Effector triggered immune response in *Arabidopsis thaliana* is a quantitative trait

Michail Iakovidis,* Paulo J. P. L. Teixeira,† Moises Exposito-Alonso,† Matthew G. Cowper,* Theresa F. Law,‡ Qingli Liu,* Minh Chau Vu,* Troy Minh Dang,* Jason A. Corwin,* Detlef Weigel,† Jeffery L. Dangl,‡,§ and Sarah R. Grant*,§,1

* Department of Biology, University of North Carolina, Chapel Hill, NC, 27599
† Department of Molecular Biology, Max Planck Institute for Developmental Biology, Tübingen, Baden-Württemberg, Germany, 72076
‡ Howard Hughes Medical Institute, University of North Carolina, Chapel Hill, NC, 27599
§ Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, NC, 27599

**Corresponding Author**

1 4258 Genome Sciences Building, 250 Bell Tower Drive, Department of Biology, CB#3280, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599
Phone number: +1 (919) 962-4470
Email: sgrant@email.unc.edu
ABSTRACT

We identified loci responsible for natural variation in Arabidopsis thaliana (Arabidopsis) responses to a bacterial pathogen virulence factor, HopAM1. HopAM1 is a type III effector protein secreted by the virulent Pseudomonas syringae strain Pto DC3000. Delivery of HopAM1 from disarmed Pseudomonas strains leads to local cell death, meristem chlorosis, or both, with varying intensities in different Arabidopsis accessions. These phenotypes are not associated with differences in bacterial growth restriction. We treated the two phenotypes as quantitative traits to identify host loci controlling responses to HopAM1. Genome Wide Association (GWA) of 64 Arabidopsis accessions identified independent variants highly correlated with response to each phenotype. Quantitative Trait Locus (QTL) mapping in a recombinant inbred population between Bur-0 and Col-0 accessions revealed genetic linkage to regions distinct from the top GWA hits. Two major QTL associated with HopAM1-induced cell death were also associated with HopAM1-induced chlorosis. HopAM1-induced changes in Arabidopsis gene expression showed that rapid HopAM1-dependent cell death in Bur-0 is correlated with effector-triggered immune responses. Studies of the effect of mutations in known plant immune system genes showed, surprisingly, that both cell death and chlorosis phenotypes are enhanced by loss of EDS1, a regulatory hub in the plant immune signaling network. Our results reveal complex genetic architecture for response to this particular type III virulence effector, in contrast to the typical monogenic control of cell death and disease resistance triggered by most type III effectors.

INTRODUCTION

Plant pathogens have evolved complex strategies to circumvent the host innate immune response and enhance virulence, including the use of effector proteins to disrupt host immune signaling (Macho and Zipfel, 2015). Plant immunity functions through extra- and intra-cellular receptor systems. Cell surface pattern recognition receptors (PRR) respond to pathogen molecules such as flagellin by activation of a complex defense signaling cascade called MTI (or PTI) for microbe (pathogen)-triggered immunity. MTI leads to production of reactive oxygen, secretion of pathotoxins and reinforcement of cell walls at the infection site, which collectively protect the plant from infection. Successful pathogens introduce into host cells effector proteins that interact with components of the MTI response to dampen it, allowing the pathogen to evade host immunity. In response, the plant immune system evolved an intracellular class of highly polymorphic immune receptors, termed NLR proteins (Nucleotide binding Leucine-rich Repeat proteins; (Bonardi and
There are two classes distinguished by their N-terminal domains, TIR-NLR’s which require the function of the defense protein EDS1 and CC-NLRs which are generally independent of EDS1 (Feys et al., 2001; Wiermer et al., 2005). These proteins can associate with targets of pathogen effectors that are presumably components of the MTI response, or decoys thereof (Dangl and Jones, 2001; Dodds and Rathjen, 2010; Kroj et al., 2016; van der Hoorn and Kamoun, 2008). When the targets are perturbed by a pathogen effector, an associated NLR can be activated, leading to an effector triggered immune response (ETI). ETI is often thought of as an amplified MTI response (Jones and Dangl, 2006; Tao et al., 2003), commonly resulting in rapid cellular desiccation and death at the site of attempted infection (Wright and Beattie, 2004). This hypersensitive cell death response (HR) can isolate microbial pathogens and halt their proliferation. Because effectors are required to suppress MTI, but can trigger ETI, successful pathogens are under evolutionary pressure to continuously ‘re-sort’ their effector collections to suppress MTI while avoiding NLR receptor activation. This evolutionary tug of war has led to bacterial pathogens with diverse collections of effectors that often function through unusual chemistries or via convergent evolution to mimic eukaryotic enzymatic functions (Cheong et al., 2014; Fu et al., 2007; Zhang et al., 2007).

MTI and ETI are accompanied by dramatic changes in host hormone signaling and altered expression of thousands of host genes (Howard et al., 2013; Lewis et al., 2015; Zipfel et al., 2006; Zipfel et al., 2004). Pathogen effector proteins interact with specific host targets that potentially influence only a sector or branch of this host immune output. Identification of host targets of pathogen effectors has been a useful method to define and study subsets of the immune system and to understand how they contribute to immunity (Deslandes and Rivas, 2012; Macho and Zipfel, 2015). Here, we investigated the genetics of natural variation in plant responses to toxic effects of the Pseudomonas syringae type III secretion system effector HopAM1. P. syringae pv. tomato DC3000 (Pto DC3000), a Gram-negative bacterium virulent on tomato and on Arabidopsis, is an extensively studied model pathogen for plant-microbe interactions (Chang et al., 2005; Cunnac et al., 2011; Lewis et al., 2015; Schechter et al., 2006; Xin and He, 2013). Pto DC3000 has at least 30 type III effector genes including two nucleotide sequence-identical copies of hopAM1, one in the genome (PSPTO_1022) and one in the plasmid (PSPTO_A0005) (Buell et al., 2003). The hopAM1 gene is sporadically dispersed among phylogenetically diverse P. syringae isolates (Arnold et al., 2001; Baltrus et al., 2011) and other Gram-negative plant pathogen groups, such as Ralstonia, Xanthomonas and Pantoea (Integrated Microbial Genomes, http://img.jgi.doe.gov/) (Nordberg et al., 2014). HopAM1 was originally identified from P. syringae pv. pisi via its ability to
trigger a presumably NLR-mediated ETI response on some pea cultivars (Cournoyer et al., 1995).

Addition of hopAM1 to the effector complement of the weakly pathogenic strain P. syringae pv. maculicola (Pma M6CdE) conferred a growth advantage on Arabidopsis (Goel et al., 2008). Furthermore, hopAM1 was one of eight type III effector genes that collectively restored nearly full virulence when added back to a completely growth-defective derivative of Pto DC3000 from which the 28 most strongly expressed type III effector genes had been removed by sequential deletion, Pto DC3000D28E (Cunnac et al., 2011). Like many other type III effectors, HopAM1 has toxic effects when over-expressed from a transgene in plants (Goel et al., 2008) and even in yeast (Munkvold et al., 2008). This latter phenotype has hampered approaches to define the biochemical function of HopAM1.

HopAM1 induces two unusual responses on Arabidopsis which are both variable across Arabidopsis accessions. HopAM1 can induce meristem chlorosis on many accessions; noted as emergence of chlorotic leaves 5 to 10 days after onset of HopAM1 expression when expressed from a conditionally induced transgene in Arabidopsis or following inoculation of plant leaves with disarmed Pseudomonas strains designed to deliver HopAM1 by type III secretion. The effect appears to be systemic since it was not possible to recover any of the inoculated bacterial strain from the chlorotic leaves that emerged following hand inoculation of more mature leaves (Goel et al., 2008). Disarmed Pseudomonas strains expressing hopAM1 can also induce local cell death in leaves at the infiltration site and the timing of onset is variable across inbred Arabidopsis accessions. We exploited this natural variation in Arabidopsis responses to HopAM1 to define host loci that control them. Using linkage analyses in both experimental crosses and the global population to describe the genetic architecture of the response to HopAM1, we identified multiple loci associated with differential response to HopAM1 for both cell death and meristem chlorosis.

MATERIALS AND METHODS

*Arabidopsis thaliana* germplasm, propagation and growth conditions: Seeds were germinated on a mix of potting soil (13:6:3 Metro-Mix 360 : baked sand : Perlite; 1.4 gr of Peters 20-20-20 “All Purpose Fertilizer” and 0.85 ml of Marathon per liter of soil mix), stratified 5 days at 4 °, transferred to growth chambers and grown for 9 hours under light (5900 lux) and 15 hours in dark at 21 ° (day) and 18 ° (night), 45-50% relative humidity. For propagation, plants were transferred to a long day (15 hours light) greenhouse with similar conditions. Plants for HopAM1-induced meristem chlorosis were prepared differently as described below. Col-0 x Bur-0
Recombinant Inbred Line (RIL) population was obtained from Christine Camilleri (INRA, France) (Simon et al., 2008). *A. thaliana* accessions, mutant and transgenic CS_ and SALK_ lines were obtained from ABRC (Ohio State University, USA). The *eds1-2* line is a null mutant introgressed from a mutant in the accession Ler-0 into the Col-0 background (Bartsch et al., 2006). Mutant lines *axr2-1*, *abi1-1* and *aba2* were obtained from Jason Reed (UNC-Chapel Hill, USA) (Nagpal et al., 2000), *ein2-5* and *ein2-50* from Joseph Kieber (UNC Chapel Hill, USA) (Wang et al., 2007), the *global* DELLA quintuple mutant from Salomé Prat (Centro Nacional de Biotecnologia, Madrid, Spain) (Cheng et al., 2004), and the triple TOC64 mutant from Úrsula Flores Pérez (Oxford University, Oxford, UK) (Aronsson et al., 2007).

**Bacterial strains and growth assay:** A list with strains and phenotypes assayed can be found in Table S1. Bacterial strains were maintained and grown as described (Chung et al., 2011) and *in planta* growth assays were done as described (Chang et al., 2005). Bacterial strains were grown in King’s B (KB) liquid media at 28 °C with shaking overnight. Antibiotic concentrations used for *P. syringae*, *P. fluorescens* and *P. maculicola* were: 50 μg/ml rifampicin, 50 μg/ml kanamycin, 10 μg/ml spectinomycin, 5 μg/ml tetracyclin. Weighted ANOVA was used to compare bacterial growth differences between strains from multiple experiments in Col-0 and Bur-0. Comparisons among the groups were done using Tukey’s post-hoc test.

**DNA extraction and PCR conditions for genotyping:** We modified the protocol from (Klimyuk et al., 1993) as follows. Plant material (1-2 young leaves) was harvested and placed in a well (Deepwell plate 96/2000 μl; Eppendorf) along with a 4mm borosilicate glass ball and 400 μl 0.25 M NaOH. Samples were then homogenized at 1200 strokes/min for 1 min (2000 GENO/GRINDER, Spex-CentriPrep). Plates were centrifuged for 1 min at 1000 rpm to precipitate plant tissue. 50 μl of supernatant were transferred into a 96-well PCR plate (Non-Skirted, MultiMax) and heated at 96° for 30 sec in a Peltier Thermal Cycler (BIO-RAD, DNA-Engine) with open lid. 50 μl of 0.25 M HCl were added to neutralize the sample, and 25 μl of alkaline lysis buffer (0.5 M Tris-HCl pH 8.0, 0.25% v/v IGEPAL CA-630) were added immediately after, mixing the samples by pipetting. The PCR plate was heated again at 96° for 120 sec and left to cool at room temperature for 5 min. 1-2 μl of DNA sample were used for PCR for genotyping. PCRs were set for a 7 min “Hot-Start” step at 95°, followed by 35 cycles of 30 sec 95° denaturation, 30 sec X (primer-specific)° annealing and 60 sec 72° extension, concluding with a 5 min 72° final extension. Optimized 10x PCR buffer was used for genotyping (0.11 M KCl, 0.11 M (NH₄)₂SO₄, 0.24 M Tris-HCl (pH 8.3),
0.022 M MgCl₂, 0.6% v/v Triton X-100, 1.6% v/v Tween 20, 1.2% v/v NP-40, 1.5% v/v formamide, 0.1 mg/ml BSA, 10% v/v glycerol, 0.02 M TMAC).

**HopAM1-induced cell death assay:** Cell death symptoms induced by bacteria were scored following trypan blue staining (Boyes et al., 1998) at various time intervals. 5-6 week old plants were hand inoculated with bacteria. Inocula with *Pto*-derived strains were adjusted to OD₆₀₀ 0.1 (~5 x 10⁷), and with *Pfo* or *Pma* strains to OD₆₀₀ 0.2 (~1 x 10⁸ cfu/ml) in 10 mM MgCl₂. For phenotyping, 3-4 leaves of 5-week old plants were marked with a Sharpie felt pen on the axial side and the abaxial side was hand inoculated with *Pto* DC3000D28E(*hopAM1*) (see Table S1), and scored for cell death response at 24, 26, 32, 48, 72, and 96 hours post inoculation (hpi) (Figure 1C). Quantitative scoring of cell death symptoms ranged from “5” (apparent by 24 hpi) to “0” (not apparent by 96 hpi) based on the time point that symptoms were first observed.

**HopAM1-induced meristem chlorosis assay:** Tissue culture plates (6-well; #353046 Falcon) were filled with sifted soil mix (prepared as described (Boyes et al., 1998; Holt et al., 2002). Each plate was then covered with nylon mesh and fastened with 4 wide rubber bands. Four plastic head pins were placed inside the corners of each plate to provide aeration during germination and support during vacuum infiltration. Five to ten seeds were sown on each soil mount; plate lids were then placed on the pins and plates were moved to 4 ° for 4-5 days. Seedlings were thinned to a single plant in the middle of each mount. Three 4-week old plants per line were vacuum infiltrated with *Pto* DC3000D28E(*hopAM1*) and scored for chlorotic rosette phenotype at 10 days post inoculation (dpi): Plates were placed inverted in a rectangular plastic container filled with bacterial inoculum (OD₆₀₀ 0.05 (~2.5 x 10⁷ cfu/ml), 10 µM MgCl₂, 60 µl/l Silwet L-77 surfactant), 4 cm deep and vacuum was applied for 100-120 seconds. Plants were transferred into growth rooms. Phenotypic scores were assigned based on visible chlorosis in rosettes at 10 dpi. The phenotype was recorded as “any response” (1) or “no response” (0).

**QTL analysis:** *Cell death.* Three F₈ plants each of 342 lines from a Col-0 x Bur-0 RIL mapping population (Simon et al., 2008) were scored for HopAM1-induced cell death, following hand inoculation with *Pto* DC3000D28E(*hopAM1*). Initial QTL analysis on the 89 markers spanning 5 chromosomes of *A. thaliana* was performed by CIM using WinQTL Cartographer 2.5.011 (Wang et al., 2012). After the detection of several QTL, the entire RIL population was reannotated using 60 additional markers to provide extra resolution for CIM. New markers were selected proximally and distally for each marker within the QTL regions detected. Mapping distances were
recalculated using MapMaker 3.0 (Lincoln et al., 1992). For detecting QTL, a LOD score of 3.2 was chosen based on 1000 permutations at $p \leq 0.01$ for CIM. Walking speed was 0.5 cM and window size 10 cM. Epistatic interactions between the QTL identified in CIM were investigated in detail using a general linear model structure (Brady et al., 2015), which provides more power than traditional pairwise $t$-tests in factorial interaction studies, by using the ANOVA framework.

**Meristem chlorosis.** Three plants per Col-0 x Bur-0 RIL (Simon et al., 2008) were scored for HopAM1-induced meristem chlorosis at 10 and 14 dpi, following vacuum infiltration with *Pto Dc3000d28E*(*hopAM1*). CIM was performed again on the original genotyping data (89 markers) and the reannotated data (89+60 markers). For detecting QTL, a LOD score of 3.2 was chosen based on 1000 permutations at $p \leq 0.01$ for CIM. Walking speed was 0.5 cM and window size 10 cM. Other parameters were set at default. The model for corrected means that was used before for HopAM1-induced cell death (see Table S2) was not suitable for the binary nature of phenotypes for HopAM1-induced meristem chlorosis and was not included in the model. Phenotypic scores for all 342 RILs were assigned as observed (with at least one responsive replicate resulting in a score of “1”). Symptoms similar to HopAM1-induced cell death and meristem chlorosis have not been observed before in untreated Col-0 x Bur-0 RILs (Chavigneau et al., 2012; Gery et al., 2011; Simon et al., 2008).

**GWA analysis:** To obtain a species-wide view of HopAM1-induced cell death and meristem chlorosis, we assayed 98 *A. thaliana* accessions (ABRC). For most of our accessions (n=64), whole-genome sequences were available (http://1001genomes.org). (1001 Genomes Consortium, 2016). We used 4,004,754 SNPs with 0.98 genotyping rate and Minor Allele Frequency $> 5\%$ to perform GWA analyses, using a linear mixed model to correct for population structure, implemented in the Efficient Mixed-Model Association eXpedited (EMMAX) package (Kang et al., 2010), as this has been proven to be efficient for Arabidopsis (Atwell et al., 2010). This model fits an additive genetic variance factor using a kinship matrix, which allows calculation of narrow sense heritability for the GWA population. To test the significance of the genetic variance factor, we used a likelihood ratio test comparing the fit of the full model against the null model without the kinship matrix. Allele effect sizes were reported as standardized regression coefficients or “beta”. They are interpreted as the difference between genotypes carrying the minor allele frequency (MAF) allele to those with the reference allele, and are expressed in standard deviations of the phenotype. Enrichment of top SNPs in QTL peaks was tested using a Fisher’s exact test with 2X2 tables of counts as follows:
**Top GWA SNPs inside QTL range:** Total SNPs inside QTL range/

**Top GWA SNPS in the chromosome of the QTL:** Total SNPs in the chromosome of the QTL.

**Confocal Microscopy:** Small leaves (5 mm diameter) were collected 14 days after vacuum infiltration with *Pto* DC3000(*hopAM1*). The abaxial side of Col-0 and Bur-0 leaves was observed using a C-Apochromat 40X/NA1.2 water immersion lens on a Zeiss LSM710 confocal laser scanning microscope. Autofluorescence was observed using a 560 nm diode laser line for excitation and the PMT (photomultiplier tube detector) collected emission bandwidth set at 568-740 nm. The confocal images were edited with ZEN 2009 software.

**RNA sequencing:** We used RNA-seq to investigate HopAM1-associated changes in the transcriptome of infected Arabidopsis leaves. Five-week-old plants grown under short days were hand-infiltrated (OD$_{600}$ = 0.1, ~2.5x10$^7$ cfu/ml) with the control strain *Pto* DC3000D28E transformed with the empty vector pJC531; or with *Pto* DC3000D28E(*hopAM1*) (Goel et al., 2008). The accessions Col-0 and Bur-0 were used in the experiment. Samples were harvested immediately before (T = 0 hours) and 2, 4, 6, 8, 10 and 12 hrs after bacteria infiltration. Three biological replicates were analyzed per condition, each consisting of three 7 mm discs collected from the inoculated leaf of one individual plant. Samples were frozen in liquid nitrogen and ground using glass beads and the Tissue Lyser II system (Qiagen). Total RNA was extracted using TRIzol (Invitrogen), DNase treated (Ambion Turbo DNase), and purified using the RNeasy Mini kit (Qiagen). 1 µg was used to prepare Illumina-based mRNA-seq libraries. Quality control and quantification of the final libraries were performed using the 2100 Bioanalyzer (Agilent) and the Quant-iT PicoGreen dsDNA Reagent (Invitrogen). Barcoded libraries were combined into a single pool and sequenced in two lanes of an Illumina HiSeq2500, resulting in an average of 2.1 million 50 bp single-end reads per library.

RNA-seq reads were mapped using TopHat (Trapnell et al., 2009), allowing only one mismatch and discarding any read that mapped to multiple positions. The TAIR10 assembly was used as the reference genome for Col-0, whereas the assembly available at [http://mus.well.ox.ac.uk/19genomes/](http://mus.well.ox.ac.uk/19genomes/) was used as the reference for Bur-0 (Schneeberger et al., 2011). Approximately 90% of reads could be mapped to the Arabidopsis genome. Reads mapped to nuclear protein-coding genes were counted using HTSeq (Anders et al., 2015) and the package edgeR (Robinson et al., 2010) was used to define differentially expressed genes between plants infiltrated with *Pto* DC3000D28E(*hopAM1*) and *Pto* DC3000D28E(EV) at each time point. Genes
with FDR lower than 0.05 and a fold-change variation greater than 2 were considered differentially expressed between conditions. All nuclear protein-coding genes were tested for differential expression without the adoption of thresholds to filter out weakly expressed genes. Gene Ontology (GO) enrichment analyses were performed with the PlantGSEA platform (Yi et al., 2013) using the sets of differentially expressed genes. Transcriptional activation of chosen gene sets was represented as the median z-score transformed expression values (in RPKM - Reads Per Kilobase of transcript per Million mapped reads).

**Data availability:** Bacterial strains are available upon request. All data necessary for reproducing QTL results and complete edgeR results for RNA-seq data can be found as Supplemental Materials. Raw RNA sequencing data are available at the NCBI Sequence Read Archive under the accession number SRP075162.

**RESULTS**

**The quantitative nature of HopAM1-induced cell death.** Previously, we cloned 93 type III secretion system effectors from *Pseudomonas syringae* isolates (Baltrus et al., 2011) and transferred them individually into an engineered strain of the non-pathogenic *P. fluorescens* bacterium carrying a heterologous type III secretion system apparatus (Thomas et al., 2009). Disease resistance responses are often qualitative, and we hoped to identify several such host responses across this survey. HopAM1 induced obvious cell death in some accessions. To better define the distribution of phenotypes across the host species and to identify accessions that would be suitable for mapping in experimental crosses, we re-screened a collection of 98 accessions delivering HopAM1 by infection with the disarmed bacterial strain *Pto* DC3000D28E (Cunnac et al., 2011) bearing a plasmid with the *hopAM1* gene with a C-terminal HA tag expressed from its own promoter (Goel et al., 2008). Although its virulence is severely reduced, *Pto* DC3000D28E stimulates MTI effectively and is at least as effective as *P. fluorescens* for delivery of type III effectors. Following hand inoculation of leaves with *Pto* DC3000D28E(*hopAM1*), we observed a range of cell death responses at the site of inoculations that varied between Arabidopsis accessions in the timing of onset of cell collapse (see Figure S1, Table S3). Bur-0 showed strong cell death symptoms at 24 hpi; and Col-0 did not suffer from any symptoms, not even microscopic lesions visible with Trypan blue staining (see Figure S2), until 6 dpi (~144 hpi) (Figure 1 A-B). Analysis of an F$_2$ population (468 plants) of Bur-0 x Col-0 demonstrated non-Mendelian
segregation of HopAM1-induced cell death (see Table S4). Therefore, we followed the timing of appearance of cell death symptoms after inoculation to derive a quantitative cell death score (Figure 1C).

**Screening and reannotation of Bur-0 x Col-0 RILs:** We explored the genetic architecture driving HopAM1-induced cell death using an established Bur-0 x Col-0 F₈ recombinant inbred line (RIL) population (Simon et al., 2008) screened with *Pto DC3000D28E(hopAM1)*. Cell death symptoms developed continuously over time after inoculation (see Figure S2) in the RIL population and phenotyping was performed following the numerical scoring system illustrated in Figure 1C. Two individual plants from each RIL were infiltrated with *Pto DC3000D28E(hopAM1)* and tissue was harvested from both for genotyping (see Table S5). The distribution of phenotypic scores among the RILs was similar (see Figure S3) to the distribution of the 98 *A. thaliana* accessions screened previously, where most lines presented with intermediate cell death phenotypes (see Figure S1) (Kolmogorov-Smirnov Test for similarity between distributions; \( D = 0.18, \ p\text{-value} = 0.014 \)). The parental accessions Col-0 and Bur-0 had the most extreme phenotypes, and there was no transgressive segregation among RILs. To evaluate the heritability of the cell death response in the RILs, a second screen of the RIL population was performed, and the initial phenotypic scores were adjusted to control for environmental effects using a general linear model (Brady et al., 2015; Chan et al., 2011) (see Table S2). The broad sense heritability \( H^2 \) within the RILs for HopAM1-induced cell death was high \( H^2=95.4\%, \ p\text{-value} < 2\times 10^{-16} \), with a very low amount of phenotypic variation being controlled by combined experimental factors \( H^2=0.06\% \) (see Table S2).

**Five Arabidopsis loci additively control HopAM1-dependent cell death in the Bur-0 x Col-0 cross:** Composite Interval Mapping (CIM) performed among the RILs identified five loci on chromosomes 1 (QTL1 = 0.0 – 8.4 Mb), 2 (QTL2 = 8.7 – 11.1 Mb), 4 (QTL4 = 0.0 – 5.79 Mb), and 5 (QTL5A = 16.3 – 21.3 Mb; QTL5B = 25.9 – 26.9 Mb) (Figure 1D, Table S6) (Wang et al., 2012). We screened putative HIFs (Heterozygous Inbred Families) derived from the RIL population for segregation at QTL4, QTL5A and QTL5B to fine map the loci controlling HopAM1-induced cell death in the Bur-0 x Col-0 background. We progeny tested several RILs with a heterozygous haplotype for the QTL region of interest to identify segregating lines and then chose a few lines for subsequent fine mapping of each QTL (see Figure S4). QTL4 was delimited within the centromeric region of chromosome 4 (in Col-0 containing many genes of unknown function, several cysteine rich receptor-like kinases, and one TIR-NLR, (At4g04110)). However, due to low
recombination near the centromere, we could not reduce the size of the associated interval further. Thus, we focused our fine mapping efforts on QTL5A and QTL5B. QTL5A was delimited within 296 kb (18.682 – 18.978 Mb) (containing genes of various function, including many TIR-NLR genes and the plasma membrane localized receptor kinase protein FLS2 (At5g46330) which recognizes bacterial flagellin peptides that stimulate MTI responses (Zipfel et al., 2004). QTL5B was delimited to within 110 kb (26.639 – 26.749 Mb) (containing 30 genes, including MAPKK kinases and CC-NLR genes). The Col-0 gene annotations for the regions in QTL4, 5A and 5B are listed in Table S7. We found only additive effects between the QTLs using an ANOVA for each QTL pair from the initial CIM mapping to identify pairwise epistasis contributing to the HopAM1-induced cell death response (see Figure S5, Table S8) (Brady et al., 2015). However, our population sizes may be too small to effectively evaluate higher order epistasis (Joseph et al., 2013; Taylor and Ehrenreich, 2014).

**HopAM1-induced cell death is affected by EDS1, HSP90.2 and SGT1B:** To assess if HopAM1-induced cell death is affected by genes known to be required for disease resistance, we made several crosses between the Bur-0 accession and Col-0 loss of function mutants in defense-related genes: sid2-1 (Wildermuth et al., 2001), sgt1b (Austin et al., 2002), rar1-21 (Tornero et al., 2002), ndr1-1 (Century et al., 1995), eds1-2 (Bartsch et al., 2006), hsp90.2-3 (Hubert et al., 2003), pad4-1 (Glazebrook et al., 1996) and adr1-1 (Grant et al., 2003). A total of 96 F2 progeny from each cross were genotyped and plants homozygous at markers flanking each Col-0-derived mutation in the respective crosses were selected for cell death phenotyping. As a comparative control, 96 plants from a Bur-0 x Col-0 F2 progeny were screened for cell death and subsequently genotyped with the same markers used for the specific mutation in each cross. In Figure 1E, the phenotypes of the F2 plants homozygous for a Col-0-derived mutant allele from the Col-0 mutant x Bur-0 cross are presented next to the phenotypes of plants from the subset of control Bur-0 x Col-0 F2s which were homozygous for the wild type Col-0 allele for the relevant gene. Our results indicated that eds1-2, hsp90.2-3 and sgt1b affect HopAM1-induced cell death symptoms from Bur-0 (Figure 1E). Homozygosity for either the hsp90.2 (hsp90.2Col-0) or sgt1b mutant allele (sgt1bCol-0) in Bur-0 x Col-0 background attenuated HopAM1-induced cell death symptoms compared to their respective Bur-0 x Col-0 control populations. Because HSP90.2 and SGT1b are part of a steady state NLR chaperone complex required for NLR ETI receptor activation (Holt et al., 2005; Hubert et al., 2003; Shirasu, 2009), this is consistent with the hypothesis that at least one of the cell death QTL loci (Figure 1D) contains a functionally relevant NLR gene. The eds1-2 mutation in the Bur-0 x Col-0 background enhanced the onset of HopAM1-induced cell death
symptoms (Figure 1E) which was unexpected because EDS1 is required for the function of TIR-NLR proteins (Feys et al., 2001). The other defense mutations we tested had no effect.

**Genome wide association reveals additional loci associated with HopAM1 cell death.** A disadvantage of QTL experiments is that the identified loci may represent rare, deleterious alleles compared to the entire population surveyed. Genome Wide Association (GWA) screens can complement QTL studies by identification of loci that do not have segregating alleles in the two RIL founder populations (Gibson, 2012; Weigel and Nordborg, 2015). We used 64 of the 98 accessions we had screened previously for a GWA study (see Table S3) because whole-genome resequencing data were available for them (http://1001genomes.org) (1001 Genomes Consortium, 2016). The most significantly associated SNPs did not overlap with the intervals defined by the Bur-0 x Col-0 QTL analysis (Figure 2) but we found enrichment in the QTL regions when we considered the 50 (or more) most significantly associated SNPs in same chromosome as the QTL (Table 1). The narrow sense heritability calculated for this study was $h^2=34.5\%$, (Likelihood Ratio Test, $p=0.430$) and the allele frequencies of the SNPs with the strongest allele effects were generally shifted towards low minor allele frequency (see Figure S6A), consistent with a polygenic architecture for the trait according to an infinitesimal model (Gibson, 2012). The SNPs most significantly associated with variation in HopAM1-induced local cell death were two peaks with multiple significantly associated single nucleotide polymorphisms (SNP) on chromosome 2, and single SNPs on chromosomes 1 and 2 (Figure 2). Five out of the 10 most significant SNPs fell within a 154 bp non-coding region between At1g50420, encoding the SCARECROW-LIKE 3 transcription factor (SCL3), a positive regulator of gibberellic acid signaling (Zhang et al., 2011) and At1g50430 (STEROL DELTA-7 REDUCTASE), which encodes a sterol reductase involved in brassinosteroid synthesis (Lecain et al., 1996) (see Table S9). The most significantly associated SNP was on chromosome 2 in the 4th exon of CYCLOPHILIN40 (At2g15790) which encodes a protein isomerase that functions in maturation of the AGO1-containing RISC complex important to epigenetic gene regulation (Iki et al., 2012; Romano et al., 2004; Smith et al., 2009). Aligning the haplotypes available from the 1001 Genomes Project that were used in our GWA over a 30 kb region spanning the CYCLOPHILIN40 gene showed a correlation between haplotype and the HopAM1-dependent cell death phenotype (see Figure S7).

**Other biparental populations have HopAM1-induced cell death QTLs that overlap with Bur-0 x Col-0 QTL5A:** To explore the differential genetic architecture driving HopAM1-induced cell death among the accessions, we screened three different biparental mapping populations to
investigate whether HopAM1-induced cell death is also a quantitative trait in other accessions. We scored plants from three other populations with parents divergent in their cell death phenotypes, but not as divergent as Bur-0 x Col-0: a Ler-0 x Col-4 RIL population (CS1899), a Sha x Col-0 F₂ population, and a Ws-2 x Col-0 F₂ population for HopAM1-induced cell death (see Table S10). Ler-0 and Ws-2 had cell death scores of 2 and the Sha score was 4 (see Table S3). All three populations exhibited a continuous phenotypic response similar to the Bur-0 x Col-0 background, but with weaker phenotypes. We again observed no transgressive segregation. We performed a CIM analysis to identify the loci controlling the trait in all three backgrounds and to search for overlap with the loci identified in the Bur-0 x Col-0 background (see Figure S8). In the Ler-0 x Col-4 background, two QTL were identified on chromosome 1 (16.1 – 22.2 Mb) and chromosome 5 (15.4 – 20.9 Mb), and in the Ws-2 x Col-0 and Sha x Col-0 backgrounds, one QTL was identified that was solely responsible for the HopAM1-induced cell death (chromosome 5, 14.7 – 23.8 Mb for Ws-2 x Col-0; 8.4 – 20.1 Mb for Sha x Col-0). The QTL identified on chromosome 5 for all three mapping populations (Ler-0 x Col-4, Ws-2 x Col-0, Sha x Col-0) overlapped with QTL5A from the Bur-0 x Col-0 RIL population (16.3 – 21.3 Mb), possibly suggesting that the common parent, Col-0/Col-4, carries an unusual allele at this locus.

**HopAM1-induced cell death and chlorosis are not associated with bacterial growth restriction in both Col-0 and Bur-0:** HopAM1 induces obvious phenotypes, but it is unclear how these might be related to the restriction of pathogen growth, the effect most relevant to the host. HopAM1 enhances the growth of a weakly virulent *P. syringae* strain *Pma* (M6CdE) in the Ws-2 accession, but not in several other accessions tested (Goel et al., 2008). Thus, HopAM1 is a weak virulence factor, at least on Ws-2. To determine if the different cell death responses of Bur-0 and Col-0 to HopAM1 were correlated with differences in the ability to restrict pathogen growth via recognition of HopAM1, we infected Bur-0 and Col-0 with *Pto* DC3000. This strain grew to levels associated with disease in both accessions (Figure 3), with slightly higher titers in Bur-0. To determine if the endogenous *hopAM1* genes affect the growth of *Pto* DC3000 in these accessions, we infected Col-0 and Bur-0 with a *Pto* DC3000 derivative, *Pto* JB206, which lacks both the chromosomal (PSPTO_1022) (Boch et al., 2002) and plasmid borne (PSPTO_A0005) (Landgraf et al., 2006) copies of *hopAM1*. We found a small, statistically significant, growth advantage of *Pto* JB206 over *Pto* DC3000 in both Bur-0 and Col-0 (Figure 3). This growth advantage disappeared from both accessions when *Pto* JB206 was complemented with the plasmid carrying *hopAM1* (Figure 3). Thus, HopAM1 is recognized by both accessions, at least to an extent sufficient to trigger growth restriction and there is no genetic difference between Bur-0 and Col-0.
for this pathogen growth restriction. We infer that both accessions recognize and respond to HopAM1 at levels sufficient to trigger weak ETI; however only in Bur-0 does the signal delivered by HopAM1 result in ETI-associated cell death.

**Overlapping and distinct QTL for HopAM1-induced meristem chlorosis and cell death:**

HopAM1 induces a chlorotic phenotype in newly developed leaves when delivered by a bacterial strain (*Pma* M6CdE) via hand infiltration or following transgenic expression of *hopAM1* in Arabidopsis (Goel et al., 2008). An improved vacuum infiltration assay was devised using *Pto* DC3000D28E(*hopAM1*) (see Materials and Methods). All 64 accessions used in our GWA for HopAM1-induced cell death response (see Table S2) were rescreened for HopAM1-induced meristem chlorosis. While most of the accessions exhibited meristem chlorosis at 7 dpi, twelve remained non-chlorotic (see Table S11). In fact, Col-0 exhibited strong meristem chlorosis and Bur-0 was non-responsive, highlighting the contrasting underlying genetics of these two accessions in response to HopAM1 (Figure 4A). Of the three accessions that exhibited the highest cell death scores (Bur-0, Hov-4, and Wil-2; see Table S11), only Bur-0 did not exhibit HopAM1-induced meristem chlorosis. Hence, HopAM1 cell death and meristem chlorosis inducing activities are not mutually exclusive. Confocal laser microscopy of Col-0 and Bur-0 newly emerged chlorotic leaves at 14 days after infection revealed that the chloroplasts in Col-0 were smaller, deformed, and exhibited a much weaker fluorescence signal, while the chloroplasts in the corresponding leaves in Bur-0 appeared normal (Figure 4B). Furthermore, Col-0 chlorotic leaf tissue had smaller, round-shaped mesophyll cells, while Bur-0 had wild-type cell morphology (Figure 4B). We used the Bur-0 x Col-0 RIL population (Simon et al., 2008) again to identify loci controlling the HopAM1-induced meristem chlorosis. To assess the heritability of the meristem chlorosis trait in the RILs, we performed a second screen of a subset of the RIL population (162 lines with the highest number of recombination events were chosen). We tested the influence of environmental effects on our meristem chlorosis phenotype scores using a general linear model as before (Chan et al., 2011) (see Table S12). The broad sense heritability of the HopAM1-induced meristem chlorosis trait was $H^2 = 60.7\%$, $p$-value $< 2e^{-16}$. Using the reannotated Bur-0 x Col-0 RILs, we performed a CIM that revealed three loci on chromosomes 4 (QTL4c1 = 0.9 – 5.2 Mb, QTL4c2 = 6.9 – 15.7 Mb) and 5 (QTL5c = 18.3 – 24.1 Mb) (Figure 4C, Table S13). The genomic regions of QTL4c1 and QTL5c overlap with the HopAM1-induced cell death QTL4 (0.0 – 5.79 Mb) and QTL5A (16.3 – 21.3 Mb), but the most significant chlorosis locus, QTL4c2, was distinct from all QTL identified for HopAM1-induced cell death.
HopAM1-induced cell death and meristem chlorosis traits are independent among wild accessions: We also performed GWA mapping for HopAM1-induced meristem chlorosis among the 64 accessions that we had screened for HopAM1-induced cell death (see Table S11). The narrow sense heritability for the GWA study was $h^2=20.9\%$ (Likelihood Ratio Test, $p=0.625$) and the most significant SNPs tended to have low minor allele frequencies (see Figure S6B). There was an enrichment of the most significantly associated SNPs with QTL4C2 (Table 2). However, the only peak above the stringent Bonferroni significance level was outside of the QTL intervals (Figure 5). This SNP and three more of the 10 most significant meristem chlorosis SNPs (FDR≤0.05; including the top hit) fell within a 235 bp region between the 3rd and 4th exon of TOC64-III (At3g17970) which encodes a protein of the chloroplast outer envelope membrane transport complex (Sommer et al., 2013) (see Table S14). In total, 27 out of 50 top hits fell within a 2 kb region of TOC64-III (3rd to 9th exon). Alignment of all the 1001 Genomes haplotypes that were used in the GWA study across a 30 kb region spanning the top SNP indicated a correlation between TOC64-III haplotype and HopAM1-induced meristem chlorosis response (see Figure S9). Single toc64-III mutants, and a toc64 triple mutant (Materials and Methods) were tested in the background of the meristem chlorosis-responsive accession Col-0. All toc64 mutants, including the triple mutant, were responsive for HopAM1-induced meristem chlorosis, indicating that lack of TOC64 is not sufficient to prevent the effect.

We also screened additional Arabidopsis mutants for lack of HopAM1-induced meristem chlorosis (see Table S15). The double mutant rbohd rbohf (CS68522) (Torres et al., 2002) was not responsive, suggesting that superoxide derived from this NADPH oxidase complex is required for the phenotype. Both eds1-2 and an ein2-1 pad4-1 sid2-2 (CS66006) triple mutant enhanced the meristem chlorosis by extending the duration of time in which plants produced chlorotic leaves compared to controls (see Figure S10). Neither the ein2-1 sid2-2 double mutant nor pad4-1 exhibited this enhanced chlorosis. EDs1 and PAD4 proteins interact and potentiate accumulation of salicylic acid (SA) in pathogen infected tissues, potentially through regulation of TIR-NLR action (Feys et al., 2001; Rietz et al., 2011; Wagner et al., 2013). SID2 encodes isochorismate synthase (ICS1) required for production of SA (Wildermuth et al., 2001). The extended chlorosis response of these two mutant lines suggests HopAM1 chlorosis is limited in timing through EDS1 function. All other Col-0 background mutant lines were similar to the parental Col-0 accession.

Host transcriptional signatures indicate that HopAM1 suppresses MTI but also induces ETI in Bur-0: To further investigate how HopAM1 interferes with host cellular functions, we performed
genome-wide transcriptome analysis of infected plants. Five week-old Col-0 and Bur-0 plants were hand-inoculated with *Pto* DC3000D28E(EV) or *Pto* DC3000D28E(*hopAM1*). RNA-seq libraries were prepared from plants harvested immediately before infiltration and at six time-points post-infiltration spanning a 12 hour period (Methods and Materials). The number of differentially expressed genes between plants inoculated with *Pto* DC3000D28E(EV) and *Pto* DC3000D28E(*hopAM1*) was relatively low at 2 hpi, a time point just preceding delivery of type III effectors (de Torres Zabala et al., 2009), for both accessions (306 in Col-0 and only 4 in Bur-0; Figure 6A). The number of differentially expressed genes greatly increased over the remainder of the infection time course, ranging from 1432 to 2981 in Col-0 and from 522 to 5762 in Bur-0 (Figure 6A). The complete edgeR results are presented in Table S16. *HopAM1*-dependent differential expression was minimal for the ten most highly associated loci identified in each GWA study. The *HopAM1* chlorosis QTL regions still include hundreds of genes and many of them showed *HopAM1*-dependent transcriptional responses at some time points. The more narrowly defined cell death related QTLs, QTL4, QTL5A and QTL5B also included multiple genes with differential expression in response to *HopAM1* (see Table S7).

Gene Ontology (GO) enrichment analyses showed that *HopAM1* down-regulated defense-related genes at most time points in Col-0, and at the early time points in Bur-0 (see Figure S11; Tables S17, S18, S19, S20, S21). We determined if the changes in expression of defense-responsive genes were related to MTI or ETI by focusing on the expression of 1418 genes induced by the well characterized MAMP flg22 (Rallapalli et al., 2014) for MTI and for ETI, on expression profiles of a set of 747 markers corresponding to genes up-regulated in a comparison of Col-0 plants infected with ETI–inducing *Pto* DC3000(*avrRps4*) (triggering the RPS4 TIR-NLR) versus plants infected with virulent *Pto* DC3000 (Howard et al., 2013). The average expression profile of the flg22-induced genes after infection with *Pto* DC3000(*hopAM1*) compared to *Pto* DC3000(EV) is consistent with *HopAM1* suppressing MTI in both accessions at early time points. At 10-12 hpi ETI-induced genes were up regulated by *HopAM1* in Bur-0 but not in Col-0 (Figure 6B). Specific genes associated with typical immune responses such as GSL5 (*At4g03550*), *RBOHD* (*At5g47910*), SAG101 (*At5g14930*), NDR1 (*At3g20600*), and SID2 (*At1g74710*) were up-regulated in Bur-0 from 10 hpi onwards, but not in Col-0 (see Figure S12). Our gene expression profiling indicates that *HopAM1* induces an ETI response in the Bur-0 accession, which supports rapid *HopAM1*-induced cell death, but not in Col-0, which does not. Notably, this difference in ETI response is not correlated with restriction of bacterial growth in Bur-0 compared to Col-0 (Figure 3).
**P. syringae** type III effectors suppress MTI transcriptional responses in the host plant (Lewis et al., 2015; Truman et al., 2007). Since HopAM1 is delivered to host plants by *Pto* DC3000 along with the rest of its suite of type III effectors, we expected that the transcriptional response to infection with *Pto* DC3000 should overlap with the response to *Pto* DC3000D28E(*hopAM1*). Lewis and colleagues, (Lewis et al., 2015) identified a set of 2325 Arabidopsis genes that were differentially expressed (769 down-regulated; 1556 up-regulated) during the infection with *Pto* DC3000, but not with the mutant *Pto* DC3000*hrpA*. Since the *hrpA* mutant does not deliver effectors into the host cells, the differential gene expression was dependent on the bacterial effectors of *Pto* DC3000. 213 of the 769 *Pto* DC3000 effector down-regulated genes (28%) were also down-regulated by HopAM1. These genes were enriched in biological processes related to nucleosome organization and chromatin assembly (FDR < 0.001; see Table S22). Furthermore, 510 of the 1556 genes (33%) activated by *Pto* DC3000 effectors were also up-regulated by HopAM1 in our experiment. These genes were enriched in biological processes related to autophagy and photoperiodism (FDR < 0.001; see Table S22). MAMP perception has been shown to trigger large-scale suppression of genes for chloroplast-localized proteins in Arabidopsis, but that suppression was attenuated in plants infected with *Pto* DC3000, due to the combined functions of its collection of type III effectors (de Torres Zabala et al., 2015). When compared to *Pto* DC3000D28E(EV), infection with *Pto* DC3000D28E(*hopAM1*) resulted in higher expression of genes encoding chloroplast-localized proteins in both Col-0 and Bur-0 at 4 and 6 hpi, followed by reduced expression in both accessions by 12 hpi (see Figure S13), indicating that HopAM1 may affect chloroplast responses to MTI (Figure 4B) in both accessions even though Bur-0 does not suffer from meristem chlorosis.

**DISCUSSION**

**HopAM1-induced local cell death is associated with HopAM1-induction of ETI response.**

Using RNA-seq, we compared gene expression changes induced by *Pto* DC3000D28E(*hopAM1*) to changes induced by *Pto* DC3000D28E(EV). We found that HopAM1 repressed gene sets related to MTI immune responses in both Col-0 and Bur-0 at early time points (Figure 6B). Our results are consistent with our previous report that HopAM1 suppresses basal defense responses (Goel et al., 2008). Genes suppressed by HopAM1 overlapped with genes suppressed by *Pto* DC3000 delivering its full suite of type III effectors that includes HopAM1 (de Torres Zabala et al., 2015; Lewis et al., 2015; Truman et al., 2007). However, at 10-12 hpi, we defined a Bur-0-specific
induction of genes previously associated with RPS4-dependent ETI (Howard et al., 2013) (Figure 6C; see Figure S11). As expected if the early onset cell death seen in Bur-0 is the result of induced ETI, it is attenuated in F2 plants from crosses of Bur-0 with Col-0 derived mutants which were homozygous for loss of function mutations in HSP90.2 and SGT1b (Figure 1E) required for NLR receptor activation (Holt et al., 2005; Hubert et al., 2003; Shirasu, 2009).

Our QTL analysis for HopAM1-induced cell death on the Bur-0 x Col-0 RIL population revealed multiple loci contributing additively to the timing of onset of cell death (Figure 1D). The three loci that account for most of the variation (QTL4, QTL5A and QTL5B) each contain genes encoding NLR proteins, ETI receptors that are often highly variable in Arabidopsis (Cui et al., 2015; Shen et al., 2006) (see Tables S3 and S7). Additional fine mapping and mutation analysis of individual candidates will be necessary to confirm candidate genes as responsible for the QTL traits. Specific alleles at each of the cell death QTL loci contribute additively to induce cell death earlier in Bur-0 than in other accessions as if each locus has an independent ETI function and the timing of cell death depends on the accumulation of effects from each locus. This is unlike the well-characterized ETI responses to pathogen effector proteins that present as gene-for-gene resistance scenarios, where one NLR gene is sufficient to mount an effective ETI response when its product recognizes the action of one type III effector (Dangl and Jones, 2001).

**GWA studies complement QTL mapping in biparental populations.** Since they work at the population level, GWA studies have maximal power to detect associations with common allele variants and might give additional clues to understand species-wide adaptation patterns. GWA studies can identify loci even when they have relatively low power (Atwell et al., 2010) or when heritability is apparently low (Fournier-Level et al., 2011), and provide much higher mapping resolution with fewer individuals than QTL analysis (Balasubramanian et al., 2009). Successful examples are GWA studies of plant fitness in field experiments in which a myriad of causal loci with environmental-dependent effects lowered heritability estimates below 50%, usually closer to zero, but loci underlying relevant ecological traits were still found (Fournier-Level et al., 2011; Fournier-Level et al., 2013). In humans, GWA with polygenic and complex disease traits such as human height or schizophrenia keep providing new gene candidates and promote advances in quantitative genetic theory (Eichler et al., 2010; Lee et al., 2014; Visscher et al., 2010; Yang et al., 2010). The most significant candidate genes from our HopAM1-induced cell death GWA have functions consistent with affecting an ETI response. The most significantly associated SNPs were in the gene for cyclophilin 40, involved in gene epigenetic gene regulation, which is important to
most cellular functions and certainly relevant to mounting effective ETI responses (Navarro et al., 2006; Shivaprasad et al., 2012; Zhai et al., 2011). Other significant cell death SNPs were located between a gene for brassinosteroid synthesis (STEROL DELTA-7 REDUCTASE) and a gene for regulation of gibberellic acid signaling (SCL3). Both hormones contribute to the interplay of plant hormones that balances plant growth and defense (Bari and Jones, 2009; Lozano-Duran and Zipfel, 2015).

**Local cell death is associated with activation of ETI responses even though it is not linked to changes in pathogen growth.** The HopAM1-induced ETI response does not result in less growth of *Pto DC3000* in Bur-0 than in Col-0 (Figure 3). Thus, while there is a clear ETI transcriptional signature from HopAM1 in Bur-0 that is cell death related, this ETI signature is decoupled from growth restriction itself. There are several examples of NLR-mediated ETI in which cell death and pathogen growth are uncoupled (reviewed in (Coll et al., 2011)) such as the extreme resistance to potato virus X by the Rx NLR protein (Bendahmane et al., 1999). Some ecotypes of Arabidopsis are resistant to *P. syringae* encoding the type III effectors AvrRps4 or HopA1 without developing obvious local lesions (Gassmann, 2005; Kim et al., 2009). Uncoupling of effector-triggered cell death and pathogen growth restriction has also been observed for a number of Arabidopsis mutants such as cyclic nucleotide gated ion channel mutants *dnd1* (Clough et al., 2000) *him1* (Balague et al., 2003) and *dnd2* (Jurkowski et al., 2004) and *metacaspase 1* (Coll et al., 2010). The extent of cell death can also vary depending on physiological conditions, such as light (Bruggeman et al., 2015; Zeier et al., 2004) and temperature (Menna et al., 2015) without loss of bacterial growth restriction.

A further hint that HopAM1 targets NLR-dependent downstream pathways comes from results showing that *EDS1* plays a role both in HopAM1-induced cell death (Figure 1E) and meristem chlorosis (see Figure S10). In most examples *eds1* loss-of-function mutants cause reduction in pathogen- or stress-induced TIR-NLR mediated ETI and cell death responses (Bartsch et al., 2006; Bhattacharjee et al., 2011; Ochsenbein et al., 2006). Interestingly, HopAM1 induced a constant transcriptional upregulation in Col-0 and Bur-0 from 6 hpi of both *EDS1* (At3g48090) and *PAD4* (At3g52430) (see Table S16). However, if HopAM1-mediated cell death is the consequence of ETI activation of a TIR-NLR, a loss of function mutant like *eds1-2* is expected to be unable to mount the ETI response. Instead, homozygosity for the *eds1-2* allele enhanced cell death in our F2 plants (Figure 1E). Such a counterintuitive observation of a loss-of-function *eds1* mutation resulting in stronger NLR-mediated cell death has been observed before: the *eds1-2*
mutation has been shown to enhance the immune response of an autoactive mutant derived from the CC-NLR gene $ADR1$ (Roberts et al., 2013). In this regard, our finding of a cluster of CC-NLR genes in QTL5B, which we reduced to an interval of 100 kb containing only 30 genes, may suggest that one or more of these genes is causally related to HopAM1-induced cell death in Bur-0. Further fine-mapping will address this possibility.

**HopAM1 may reduce MTI responses through changes in chloroplast metabolism.** Reactive oxygen, specifically superoxide, is required for HopAM1-induced meristem chlorosis since the double mutant of $RBOHD$ and $RBOHF$ did not support it (see Figure S10). Reactive oxygen species (ROS) accumulation is an integral part of MTI and ETI responses and high levels of ROS damage chloroplasts (Kim et al., 2012; Wagner et al., 2004). Chloroplasts play a key role in defense by producing ROS (Shapiguzov et al., 2012) and balancing hormone levels (Robert-Seilaniantz et al., 2011), thus constituting a target of preference for pathogens. $Pto$ DC3000 effectors can suppress the expression of nuclear-encoded chloroplast genes in Arabidopsis and target the chloroplast (de Torres Zabala et al., 2015). Our expression analyses demonstrate that HopAM1 modifies the transcription of genes for chloroplast-localized proteins (see Figure S13). Our GWA study identified $TOC64III$, as significantly associated with variation in chlorosis phenotype (Figure 4, Table S14). $TOC64III$ encodes a chloroplast TOC transporter accessory protein (Aronsson et al., 2007; Sohrt and Soll, 2000) which modulates the translocation efficiency of proteins into the chloroplast (Sommer et al., 2013). TOC64 protein is not essential for chloroplast function; no detrimental phenotype is observed under standard growth conditions for Arabidopsis insertion mutants defective in $TOC64III$. (Aronsson et al., 2007). Likewise, TOC64 protein is not necessary for response to HopAM1 although allelic variation is associated with different chlorotic responses to HopAM1. We suggest that allelic variation in TOC64 could render chloroplasts more or less resistant to damage caused by ROS induced during MTI defense responses.

**Do meristem chlorosis and HopAM1 local cell death share a common mechanism?** HopAM1 induced local cell death and meristem chlorosis are not mutually exclusive. Both early cell death and chlorosis were observed in the Hov-4 and Wil-2 accessions (see Figure S7). Nevertheless, two of the three QTL loci related to meristem chlorosis from the Bur-0 X Col-0 RIL population overlap. Until the genes responsible for each phenotype have been identified, it is possible that independent genes and independent processes are responsible for chlorosis or cell death. However, the fact that $eds1-2$ mutants both enhance cell death and meristem chlorosis
suggests that EDS1 is a negative regulator of both HopAM1 responses. EDS1 regulates cell death by balancing phytohormone levels that lead to enhanced ROS production, likely involving modulation of TIR-NLR activation (Kim et al., 2012; Muhlenbock et al., 2008; Ochsenbein et al., 2006; Wiermer et al., 2005). Although our QTL and GWA analysis showed that HopAM1-induced chlorosis and cell death require distinct loci, EDS1 appears to play an integral role in a critical biochemical junction where these two responses converge.

CONCLUSION

Pathogen effectors interact with and alter components of protein complexes important to plant immunity (Macho and Zipfel, 2015; Mukhtar et al., 2011; Weßling et al., 2014). Therefore, individual pathogen effector proteins can serve as probes to identify interacting partners in immune complexes. To our knowledge, this is the first time that responses to a single pathogen effector have generated quantitative trait responses in the host. Previously defined ETI-related gene transcriptional alterations were also not associated with HopAM1-dependent bacterial growth restriction, which challenges the concept of what constitutes an ETI response, and more importantly, what is required to halt pathogen proliferation. Our study demonstrates that dissection of natural variation of host plants for quantitative responses to individual effectors, such as HopAM1, can reveal multiple genes involved in plant immune responses and help to dissect the quantitative features of what is often assumed to be a qualitative response.

ACKNOWLEDGEMENTS

We would thank Marc Nishimura for immeasurable assistance with materials and advice. We would also like to thank Farid El Kasmi, Eui-Hwan Chung, Freddy Monteiro, Scott Yourstone, and Sur Herrera-Paredes for general assistance and advice; Joseph Kieber, Jason Reed, Li Yang, Christine Camilleri, Salome Prat, and Úrsula Flores Pérez for providing various mutant lines used in this study. MI, MGC, C-M V, TD and SRG were supported by NSF grant IOS-1022286. Work at the Max Planck Institute was supported by ERC Advanced Grant IMMUNEMESIS. JLD is an Investigator of the Howard Hughes Medical Institute, supported by the HHMI and the Gordon and Betty Moore Foundation (GBMF3030) and was also supported by NSF grant IOS-1257373. PJPLT was supported by a fellowship from the Pew Latin American Fellows Program in the Biomedical Sciences.
LITERATURE CITED


effector genes from *Pseudomonas syringae*. Proceedings of the National Academy of Sciences of the United States of America 102, 2549-2554.


Effects of RNAi-induced CBF depletion and QTL localisation vary among accessions. Plant Science 180, 12-23.


gene for which mutation causes the "defense, no death" phenotype. Molecular plant-microbe interactions : MPMI 17, 511-520.


Truman, W., Bennett, M.H., Kubigsteltig, I., Turnbull, C., and Grant, M. (2007). Arabidopsis systemic immunity uses conserved defense signaling pathways and is mediated by


<table>
<thead>
<tr>
<th>Number of top SNPS</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>500</th>
<th>1000</th>
<th>10000</th>
</tr>
</thead>
<tbody>
<tr>
<td>QTL1</td>
<td>0.489</td>
<td>0.995</td>
<td>0.579</td>
<td>0.643</td>
<td>0.909</td>
<td>1</td>
</tr>
<tr>
<td>QTL2</td>
<td>0.818</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>QTL4</td>
<td>0.0906</td>
<td>0.0278</td>
<td>3.10E-13</td>
<td>2.88E-46</td>
<td>2.14E-66</td>
<td>1.52E-08</td>
</tr>
<tr>
<td>QTL5A</td>
<td>0.537</td>
<td>0.0434</td>
<td>1.55E-06</td>
<td>0.00942</td>
<td>0.0088</td>
<td>3.73E-07</td>
</tr>
<tr>
<td>QTL5B</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>7.04E-06</td>
<td>0.00021</td>
<td>0.00021</td>
</tr>
</tbody>
</table>

**Table 1.** *p*-values of enrichment tests of increasing numbers of most significant SNPs for each HopAM1-induced cell death QTL calculated using a Fisher’s exact test with a 2 X 2 test table design.

<table>
<thead>
<tr>
<th>Number of top SNPS</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>500</th>
<th>1000</th>
<th>10000</th>
</tr>
</thead>
<tbody>
<tr>
<td>QTL4C1</td>
<td>1</td>
<td>0.982</td>
<td>0.959</td>
<td>0.93</td>
<td>0.502</td>
<td>0.993</td>
</tr>
<tr>
<td>QTL4C2</td>
<td>0.114</td>
<td>0.0253</td>
<td>0.0145</td>
<td>0.0006</td>
<td>0.0002</td>
<td>0.0002</td>
</tr>
<tr>
<td>QTL5C</td>
<td>0.898</td>
<td>0.999</td>
<td>1</td>
<td>7.93E-01</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 2.** *p*-values of enrichment tests of increasing numbers of most significant SNPs for each HopAM1-induced chlorosis QTL calculated as in Table 1.
Figure 1 | HopAM1 induces variable, cell death symptoms of multigenic inheritance in *A. thaliana* accessions. (A) HopAM1-induced cell death in Bur-0, Col-0 and F1 progeny at 48 hpi.
after hand inoculation with *Pto* DC3000D28E(*hopAM1*). OD<sub>600</sub> = 0.1 (~5x10<sup>7</sup> cfu/ml). Bur-0 exhibits one of the strongest onsets of symptoms as early as 23 hpi, while Col-0 exhibits only chlorosis starting around 96 hpi. F<sub>1</sub> progeny become symptomatic around 48 hpi. (B) Cell death shown with Trypan blue staining on the same leaves as above (48 hpi). (C) Sigmoid distribution of cell death symptoms representative of quantitative traits. The quantification of cell death at specific time points post-inoculation is shown. Curve shows the percentage of plants in a Bur-0 x Col-0 RIL population that exhibit cell death symptoms at a given time point. “Black circle” = Bur-0; “open circle” = Col-0. (D) Composite Interval Mapping (CIM) on 342 re-annotated RILs from a Col-0 x Bur-0 collection. The x-axis displays chromosomes 1 through 5 with map distances in cM. Peak LOD scores are shown by black triangles along with their values. Effects on phenotype variation are shown as percentages for QTL4 and QTL5A. The global permutation level of significance was set on LOD 3.2. (E) Distribution of HopAM1-induced cell death scores among different F<sub>2</sub> progeny between Bur-0 and mutant lines. Y-axis shows number of plants selected from each population with each phenotype score for HopAM1-induced cell death (“5” = Bur-0, “0” = Col-0). Statistical significance between distributions was based on two-way ANOVA tests for each pair (statistical significance: ‘****’=P≤0.001; ‘***’=P≤0.01; ‘**’=P≤0.05).
Figure 2 | The genetic architecture of HopAM1 cell death response defined by GWA depends on several loci with strong to moderate effects. HopAM1-induced cell death response was scored in a collection of 64 Arabidopsis accessions. EMMAX was used for genome-wide association mapping based on whole genome sequence data of over 4 million SNPs. The Manhattan plot shows the association of each SNP and its $p$-value across the five Arabidopsis chromosomes (1 through 5 from left to right, separated by vertical lines). The dotted line designates the significance threshold for Bonferroni correction ($p \leq 1.2 \times 10^{-8}$). All SNPs over the FDR threshold are highlighted with a circle. Grey outline reprises the QTL regions identified in Figure 1D in the Bur-0 x Col-0 background (cM converted to Mb only for each genetic marker’s coordinates on the genome and shows LOD score at that specific location; not identical, but very similar to peak LOD scores shown in previous QTL analysis in Figure 1D). Right y-axis: LOD score; yellow-dotted line: significance threshold for CIM.
Figure 3 | HopAM1 causes reduced growth of *P. syringae* in Col-0 and Bur-0 accessions, independent of cell death symptoms. Bacterial growth in Col-0 and Bur-0 accessions. The experiment is representative of five independent replicates; two times the Standard Error between the means is noted by error bars. A weighted ANOVA test was applied to the difference in growth of the strains carrying *hopAM1* (*Pto* DC3000 and *Pto* JB206+AM1) compared with the growth of the *hopAM1* deleted strain (*Pto* JB206). Statistical significance is indicated by letters based on the weighted ANOVA test ($p < 0.1$).
Figure 4 | HopAM1 induces rosette chlorosis affecting chloroplast development and cell morphology controlled by three QTLs in Col-0 x Bur-0. A) Meristem chlorosis response following bacterial vacuum infiltration with Pto DC3000D28E(hopAM1). Col-0 exhibits strong chlorosis symptoms, while Bur-0 does not. F1 progeny exhibit mild symptoms. Chlorosis appears in newly emerging leaf and meristem in Col-0 at 10 dpi following vacuum infiltration. Chlorosis can be observed up to 21 dpi. B) Confocal laser scanning microscopy of Arabidopsis accessions Col-0 and Bur-0 leaf tissue, harvested 14 dpi following vacuum infiltration with Pto DC3000D28E(hopAM1). Chlorophyll autofluorescence (enlarged and brightened sections with white dotted outline shown on the top left corner of respective pane) (red channel), plant cell shape (black dotted outline of cells) (green channel), and merged channels are shown for each accession. Scale bar = 10 μm. Upper and lower panels taken at equal light intensity. C) CIM on 342 reannotated RILs from a Col-0 x Bur-0 collection. The x-axis displays chromosomes 1 through 5 with map distances in cM. Peak LOD scores are shown by black triangles along with their values. Effects on phenotype variation are shown as percentages for all three QTLs. The global permutation level of significance was set on LOD 3.2.
Figure 5 | The genetic architecture of the HopAM1 chlorosis response defined by GWA is associated with a single locus on chromosome 3. HopAM1-induced chlorosis was scored in a collection of 64 Arabidopsis accessions as for Figure 2. Nearly all top 10 hits were inside genes. Grey outline reprises the QTL regions identified in Figure 4C in the Bur-0 x Col-0 background (cM converted to Mb only for each genetic marker’s coordinates on the genome and shows LOD score at that specific location; not identical, but very similar to peak LOD scores shown in previous QTL analysis in Figure 4C). Right y-axis: LOD score; yellow-dotted line: significance threshold for CIM.
Figure 6 | HopAM1 induces intense transcriptional reprogramming for thousands of genes in Col-0 and Bur-0, results in initial suppression of MTI in both ecotypes and later induction
of ETI in Bur-0 only. (A) Number of genes (y-axis) differentially expressed as a result of HopAM1 delivery in Col-0 and Bur-0 every two hours (x-axis) in the first 12 hours post infiltration. Red bar – down-regulated; blue bar – up-regulated. (B) (upper lane) Average expression profile of flg22-induced genes within the same time period in Col-0 and Bur-0; (lower lane) average expression profile of genes up-regulated by ETI induced upon recognition of the avrRps4 avirulence factor by the NLR protein Rps4 in Col-0. Gene expression is shown as the median z-scored transformed RPKM values of the 1418 and 718 marker genes for MTI and ETI respectively. (C) Overlap between genes differentially expressed by Pto DC3000D28E(hopAM1) infection and those specifically misregulated by Pto DC3000 effectors. A total of 5610 genes that were differentially expressed in Col-0 in at least one time-point of our experiment were included in the analysis. A set of 517 genes showing ambiguous regulation (i.e. both up- and down-regulation at different time points) was discarded. (D) Venn diagrams showing number of genes up-regulated and down-regulated at 12 hpi for both Bur-0 and Col-0. Gene Ontologies for the top five sets are shown for each group.

SUPPLEMENTAL TABLES

Table S1 | List of bacterial strains and plasmid used in this study.
Table S2 | ANOVA tests and adjusted means of phenotypic scores for HopAM1-induced cell death response per RIL.
Table S3 | HopAM1-dependent cell death response is variable in Arabidopsis thaliana germplasm.
Table S4 | Bur-0 x Col-0 F₂ segregation ratios for HopAM1-induced cell death.
Table S5 | Reannotated Bur-0 x Col-0 RIL population.
Table S6 | Composite Interval Mapping result data for HopAM1-induced cell death on a Bur-0 x Col-0 RIL population.
Table S7 | edgeR results (infected vs mock in Bur-0 and Col-0) for the differential expression of genes in the cell death QTL intervals QTL4, QTL5A and QTL5B.
Table S8 | ANOVA tables for pairwise interactions between QTLs for HopAM1-induced cell death.
Table S9 | Lists of top SNP hits for HopAM1-induced cell death.
Table S10 | Segregation ratios for HopAM1-induced cell death in three different mapping populations.
Table S11 | HopAM1-induced rosette chlorosis response in Arabidopsis thaliana germplasm.
Table S12 | ANOVA tests for HopAM1-induced chlorosis response per RIL.
Table S13 | Composite Interval Mapping result data for HopAM1-induced chlorosis on a Bur-0 x Col-0 RIL population

Table S14 | List of top SNP hits for HopAM1-induced chlorosis.

Table S15 | List of mutant lines screened for HopAM1-induced chlorosis.

Table S16 | Complete edgeR results for the differential expression analysis (infected vs mock) in Bur-0 and Col-0 for all time points.

Table S17 | List of differentially regulated gene sets identified by ontology enrichment analysis at 4 hpi following inoculation with Pto DC3000D28E(hopAM1).

Table S18 | List of differentially regulated gene sets identified by ontology enrichment analysis at 6 hpi following inoculation with Pto DC3000D28E(hopAM1).

Table S19 | List of differentially regulated gene sets identified by ontology enrichment analysis at 8 hpi following inoculation with Pto DC3000D28E(hopAM1).

Table S20 | List of differentially regulated gene sets identified by ontology enrichment analysis at 10 hpi following inoculation with Pto DC3000D28E(hopAM1).

Table S21 | List of differentially regulated gene sets identified by ontology enrichment analysis at 12 hpi following inoculation with Pto DC3000D28E(hopAM1).

Table S22 | List of genes up-regulated and down-regulated by either Pto DC3000 effectors or HopAM1 alone in Col-0 at 12 hpi.