Fig. 5D).

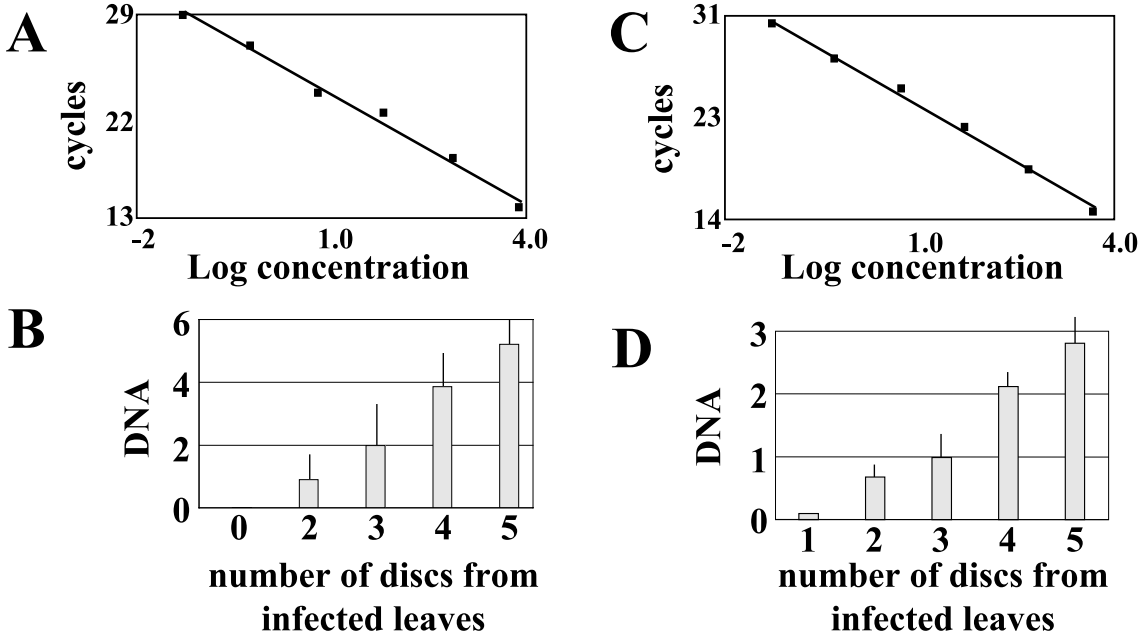

Fig. 5. Quantification of Arabidopsis bacterial pathogens in real-time PCR applications. A: Regression line for real-time PCR analysis of a 10-fold dilution series of *E. carotovora* genomic DNA starting from 10 ng using the primer pair AFP16–AFP17. B: Relative amount of *E. carotovora* DNA for samples containing 10 leaf discs of which up to five were collected from pathogen-inoculated plants. Data represent average plus standard deviation of three samples. C: Regression line for real-time PCR analysis of a 10-fold dilution series of *P. syringae* genomic DNA starting from 100 ng using the primer pair OWB575–OWB576. D: Relative amount of *P. syringae* DNA for samples containing 10 leaf discs of which up to five were collected from pathogen-inoculated plants. Data represent average plus standard deviation of three samples.

4. Discussion

Over the years, Arabidopsis has become subject to many studies focusing on all kinds of plant processes. This interest has even deepened since the genomic sequence of this plant was released [29]. Also in the field of disease resistance Arabidopsis has become a preferred model to study defense responses [22,30]. And although much effort is dedicated to studying and altering disease signaling networks, the actual outcome of altered signaling cascades (enhanced resistance or enhanced susceptibility) is often poorly assessed. Most studies concentrate on gene-for-gene resistance where the interaction is governed by pathogen avirulence genes and host resistance genes, and the outcome often is either complete resistance or susceptibility. For this kind of interaction a qualitative assessment of disease resistance is often sufficient. For many other plant–pathogen interactions, this is not the case. In this manuscript we demonstrate the use of real-time PCR for quick and reliable assessment of pathogen colonization on Arabidopsis. Though there are a handful of studies demonstrating the use of real-time PCR for detection of a single pathogen on a single plant species [31–34], this is the first report describing the use of this technique for a range of different pathogens of *Arabidopsis thaliana*. We have shown here that dilution series of pathogen DNA can be accurately quantified over a large concentration range, in the absence or presence of host DNA, using real-time PCR. This offers the opportunity for a sensitive pathogen detection. In addition we have demonstrated the utility of real-time PCR for assessing the susceptibility of different Arabidopsis genotypes towards three different fungal pathogens, corroborating susceptibility phenotypes as published previously. Quantification also accurately worked for necrotrophic pathogens, often causing heavy tissue necrosis. However, extracts from very heavily necrotized tissues may result in aberrant PCR kinetics, disturbing the quantitative character of the technology. In this study, quantification of infections with *E. carotovora* and *B. cinerea*, the pathogens causing the most severe necrosis, has proven to be sometimes difficult. In such cases, aberrant PCR kinetics could be circumvented assessing time points in the infection process before tissues were totally macerated. Alternatively, using relatively low inoculation densities, one could try slowing down the infection process.

There are a number of specific advantages of the techniques used in this study. First, the LightCycler™ PCR technology provides the basis for very rapid quantitative PCR assays. Typically, 32 parallel PCRs can be processed within 40 min including quantification and melting analysis, due to the use of glass capillaries as reaction tubes. Second, due to the use of SYBR®Green I, the reaction cost is limited compared to real-time PCRs that depend on hybridization of labeled sequence-specific probes. Third, this technique allows a very sensitive detection of pathogens. DNA amounts as little as 0.1 pg could easily be detected.

There are, however, also specific disadvantages to the use of this technique. Due to the high sensitivity and the small reaction volumes some optimizing is needed to know how much plant material should be extracted and included in the reaction mixture. Often, extracts need to be seriously diluted and only small volumes are included in the sample, which can give rise to high sample-to-sample variation.

In this study we have chosen not to normalize the pathogen DNA concentration to a constitutively expressed plant gene because it can be anticipated that tissue necrosis caused by a pathogen infection leads to decreased transcriptional activity of host cells. Normalizing pathogen DNA concentrations to the activity of host genes thus can lead to an overestimation of pathogen biomass. As a solution to this problem we chose to analyze equal areas (or weights alternatively) of host tissue treated with equal densities of pathogen inoculum, thus allowing to accurately assess the increase in pathogen colonization.

Overall, this technique has proven to be very efficient for the detection of pathogen biomass at early stages of infections.

Acknowledgements

This research was financially supported by the 'Vlaams Instituut voor de bevordering van het Wetenschappelijk-Technologisch Onderzoek-Vlaanderen' (IWT-010121), the 'Fonds voor Wetenschappelijk Onderzoek-Vlaanderen' (FWO-G.0277.00N) and by De Ceuster Corp., Sint-Katelijne-Waver, Belgium. B.P.H.J.T. received a postdoctoral fellowship of the 'Fonds voor Wetenschappelijk Onderzoek-Vlaanderen'.

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