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Technical advance

A PCR assay for the quantification of growth of the oomycete pathogen *Hyaloperonospora arabidopsidis* in *Arabidopsis thaliana*

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SUMMARY

The accurate quantification of disease severity is important for the assessment of host–pathogen interactions in laboratory or field settings. The interaction between *Arabidopsis thaliana* and its naturally occurring downy mildew pathogen, *Hyaloperonospora arabidopsidis* (*Hpa*), is a widely used reference pathosystem for plant–oomycete interactions. Current methods for the assessment of disease severity in the *Arabidopsis–Hpa* interaction rely on measurements at the terminal stage of pathogen development; namely, visual counts of spore-producing structures or the quantification of spore production with a haemocytometer. These assays are useful, but do not offer sensitivity for the robust quantification of small changes in virulence or the accurate quantification of pathogen growth prior to the reproductive stage. Here, we describe a quantitative real-time polymerase chain reaction (qPCR) assay for the monitoring of *Hpa* growth *in planta*. The protocol is rapid, inexpensive and can robustly distinguish small changes in virulence. We used this assay to investigate the dynamics of early *Hpa* mycelial growth and to demonstrate the proof of concept that this assay could be used in screens for novel oomycete growth inhibitors.

Keywords: *Arabidopsis*, *Hyaloperonospora arabidopsidis*, oomycete, real-time PCR.

INTRODUCTION

Oomycete pathogens cause disease on many important crops and have a high evolutionary potential to circumvent host resistance and chemical control (Kamoun *et al*., 2015). *Hyaloperonospora arabidopsidis* (*Hpa*) causes downy mildew disease of *Arabidopsis thaliana*, which is characterized by the formation of aerial sporangiophores (asexual fruiting bodies) (Holub, 2008; Koch and Slusarenko, 1990). Like other downy mildew pathogens, *Hpa* is an obligate biotroph that extracts nutrients from living *Arabidopsis* cells and cannot be cultured apart from its host. *Hpa* is one of a few eukaryotic microbes that are specifically adapted to *Arabidopsis* (Holub, 2008; Koch and Slusarenko, 1990). The *Arabidopsis–Hpa* interaction occurs frequently in nature, exhibiting a high level of polymorphism for resistance/susceptibility, lending itself as a useful pathosystem to exploit *Arabidopsis* for a better understanding of the molecular basis of plant–pathogen interactions and the evolution of obligate biotrophy (Baxter *et al*., 2010; McDowell, 2014).

Many *Arabidopsis* accessions have been documented as resistant or susceptible to isolates of *Hpa* collected from field populations of *Arabidopsis* (Crute *et al*., 1993; Dangl *et al*., 1992; Holub and Beynon, 1996). Resistance is conditioned by genes encoding nucleotide-binding, leucine-rich repeat (NB-LRR) immune surveillance proteins. Moreover, many induced mutants with altered responses to *Hpa* have been described (Coates and Beynon, 2010; Slusarenko and Schlaich, 2003). For example, loss-of-function mutations in several immune signalling genes produce an 'enhanced disease susceptibility' (*eds*) phenotype, in which the growth of virulent isolates is enhanced compared with that in the wild-type host (Parker *et al*., 1996).

Typically, *Hpa* growth is quantified by the counting of sporangiophores (e.g. McDowell *et al*., 2011; Tome *et al*., 2014) or by the quantification of asexual spore production (e.g. Feys *et al*., 2001). Both techniques offer limited precision when determining small changes in host susceptibility. Furthermore, pathogen growth cannot be monitored with these techniques at the early stages of the host–pathogen interaction.

Quantitative real-time polymerase chain reaction (qPCR) has been successfully utilized for plant pathogen genotyping and diagnostics, and for the quantification of pathogen growth *in planta*. Previous publications have described PCR assays for oomycetes (Alonso *et al*., 2010; Kokko *et al*., 2006; Landa *et al*., 2011), including *Hpa* (Brouwer *et al*., 2003; Lapin *et al*., 2012). Here, we describe a protocol utilizing qPCR of a single-copy *Hpa* gene as a proxy for pathogen biomass. The assay employs standard qPCR primers and rapid genomic DNA (gDNA) preparations, with low cost and high throughput. The procedure also includes a qPCR assay for a single-copy *Arabidopsis* gene, enabling normalization to host biomass and accurate resolution of small differences in susceptibility (e.g. Alonso *et al*., 2010; Kokko *et al*., 2006). We use this assay to investigate the dynamics of *Hpa* mycelial growth in an *eds* mutant, and demonstrate a practical application of this

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method by monitoring *Hpa* growth in a defence-compromised *Arabidopsis* plant treated with the systemic benzenoid fungicide metalaxyl (Kerkenaar and Sijpesteijn, 1981).

RESULTS

qPCR requires robust primers that amplify the target sequence with high efficiency. Our target genes were actin in *Hpa* (*Hpa* gene_ID_807716) and in *Arabidopsis* (AtActin2, At1g49240) (Table S1, see Supporting Information). To determine the efficiency of these primer sets, a dilution series of gDNA from infected tissue was used as a template for qPCR (Fig. S1, see Supporting Information). The *Hpa* and *Arabidopsis* primer sets exhibited efficient amplification over a 200-fold range of template input (5 ng to 1 μg) (Fig. S1a). A standard curve of C_t values plotted against the logarithmic value of gDNA yielded slope values (*M*) of −2.322 and −2.895 for AtActin and HpaActin, respectively (Fig. S1b). Both primer sets yielded linear amplification over the range of template concentration (correlation coefficient R^2 > 0.99) with similar slopes, indicating matching efficiencies for both primer sets (Fig. S1b) (Livak and Schmittgen, 2001).

To confirm primer specificity, a dissociation curve analysis was performed on products amplified from infected and uninfected tissue. HpaActin primers did not amplify any products from uninfected *Arabidopsis* tissue, as expected. Conversely, HpaActin primers produced a single peak (T_m = 82.93 °C) from *Arabidopsis* tissue infected with *Hpa* (Fig. S2, see Supporting Information). The AtActin primer set produced a single peak, distinct from the HpaActin peak (T_m = 79.77 °C) from infected and uninfected templates. These results demonstrate that both primers sets are specific for their respective target genes.

Seedlings of *Arabidopsis* genotypes Col-0, Oy-1 and the mutant Ws *eds1-1* were inoculated with the *Hpa* isolate Emoy2. These genotypes are resistant, moderately susceptible and highly susceptible, respectively, to *Hpa* Emoy2 (Aarts *et al*., 1998; van der Biezen *et al*., 2002; Holub *et al*., 1994). We developed a protocol to homogenize tissue with a bead beater, to increase sample throughput and reduce variability in the efficiency of tissue disruption. We used DNA from this protocol and HpaActin and AtActin primer sets to monitor *Hpa* growth over the course of infection up to 6 days post-inoculation, just prior to the onset of sporulation (Fig. 1). In addition, we stained plants from the same time course with trypan blue, which highlights pathogen hyphae and host cell death (McDowell *et al*., 2011). These samples were used to visually compare hyphal growth at each time point with the values from the qPCR assay.

Col-0 resistance to *Hpa* Emoy2 is caused by the *RPP4* gene, which encodes a Toll/interleukin-1 receptor, nucleotide-binding, leucine-rich repeat (TIR-NB-LRR) protein that recognizes the Atr4 effector from Emoy2 (van der Biezen *et al*., 2002). *RPP4*-mediated hypersensitive response (HR) was macroscopically visible at 3 dpi

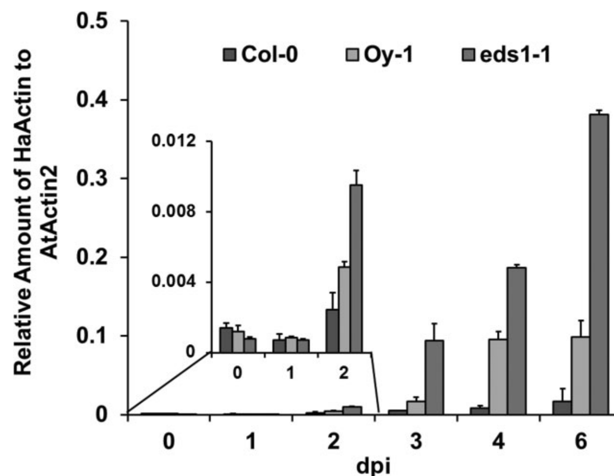

Fig. 1 Time course of *Hyaloperonospora arabidopsidis* (*Hpa*) Emoy2 growth during infection and colonization of resistant (Col-0), moderately susceptible (Oy-1) and highly susceptible (Ws *eds1-1*) *Arabidopsis*. *Hpa* growth was monitored over a 6-day time course on three different genotypes using real-time polymerase chain reaction (PCR). Growth is plotted as the relative quantity of HpaActin to AtActin [2-ΔΔCT]. Black, Col-0; pale grey, Oy-1; dark grey, *eds1-1*. Error bars represent standard deviation of three technical replicates. This experiment was replicated three times.

in cotyledons stained with trypan blue (Fig. 2), became more pronounced by 4 dpi and was profuse at 7 dpi. Correspondingly, the accumulation of *Hpa* Emoy2, as measured by qPCR, in Col-0 plateaued by 2–3 dpi (Fig. 1). qPCR indicated that *Hpa* Emoy2 growth was insignificant by 7 dpi.

Oy-1 is a naturally occurring ecotype of *Arabidopsis* that is susceptible to *Hpa* Emoy2 (Holub *et al*., 1994). Growth was steady over the 7-day period (Fig. 1) and little to no cell death was visible in samples stained with trypan blue. Oospore (sexual spores) production was apparent by 4 dpi. As expected, *Hpa* biomass was much higher in Oy-1 than in the resistant ecotype Col-0.

The third genotype tested was the immunocompromised mutant Ws *eds1-1*. *EDS1* is an important regulator of effector-triggered immunity and of basal resistance that restricts the growth of virulent *Hpa* strains (Wiermer *et al*., 2005). Loss-of-function mutations in this gene cause a phenotype of enhanced susceptibility, compared with the wild-type and susceptible *Arabidopsis* genotypes (Parker *et al*., 1996). We included Ws *eds1-1* to test whether the qPCR assay could accurately resolve small increases in *Hpa* virulence in heavily colonized tissue. The Ws *eds1-1* mutant supported higher levels of *Hpa* Emoy2, and the difference in growth compared with Oy-1 was clear by 2 dpi. At 6 dpi, the overall levels of *Hpa* Emoy2 were approximately three-fold higher in Ws *eds1-1* than in Oy-1. Examination of leaves stained with trypan blue revealed abundant hyphal growth at 2 dpi that was clearly greater in Ws *eds1-1* than in Oy-1 and Col-0 (Figs 1 and 2). These results demonstrate that the qPCR assay resolved small changes in virulence at early time points. This is not

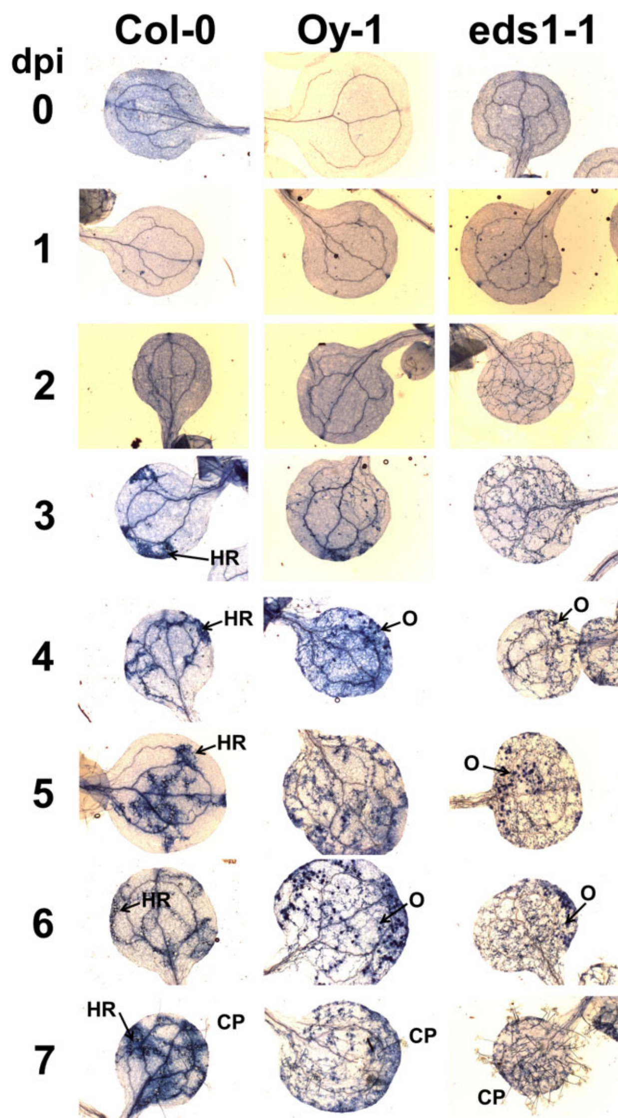

Fig. 2 *Hyaloperonospora arabidopsidis* (*Hpa*) Emoy2 growth correlates with real-time results. *Hpa*-infected tissue was stained with trypan blue and visualized with a light microscope during a time course of 7 days. CP, conidiophore; dpi, days post-inoculation; HR, hypersensitive response; O, oospore. This experiment was replicated three times.

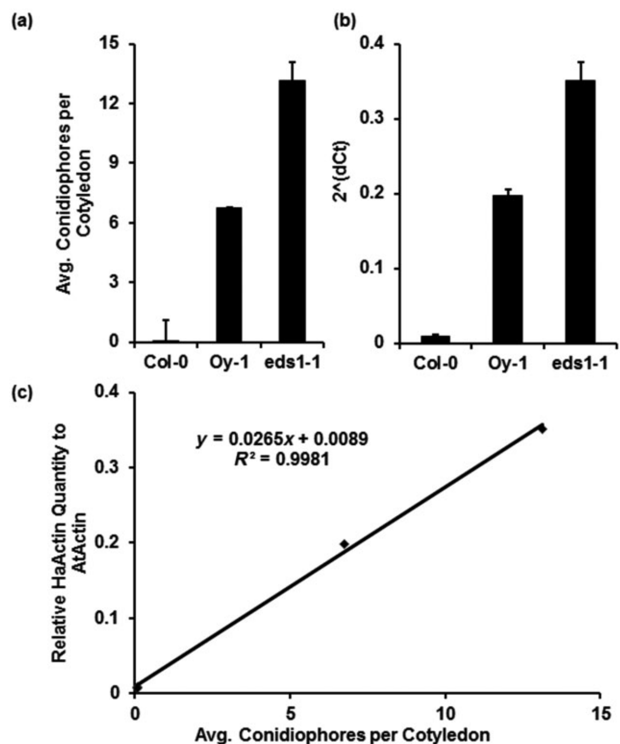

Fig. 3 *Hyaloperonospora arabidopsidis* (*Hpa*) sporulation correlates with polymerase chain reaction (PCR)-based quantification of hyphal growth in infected tissue. (a) Sporangiophores counted at 7 days post-inoculation (dpi). (b) Relative abundance of *HpaActin* to *AtActin* of infected plants at 6 dpi. (c) Results of real-time PCR quantification depicted in (b) plotted against the spore counts depicted in (a). R^2, correlation coefficient. Error bars represent standard error in (a) and standard deviation in (b). This experiment was replicated three times.

possible with assays that are based on pathogen reproduction at the terminal stage of the interaction.

To further validate the qPCR assay, we compared the measurements acquired by qPCR with estimates of growth based on traditional sporangiophore counts. We counted sporangiophores per cotyledon at 7 dpi from the same experiments as used for the qPCR assays. The qPCR assay of *Hpa* growth at 6 dpi correlated with previous time points, with little biomass in Col-0, moderate biomass in Oy-1 and enhanced growth in Ws *eds1-1* (Fig. 3b). As expected, sporulation was low on resistant Col-0, moderate on Oy-0 and high on Ws *eds1-1* (Fig. 3a). The measurements derived from qPCR and sporangiophore counts correlated well (correlation coefficient of 0.99, Fig. 3c).

The identification of novel compounds that can inhibit pathogen growth is valuable for disease control. qPCR is potentially useful for the accurate quantification of the effects of growth inhibitors. As a proof of concept, we infiltrated 4-week-old Ws *eds1-1* plants with the systemic benzenoid fungicide metalaxyl and subsequently inoculated these plants with *Hpa* Emoy2. Leaf punches were collected at 6 dpi and gDNA was extracted and used as the template for qPCR. The assay demonstrated that metalaxyl inhibited *Hpa* growth on Ws *eds1-1* plants compared with control Ws *eds1-1* plants infiltrated with water (Fig. 4). This demonstrates that the qPCR assay is potentially useful for the identification of novel growth inhibitors.

DISCUSSION

The *Hpa–Arabidopsis* interaction is a valuable reference pathosystem, and its utility has been enhanced by recent sequencing of the *Hpa* genome, the identification of effector gene candi-

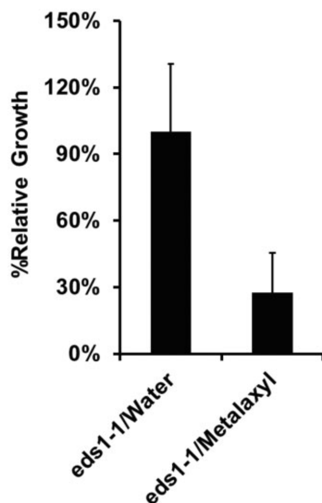

Fig. 4 Polymerase chain reaction (PCR)-based quantification of the extent to which metalaxyl inhibits *Hyaloperonospora arabidopsidis* (*Hpa*) growth. *Arabidopsis* Ws *eds1-1* plants were infiltrated with water or the fungicide metalaxyl and subsequently inoculated with *Hpa* Emoy2. Growth was quantified at 5 days post-inoculation (dpi) with quantitative real-time PCR. Error bars represent standard deviation. This experiment was replicated three times.

dates and screens for effector targets (Baxter *et al*., 2010; Cabral *et al*., 2011; Caillaud *et al*., 2011; Fabro *et al*., 2011; Mukhtar *et al*., 2011; McDowell, 2014). These and other advances have created a critical need for new assays that accurately measure small differences in *Hpa* growth *in planta*. Quantification of sporangiophores can be used to accurately distinguish differences in pathogen growth when sporangiophore density is relatively low, such as when testing mutants that incrementally disable resistance to an incompatible isolate. However, sporangiophores are difficult to count accurately when they grow densely from the surface of heavily infected leaves. Alternatively, spores can be collected, counted with a haemocytometer and normalized to fresh tissue weight. In our hands, this method has potential for high variability introduced during spore collection, as *Hpa* conidia are easily dispersed under ambient conditions. Thus, this method is less robust for distinguishing small changes in virulence. Recent protocols to quantify pathogen growth with qPCR have shown sensitivity, precision and accuracy. qPCR procedures offer the added advantage of quantifying pathogen growth at early time points in the interaction.

For these reasons, we developed an inexpensive, high-throughput procedure for qPCR quantification of *Hpa* growth. This procedure complements and extends a previously published qPCR method for *Hpa* quantification in several respects (Brouwer *et al*., 2003): First, the qPCR protocol includes an assay for a single-copy Arabidopsis gene, which enables accurate normalization of pathogen biomass to plant biomass, and eliminates the need for normalization based on tissue weight or other measures. In addition, we used a simple method for gDNA extraction that employs common, low-cost reagents. The gDNA extraction uses a leaf punch to collect tissue and a bead beater for tissue disruption, allowing 32 samples to be processed in parallel (96 tubes in the bead beater/three replicate tubes per sample). Thus, this procedure enables large numbers of samples to be processed simultaneously with minimal expense.

Our optimization experiments demonstrate that the AtActin and HpaActin primers are specific for their targets and provide linear amplification through a large concentration range of gDNA template. Importantly, the slopes generated by the standard curve of each primer set are similar, allowing a relative quantification of *Hpa* biomass (Livak and Schmittgen, 2001). The estimates of *Hpa* biomass from the qPCR method correlated well with hyphal growth observed with trypan blue and with sporangiophore production. These correlations held true in resistant, moderately susceptible and highly susceptible interactions. This indicates that amplification from the single-copy HpaActin target serves as an unbiased and faithful proxy for *Hpa* growth *in planta*. The qPCR method is also sensitive, as demonstrated during the early time points of the *Hpa–Arabidopsis* interaction. RPP4 resistance to *Hpa* Emoy2 is manifested through HR and is visible with trypan blue staining by 3 dpi. However, differences in *Hpa* growth rate were clear as early as 2 dpi with qPCR, suggesting that RPP4 resistance is established earlier than can be visually detected.

Our time course experiment comparing *Hpa* growth in *Arabidopsis* lines with moderate and high susceptibility provides a previously unattainable quantification of *Hpa* growth from the beginning to the end of the infection cycle. The amount of growth in Ws *eds1-1* and Oy-1 was similar during the first day after inoculation. During this interval, the *Hpa* spores germinate and hyphae emerge to penetrate between epidermal cells into the mesophyll layer. The first haustoria are formed during this time interval. At 2 dpi, hyphae have extended further into the mesophyll. This was the time point at which differences in *Hpa* biomass became apparent between Oy-1 and Ws *eds1-1*, suggesting that *EDS1*-dependent basal defences are activated after the initial penetration steps. It should be noted that Ws *eds1-1* is in a different genetic background and is therefore not isogenic with Oy-1. Thus, relative rates of *Hpa* Emoy2 growth in the two host backgrounds could be affected by other genetic differences in addition to the *eds1-1* mutation. However, this experiment still provides proof of concept that the qPCR assay can enable accurate measurements of small differences in growth *in planta*.

This assay allows the possibility to infiltrate candidate anti-oomycete compounds and to observe the effect on *Hpa* growth. As a proof of concept, we infiltrated adult leaves of *Arabidopsis* with metalaxyl, a systemic benzenoid fungicide that inhibits nucleic acid synthesis. A large reduction in growth was observed, even in immunocompromised Ws *eds1-1*, demonstrating that metalaxyl's inhibitory effects are caused entirely by its inherent toxicity rather than the activation of plant immunity. This general approach could

be extended to recombinant proteins or extracts that cannot be applied with a foliar spray.

EXPERIMENTAL PROCEDURES

Plant growth and maintenance of *Hpa*

Arabidopsis plants for pathogen assays were grown under 8 h of light at 22 °C, 16 h of dark at 20 °C in Sunshine Mix #1. The *Hpa* isolate Emoy2 was maintained on Oy-1 and *eds1-1 Arabidopsis* plants as described by McDowell *et al*. (2011). Conidial suspensions of 5×10^4 spores/mL were prepared from sporulating plants and applied with a Preval™ spray unit (Preval, Inc., Coal City, IL) to 10–12-day-old seedlings. Inoculated seedlings were covered for 24 h, and then uncovered and kept under short-day conditions. At 6 dpi, infected seedlings were covered and the relative humidity was raised to 100% to trigger sporulation.

Sample collection and gDNA extraction

Five seedlings from each genotype were pooled in a 1.2-mL library tube (VWR 83009-678). This constituted one sample. Three samples were collected per treatment, constituting one biological replicate. Three 2-mm glass balls were added to each library tube with 50 μL of extraction buffer [200 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5, 25 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5, 250 mM NaCl, 0.5% sodium dodecylsulfate (SDS)]. Samples were placed into the corresponding library tube rack and homogenized for 2 min in a Mini Beadbeater 96+ (BioSpec, Bartlesville, OK, USA). Samples were subjected to a second round of homogenization if needed, based on visual inspection. Samples were then spun at 21,000 X g for 1 min. Library caps were removed and discarded. Extraction buffer (400 μL) was added to each sample, new library caps were added and the samples were briefly mixed. Samples were then spun at 21,000 X g for 3 min to pellet the cell debris. Supernatant (200 μL) was added to equal parts of 100% isopropanol in a 1.5-mL plastic tube, mixed gently and incubated at room temperature for 2 min. Samples were spun at 21,000 X g for 5 min and the supernatant was discarded. Pellets were air dried for 5 min and resuspended overnight at 4 °C in 30 μL of sterile water. gDNA samples were quantified with a NanoDrop spectrophotometer (Thermo Scientific Waltham, MA USA), diluted to 10 ng/μL and stored at –20 °C.

qPCR

Samples (25 μL) were prepared by mixing 5 μL (50 ng) of gDNA with 12.5 μL of $2 \times$ Sybr Green Mastermix (ABI, Carlsbad, CA, USA), with the appropriate primers added to a final concentration of 5 μM each and water. PCRs were performed in triplicate for each biological sample, using the default relative quantification program of the ABI 7300 device. Stage 1, 50.0 °C for 2 h, followed by 95.0 °C for 10 h in stage 2. Following the initial activation and denaturation steps, 40 cycles of stage 3 were performed at 95.0 °C for 15 min, followed by 53.0 °C for 1 h. C_t values were determined using the included ABI software. *Hpa* primers were derived from Huibers *et al*. (2009). Relative abundance to AtActin was calculated as 2-ΔΔCT (Livak and Schmittgen, 2001).

Trypan blue staining

Trypan blue staining was used to visualize hyphal growth and regions of cell death, as described by McDowell *et al*. (2011), and imaged with a Zeiss Axio Imager M1 (Carl Zeiss, Jena, Germany).

Metalaxyl treatment

Four-week-old *eds1-1* plants, grown under 8 h light/16 h dark, were infiltrated with water or metalaxyl (0.1 g/L) using needle-less syringes, allowed to dry for 1 h and inoculated with Emoy2. Tissue was collected at 6 dpi using a hole punch. Two hole punches were collected per leaf from six leaves in total and pooled into three samples. gDNA was extracted for qPCR analysis as described above.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Quantification of *HpaActin* and *AtActin* genes in genomic DNA extracted from *Arabidopsis* infected with *Hyaloperonospora arabidopsidis* (*Hpa*). (a) Quantitative real-time PCR amplification profiles of normalized florescence (R_n) plotted against cycle number with AtActin and HpaActin primers. Black, 500 ng; green, 250 ng; orange, 100 ng; red, 50 ng; purple, 25 ng; blue, 5 ng. (b) Standard curve assays to measure the efficiency of AtActin and HpaActin primers from dilution series of genomic DNA extracted from *Arabidopsis* infected with *Hpa*. *M*, slope; R^2, correlation coefficient. This experiment was replicated three times.

Fig. S2 Dissociation curve analysis of PCR amplicons generated by the AtActin and HpaActin primers demonstrate target specificity. Inverse fluorescence (derivative reporter) plotted against temperature. Light blue, uninfected *Arabidopsis*; dark blue, infected *Arabidopsis*; yellow, uninfected *Arabidopsis*; green, infected *Arabidopsis*. This experiment was replicated three times.

Table S1 Primers used in this study.