Multiple Candidate Effectors from the Oomycete Pathogen *Hyaloperonospora arabidopsidis* Suppress Host Plant Immunity

Georgina Fabro1,*, Jens Steinbrenner2, Mary Coates2, Naveed Ishaque1, Laura Baxter2,3, David J. Studholme1,4, Evelyn Körner1,5, Rebecca L. Allen2, Sophie J. M. Piquerez1, Alejandra Rougon-Cardoso1,6, David Greenshields1,7, Rita Lei1, Jorge L. Badel1, Marie-Cecile Caillaud1, Kee-Hoon Sohn1, Guido Van den Ackerveken6, Jane E. Parker9, Jim Beynon2, Jonathan D. G. Jones1*

1 The Sainsbury Laboratory, John Innes Centre, Norwich, United Kingdom, 2 School of Life Sciences, Warwick University, Wellesbourne, United Kingdom, 3 Warwick Systems Biology, Warwick University, Coventry, United Kingdom, 4 Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter, United Kingdom, 5 John Innes Centre, Norwich, United Kingdom, 6 Laboratorio Nacional de Genomica para la Biodiversidad, CINVESTAV Irapuato, Mexico, 7 National Research Council Canada, Plant Biotechnology Institute, Saskatoon, Canada, 8 Plant-Microbe Interactions, Department of Biology, Utrecht University, Utrecht, and Center for Biosystems Genomics, Wageningen, The Netherlands, 9 Department of Plant-Microbe Interactions, Max-Planck Institute for Plant Breeding Research, Cologne, Germany

Abstract

Oomycete pathogens cause diverse plant diseases. To successfully colonize their hosts, they deliver a suite of effector proteins that can attenuate plant defenses. In the oomycete downy mildews, effectors carry a signal peptide and an RxLR motif. *Hyaloperonospora arabidopsidis* (Hpa) causes downy mildew on the model plant *Arabidopsis thaliana* (*Arabidopsis*). We investigated if candidate effectors predicted in the genome sequence of Hpa isolate Emoy2 (HaRxLs) were able to manipulate host defenses in different *Arabidopsis* accessions. We developed a rapid and sensitive screening method to test HaRxLs by delivering them via the bacterial type-three secretion system (TTSS) of *Pseudomonas syringae* pv *tomato* DC3000-LUX (Pst-LUX) and assessing changes in *Pst-LUX* growth in planta on 12 Arabidopsis accessions. The majority (~70%) of the 64 candidates tested positively contributed to *Pst-LUX* growth on more than one accession indicating that Hpa virulence likely involves multiple effectors with weak accession-specific effects. Further screening with a Pst mutant (ΔCEL) showed that HaRxLs that allow enhanced Pst-LUX growth usually suppress callose deposition, a hallmark of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). We found that HaRxLs are rarely strong avirulence determinants. Although some decreased Pst-LUX growth in particular accessions, none activated macroscopic cell death. Fewer HaRxLs conferred enhanced Pst growth on turnip, a non-host for Hpa, while several reduced it, consistent with the idea that turnip’s non-host resistance against Hpa could involve a combination of recognized HaRxLs and ineffective HaRxLs. We verified our results by constitutively expressing in *Arabidopsis* a sub-set of HaRxLs. Several transgenic lines showed increased susceptibility to Hpa and attenuation of *Arabidopsis* PTI responses, confirming the HaRxLs’ role in Hpa virulence. This study shows TTSS screening system provides a useful tool to test whether candidate effectors from eukaryotic pathogens can suppress/trigger plant defense mechanisms and to rank their effectiveness prior to subsequent mechanistic investigation.

Citation: Fabro G, Steinbrenner J, Coates M, Ishaque N, Baxter L, et al. (2011) Multiple Candidate Effectors from the Oomycete Pathogen *Hyaloperonospora arabidopsidis* Suppress Host Plant Immunity. PLoS Pathog 7(11): e1002348. doi:10.1371/journal.ppat.1002348

Editor: Frederick M. Ausubel, Massachusetts General Hospital, Harvard Medical School, United States of America

Received February 17, 2011; Accepted September 17, 2011; Published November 3, 2011

Copyright: © 2011 Fabro et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the following grants: The ERA-PG Effectoromics project funded by the British Biotechnological and Biological Sciences Research Council (BBSRC), the German Research Foundation (Deutsche Forschungsgemeinschaft, JEP/DFG) and the Germany-Netherlands Genomics Initiative/Netherlands Organization for Scientific Research (NIGI/NOW) to GF, JS, MC, RLA, JEP, GV, JB and JDGJ. The HFSP grant RGP0057/20067-C to DG, JLB and JDGJ; The BBSRC grants BB/E024815/1, BB/G015066/1, BB/F005806/1 supported JB. The funders had no role in experimental design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: jonathan.jones@sainsbury-laboratory.ac.uk
* Current address: CIQUIBIC-CONICET, Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina

Introduction

Plants face constant attacks by a wide array of microorganisms including bacteria, fungi and oomycetes. Obligate biotrophic pathogens are particularly interesting because they can effectively evade or suppress host recognition, thus thwarting host defenses and enabling pathogen growth and reproduction [1].

In natural environments, plant disease is rare because plants activate a multilayered defense to most potential pathogens [2]. Relatively conserved molecules, called pathogen (or microbe)-associated molecular patterns (PAMPs), are recognized by the plants via pattern recognition receptor proteins (PRRs) [3,4]. This interaction results in pattern-triggered immunity (PTI). Successful pathogens target effector proteins to the host cell cytoplasm to...
Author Summary

Hyaloperonospora arabidopsidis (Hpa) is an obligate biotroph whose population coevolves with its host, Arabidopsis thaliana. The Hpa isolate Emoy2 genome has been sequenced, allowing the discovery of dozens of secreted candidate effectors. We set out to assign functions to these candidate effectors, investigating if they suppress host defenses. We analyzed a sub-set of Hpa candidate effectors (HaRxLs) that carry the RxLR motif, using a bacterial system for in planta delivery. To our surprise, we found that most of the HaRxLs enhanced plant susceptibility on at least some accessions, while few decreased it. These phenotypes were mostly confirmed on Arabidopsis transgenic lines stably expressing HaRxLs that became more susceptible to compatible Hpa isolates. Furthermore, effectors that conferred enhanced virulence generally suppressed callose deposition, a hallmark of plant defense. This indicates that the “effectorome” of Hpa comprises multiple distinct effectors that can attenuate Arabidopsis immunity. We found that many HaRxLs did not confer enhanced virulence on all host accessions, and also that only ~50% of the effectors that conferred enhanced Pst growth on Arabidopsis, were able to do so on turnip, a non-host for Hpa. Our data reveal interesting HaRxLs for detailed mechanistic investigation in future experiments.

RxLR (for arginine (Arg), any amino acid, leucine (Leu), Arg) and EER (for glutamine (Glu), Glu, Arg) [29]. Functional analysis of Avr3a demonstrated that it accumulates in and is secreted from P. syringae bacteria before its translocation into the host cell and its RxLR and EER motifs are required for delivery [29]. Avr1b requires its RxLR and EER motifs for uptake independently of the presence of the pathogen [30]. Binding of the RxLR EER and RxLR-like motifs of several fungal and oomycete proteins to phosphatidylinositol 3-phosphate (PI-3-P) has been proposed to mediate their entry into host cells [31]. In summary, the oomycete and fungal RxLR-like motifs, and the recently described LXLFLAK motif in Crinkler proteins [32] are conserved sequences involved in effector translocation into the host [33,34]. For Hpa, no apoplastic effectors have been reported and the few effector candidates of Hpa that have LXLFLAK motifs, also carry overlapping RxLR motifs. For that reason we focused on our “effectormics” studies on predicted HaRxL-type effector candidates.

Unlike Phytophthora spp., Hpa is not transformable [35,36]. Previous reports indicate that the bacterial type-three secretion system (TTSS) can be used to study how non-bacterial effectors can manipulate host cell functions [37,38]. The phytopathogenic bacterium Pseudomonas syringae possesses a TTSS that translocates effectors to the host cell cytoplasm [39] via signals located on their N-termini [40]. P. syringae pv tomato DC3000 (Pst DC3000) grows on multiple Arabidopsis accessions [41]. Its growth in planta increases in PTI-compromised mutants like f52, eork1, sdp2, and eot3 [42,43,44,45], and decreases due to ETI when it delivers bacterial AVRs in plants carrying the cognate R proteins [46,47,48,49]. The Hpa effectors ATR1 and ATR13 can be delivered from P. syringae using fusions to the N-terminus of the bacterial effectors AvrRps4 and AvrRpm1 [37,50]. This technique has enabled the study of Hpa cytoplasmic effectors by monitoring growth in planta of P. syringae delivering different alleles of ATR1 and ATR13 into Arabidopsis accessions that carry (or not) the cognate R proteins RPP1 and RPP13. Although enhanced pathogen growth due to interference with host defence can be detected, it is likely that effectors whose prime role is to promote the elaboration of haustoria would be missed in this kind of assay.

By genomic and expression analysis of the Hpa isolate Emoy2 we defined 140 HaRxLs that carry a signal peptide and RxLR motif, and ranked them taking into account allelic diversity and expression level. Our aim was to survey a broad set of candidate HaRxLs to investigate if they might play a role in suppressing PTI and/or ETI. For this purpose the Effector Detector Vector (EDV) system [37], with a luciferase-expressing Pst DC3000 strain (Pst-LUX), was used for an initial assessment of whether 64 of these HaRxLs could enhance Pst-LUX growth on at least some Arabidopsis accessions. The majority of HaRxLs were found to increase host susceptibility on multiple accessions revealing a correlation with increased callose suppression. Interestingly, many HaRxLs were not effective on all accessions, implying that host effector targets might evolve to be refractory to effector action. However, although a few HaRxLs reduced bacterial growth on certain accessions, avirulence was rare. Selected HaRxLs were studied in more detail in transgenic plants, confirming their disease-promoting activities. On turnip, a non-host plant for Hpa, fewer HaRxLs enhanced Pst-LUX growth, and more reduced it, providing interesting clues into mechanisms that underpin non-host resistance. In addition to providing novel insights into how parasites impose host susceptibility, these data reveal several high priority HaRxLs for future mechanistic investigations.
Results

Identification of HaRxLs in the Hpa Emoy2 genome

To establish an inventory of the RxLR effector secretome of Hpa, we scanned the draft genome of Emoy2 (http://vmd.ybi.vt.edu) for all possible open reading frames (ORFs) encoding putative proteins longer than 100 amino acids. We then searched these sequences for the presence of signal peptides and from those we extracted gene models carrying RxLR-like motifs (RxLR/Q; RxL) with the sequence and positional constrains defined in Figure 1 (see Materials and Methods). Different sets of HaRxLs were identified depending on the version of the genome used (versions 3.0, 6.0 and 8.3.2). We merged different lists to define a set of 191 HaRxL genes that included the known effector genes ATR1 [26] and ATR13 [27]. Most of the encoded proteins were smaller than 300 amino acids. Approximately 37% had an acidic motif (EE/EER) after the RxLR and ~6% had a predicted nuclear localization signal (data not shown). This collection included HaRxLs identified by others using similar search algorithms [9,28].

![Figure 1. Bioinformatic pipeline used for the identification of Hyaloperonospora arabidopsidis (Hpa) HaRxLs. (*) The genome browser is maintained at the Sainsbury Laboratory (gbrowse2.tsl.ac.uk/cgi-bin/gb2/gbrowse/hpa_emoy2_publication). doi:10.1371/journal.ppat.1002348.g001](image)

We next tested which HaRxLs were expressed during the oomycete life cycle and whether these were correctly predicted. A set of Sanger ESTs from germinating Hpa spores [9] and two different cDNA libraries from infected Arabidopsis plants at 3 and 7 days post inoculation (dpi) were used (see Materials and Methods). We verified expression of 140 of the 191 predicted HaRxLs. Ninety of them were expressed from the asexual spore stage, perhaps ensuring their early availability upon initiation of infection, and remained expressed in planta until 7 dpi. The remaining 51 HaRxLs were expressed either at 3 dpi, 7 dpi or both. Data in column M of Table S1 illustrate the expression pattern of the sub-sets of effectors tested in this work. Of those for which we could confirm expression, the majority (90%) of the HaRxLs was correctly predicted and none had introns (data not shown). Highly expressed and accurately predicted HaRxLs were prioritized for cloning.

We then looked for evidence of polymorphism in effector candidates between seven Hpa isolates (Cali2, Emco5, Emoy2, Hind2, Mako9, Noco2, Waco9). Single Nucleotide Polymorphisms (SNPs) were detected either on PCR products (Baxter et al., unpublished data) or partial assemblies of Illumina short reads (N. Ishaque, unpublished data). Our results indicated that 12% of the HaRxLs were not polymorphic, 56% had between 1 and 10 SNPs, and 31% showed more than 10 and up to 30 SNPs. We classified them as not polymorphic (0 SNPs), low (≥1 SNPs ≤5), medium (≥6 SNPs ≤15) and high polymorphic (>16 SNPs) candidates (Column L, Table S1). For some HaRxLs it was difficult to distinguish heterozygosity from paralogous family members. In consequence, the real level of polymorphism might be underestimated. Recognized Hpa effectors like ATR1 and ATR13 show high levels of polymorphism [26,27] while we hypothesize that non-recognized virulent effectors, adapted to interact with a specific host target, might have low sequence variability. Hence, candidates belonging to all four above described categories were used in this study.

Construction and verification of an HaRxL library for functional screening using the EDV system

The Effector Detector Vector (EDV) delivers individual effector candidates to host plant cells using the TTSS of Pseudomonas syringae [37]. Seventy-four HaRxLs were cloned into pENTR/pEDV vectors (pEDV-HaRxLs) (Table S1). We obtained 71 fusion proteins (AvrRPS4N1–136–HA tag-HaRxL). Two candidate effectors could not be cloned correctly in pEDV, and another one was truncated and further used as a negative control (NC2, Table S1). Correct in-frame constructs were introduced by conjugation into Pst DC3000 and derivative strains, particularly one expressing the luciferase (luxCDABE) operon of Photorhabdus luminescens (Pst-LUX) [41] (see Materials and Methods for full details). No differences in bacterial growth (either in liquid or solid media) were observed in Pst-LUX clones carrying any of the 71 fusion proteins (AvrRPS4N1–136–HA tag-HaRxL) fusion proteins regarding the growth of Pst-LUX harbouring AvrRPS4N1–136–HA tag-GFP/AvrRPS4AAAA (data not shown).

We performed in vitro secretion assays to check that the 71 fusion proteins obtained were made in bacteria and secreted to the medium in TTSS-inducing conditions. Secreted protein could be detected as illustrated in Figure S1A. Proteins of the expected size were produced by Pseudomonas for 64 of the pEDV/6-HaRxLs closed. No proteins, or protein bands of incorrect size, were observed for the remaining 7 HaRxLs, which were not used in further assays (Table S1, column H). Thus, our library comprised 64 Emoy2 pEDV-HaRxLs.
In *Pst* DC3000, the TTSS, encoded by the *hrp-hru* (hypersensitive response [HR] and *pathogenicity-hru* conserved) gene cluster, is required for elicitation of HR in non-host plants like tobacco and for full pathogenicity in host plants like tomato [51,52]. To verify that the HaRxLs proteins did not alter *Pseudomonas* growth in planta by blocking the TTSS, we performed HR cell death tests in tobacco (*Nicotiana tabacum* cv. Petit Havana) and disease assays in tomato (*Solanum lycopersicum* cv. Moneymaker). Of the 64 pEDV-HaRxLs delivered by *Pst*-LUX in tobacco, only 1 attenuated HR in tobacco while four reduced disease symptoms in tomato. No candidate impaired both activities or completely abolished HR or disease (Table S2 columns C, D and representative examples in Figure S1 B, C). We infer from these results that none of the pEDV-HaRxLs constructs blocked *Pst*-LUX TTSS translocation of effectors.

The *Pst*-LUX strain was designed for screening multiple *Arabidopsis* mutants/accessions that vary in resistance to *Pst* DC3000 [41]. To evaluate the sensitivity of this system, we carefully validated the correlation between the level of bacterial bioluminescence and bacterial growth in planta using ATR1 and ATR13 (Figure S2). ATR1 Emoy2/Col-0 and ATR13 Emoy2/Emco5 conferred enhanced growth to *Pst*-LUX in the susceptible genotype Col-0, as did ATR13 Emoy2 on Nd-0 plants. This phenotype could be detected as an increase in bioluminescence that correlated with higher numbers of bacteria (colony forming units (cfu)/cm²) (Figure S2). We were also able to detect decreased growth conferred to *Pst*-LUX by ATR13 Emcy2 or ATR1 Emoy2 in Nd-0 plants (Figure S2).

Several HaRxLs delivered via *Pst*-LUX increase bacterial growth on multiple *Arabidopsis* accessions

Using spray inoculation, a protocol was developed to assay bacterial growth in a sub-set of the 96 accessions described by Nordborg et al., [53], selected to maximize variability (Bay-0, Br-0, Col-0, Ksk-1, Ler-0, Nd-0, Oy-0, Shakdara, Ts-1, Tsu-0, Wei-0, Ws-0) (Figure 2). Plants were spray-inoculated with *Pst*-LUX carrying EDV constructs that delivered either a control protein or an HaRxL via TTSS. At 3 dpi, the bioluminescence (photons/fresh weight) emitted by the bacteria was quantified as a measure of bacterial growth (Figure 2, see details in Materials and Methods). The growth of *Pst*-LUX in *planta* carrying each HaRxL was compared to a control (see below) and expressed as a ratio. This assay allowed us to establish whether a given HaRxL was able to increase or decrease *Pst*-LUX growth, manifested as quantitative differences in bioluminescence, on multiple host accessions in parallel.

Sixty-four pEDV-HaRxLs and 3 control proteins (EDV5:HA-AvrRPS4, 136, EDV6:HA-YFP, EDV6:HA-AvrRps4 AAAA) were delivered via *Pst*-LUX to 12 different *Arabidopsis* accessions. At three days post spray-inoculation, the photons/second/g fresh weight (CPS/Fw) were scored for five plants of each accession and averages, standard deviations and errors calculated. The ratio of increase or decrease in the CPS/Fw emitted by a *Pst*-LUX strain delivering a given pEDV-HaRxL, versus control (in the corresponding EDV5 or EDV6 backbone) was determined, as well as its statistical significance (one tailed T-test, unequal variances assumed) (Figure 2, Table S2). Experiments were repeated at least three times. Given the variability between experiments, the final outcome of each pEDV-HaRxL effect per accession was assessed across experiments and categorized according to the following criteria: i) a reproducible ratio higher or lower than one, showing the same trend on at least two experiments with a minimum statistical significance of p<0.05 on each of them, was considered as either “Enhanced” or “Decreased” growth and labeled with (+) or (−) respectively; ii) a non-reproducible ratio showing opposite statistically significant trends or the same trend but not statistically significant was considered as “No Change” and scored as (=) (see Table S2, columns R, S, T). A graphical synopsis of the screening outcome per effector across the 12 host accessions is presented in Figure 3, with the most effective pEDV-HaRxLs (HaRxL62) at the top, conferring enhanced *Pst* growth on all 12 accessions.

To distinguish the effector-driven Enhanced/Decreased *Pst*-LUX growth patterns from the random phenotypes that can be obtained by delivering any given protein into the plant via the EDV system, we included four internal controls (negative controls, NCs). These constructs were truncated versions of HaRxLs (NC2 and NC3), non-secreted proteins with an RxLR-like motif (NC1) or a small bacterial protein with similarity to a xylosidase (NC4, genebank: AP12301.1). NC1 is a part of a larger *Hpa* ORF encoding a putative transposable. NC2 is an early C-terminal truncated version of HaRxL143 (before the RxLR motif), while NC3 is a frame-shift version of HaRxL77 (Table S1). Functional ATR13 Emoy2 and ATR13 Emcy2 alleles were also included. The pattern shown by these internal controls allowed us to establish threshold levels to assess whether a given HaRxL had a credible effect on *Pst*-LUX growth (Figure 3, Table S2). NC3 and NC4 did not impact *Pst*-LUX bioluminescence. We attributed the residual effect of NC1 and NC2 on *Pst*-LUX growth to the random variability of the system (Figure 3) and therefore we set the thresholds as follows: for an effector to be considered as an “enhancer” of *Pst*-LUX growth it had to show an increased significant change in bioluminescence in four or more accessions. Conversely, as the control ATR13 Emcy2 was recognized in only 1 accession out of the 12 tested, any effector that decreased the growth of *Pst*-LUX on one or more accessions was classified as capable of being recognized (Figure 3).

Our results indicate that 43 pEDV-HaRxLs enhanced *Pst*-LUX growth in planta, while 28 decreased *Pst*-LUX activity (Figure 3). The majority (72%) of the pEDV-HaRxLs that increased growth in ≥4 accessions did not decrease it in any accession (Green/black bars, Figure 3). This suggests most pEDV-HaRxLs can suppress plant defenses and avoid recognition by the host, although their effectiveness varies between accessions. We found only one pEDV-HaRxL capable of enhancing *Pst*-LUX growth in all accessions tested (HaRxL62); we infer that its plant target(s) might have little natural variation between accessions and that no R gene(s) recognize it in these accessions. In addition, pEDV-HaRxL9, 17, 21, 45b, 53, 73 and HaRxL1464 were able to increase *Pst*-LUX growth in 8 or more accessions and had no negative effect on the remaining ones. The pEDV-HaRxLs that decreased *Pst*-LUX growth did so mainly in ≤3 accessions (68%). This pattern of accession-specific *Pst*-LUX growth reduction was observed for both alleles of ATR13, and also for pEDV-HaRxL4, 44, 45, 57, 106, 108, HaRxL445, 483 and 495. Only nine pEDV-HaRxLs conferred decreased bacterial growth in >3 and <8 accessions. Given the lack of accession-specificity of their phenotype we speculate these HaRxLs might affect the virulence activity of *Pst* effectors (Magenta areas, Figure 3).

Verification via growth curves of HaRxL-induced changes in *Pst*-LUX growth highlights a subset of effective HaRxLs

We extended the concordance analysis developed with ATR1 Emoy2/Col-0 and ATR13 Emoy2/Emco5 (Figure S2) to 13 other pEDV-HaRxLs delivered from *Pst*-LUX by conducting growth curve assays using seven *Arabidopsis* accessions (Table S3). For this test we selected some HaRxLs representative of the different patterns we observed in Figure 3. Briefly, we tested HaRxL62...
because it enhanced Pst-LUX luminescence in all accessions; HaRxL14, HaRxL21, HaRxL160, HaRxL164, and HaRxL492 because they enhanced Pst-LUX luminescence in ≥6, but did not decreased it in any accession. HaRxL14, 45, 57 and 106 also increased bacterial luminescence in ≥6 accessions but decreased it in 1–3 accessions. HaRxL70 was selected among the group of “non-effective” effectors, and HaRxL79 because it reduced Pst-LUX bioluminescence in ≥3 accessions.

For 32 of 35 combinations (pEDV-HaRXL×accession) we confirmed the correlation between enhanced bioluminescence and increased bacterial growth. These data verified that Pst-LUX bioluminescence reveals the effect of HaRXLs on Pst-LUX growth. We also observed that some HaRXLs have a substantial positive effect on bacterial growth on multiple accessions, and can increase Pst-LUX growth ~10-fold (Table S3 and Figure S3). In particular, we confirmed that HaRxL62 and HaRxL14 render multiple host accessions more susceptible to bacterial infection (Figure S3). Accession-specific effects were verified for HaRxL164 and HaRxL21 while putative recognition events, leading to a decrease in bacterial growth, were verified for HaRxL44 in Ler-0, HaRxL57 in Ksk-1 (Figure S3, Table S3), and HaRxL106 in Col-0 (Table S3). No effect was observed for HaRxL70 in Col-0 while the decrease in bacterial growth caused by HaRxL79 was only observed when plants were spray inoculated (Table S3). These data reinforced the usefulness of the EDV Pst-LUX assay for selecting candidates for further work.
and confirmed several candidates as a high priority for further investigation.

Host genotypes and levels of HaRxL polymorphism are not correlated with effector-induced changes in Pst-LUX growth

To evaluate if host genotypes influenced the pattern of Arabidopsis responsiveness to the set of HaRxLs tested, the spectrum of effective HaRxLs per accession was analyzed. We found that an average of 42% of the pEDV-HaRxLs enhanced Pst-LUX growth on any given accession, while only ~11% reduced Pst-LUX growth. Many combinations (46%) did not cause any change in Pst-LUX growth (Figure S4). Enhancement or decrease of susceptibility was not restricted to a particular set of accessions, and did not correlate with those accessions showing resistance or susceptibility to the infection by the Hpa isolate Emoy2 (Figure S4). The only deviations from this pattern were Nd-0, in which most of the pEDV-HaRxLs (73%) increased Pst growth and only ATR13Emco5 was able to decrease it, and Br-0 in which fewer pEDV-HaRxLs in total were effective (31% compared to the average of 42% for all other accessions) (Figure S4). These results are consistent with the idea that some effector targets are widely conserved while others vary between accessions.

The level of polymorphism of HaRxLs did not correlate with the capacity to enhance Pst-LUX growth. Among the 64 candidates tested, 11 were highly polymorphic, 21 had a medium level and 32 showed low polymorphism. HaRxLs categorized in these three groups showed ability to increase bacterial luminescence in an average of 6.62 ± 2.54, 6.2 ± 3.15 or 5.2 ± 2.66 accessions, respectively. For example, HaRxL464 and HaRxL57 showing low or no polymorphism, and the highly polymorphic HaRxL106 and HaRxL21 were all capable of increasing Pst-LUX growth in 8 or more host accessions.

HaRxLs did not trigger hypersensitive recognition in any Arabidopsis accession after EDV delivery

Isolate Emoy2 is recognized by certain Arabidopsis accessions, indicating effector recognition by R protein(s). In order to identify avirulent HaRxLs in the library, we analysed in detail Pst-LUX growth assays in each of the 12 Arabidopsis accessions (Figure 3, Figure S4). Possible recognition of pEDV-HaRxL strains in our assays was indicated by the decrease in Pst-LUX growth, usually in an accession-specific manner (Figure 3, Table S2). Potentially novel ATR proteins may have been detected in interactions with accessions Col-0, Ler-0, Br-0 and Ksk-1 (Figure S4).

ETI is strongly correlated with HR-like cell death [54,55] although HR is not always required for resistance [49,56]. We tested possible recognitions using a weakly virulent Pst DC3000 ΔACEL (Pst-ΔACEL) strain and a modified P fluorescens carrying a functional TTSS (Pf0-1) [57] to deliver potentially recognized HaRxLs to the corresponding “resistant” accessions. We performed localized leaf infiltrations using high doses of bacteria and looked for macroscopic (leaf collapse) and microscopic (dead cells

![Figure 3. Hpa HaRxLs can promote or decrease Pst-LUX growth in different Arabidopsis accessions.](https://www.plospathogens.org/article/10.1371/journal.ppat.1002348.g003)
stained with trypan blue) indicators of HR-like cell death 24 h post infiltration.

Surprisingly, no HaRxLs besides the positive control (ATR13Emco5 in Nd-0) provoked clear signs of macroscopic HR. We then stained infiltrated leaves with trypan blue and examined for microscopic lesions. All micro-HR lesions were much smaller and weaker than those triggered by bacterial effectors like AavrPmr1 or AavrRpo2 (data not shown) or by the Hpa effector ATR13Emco5 in Nd-0 (Table S4). In 78 pEDV-HaRxL/accession combinations, we saw micro-HR-like cell death in only 7 interactions, comprising just 4 candidate effectors (HaRxLA, 18, 70 and 80; in bold in Table S4). Similar results were obtained with both P0-1 and Pst-CEL strains, except for HaRxL106 where no HR was detected in Col-0 and Ksk-1 when delivered through P0-1 (Table S4). Nevertheless, the decrease in bacterial growth observed for Pst-LUX delivering each of these candidate effectors in the corresponding accessions was confirmed by reduction in disease symptoms and bacterial growth using Pst-CEL (data not shown).

None of the mild recognition patterns matched with profiles expected for ATR4, ATR5 and the putative ATR(s) recognized in Ksk-1 and Br-0 (Figure S4). Interestingly, two HaRxLs (HaRxL18 and 70) were weakly recognized in Bay-0, an accession susceptible to isolate Emoy2. These results suggest that the decreases in Pst growth we see in some HaRxL/ accession combinations are not due to strong R/AVR interactions. Also, weak recognition of some HaRxLs might not result in HR [49,56].

**Importance of PTI suppression for Hpa infection**

Many HaRxLs delivered in planta by Pst-LUX confer increased growth of an already virulent pathogen. Enhanced susceptibility to adapted pathogens is often a result of PTI suppression [42,43,44,45]. PTI- responses, like callose deposition, likely limit the growth of Hpa during infection [58,59,60]. Also, ATR13Emco2/Emco5 can complement HopM1-mediated suppression of callose deposition when delivered by Pst-CEL [37]. Therefore, we investigated if PTI affects Hpa growth, and whether Hpa is able to actively suppress PTI. As no known PAMP has been identified for Hpa, we tested whether Hpa infection alters responses to known PAMPs.

To test if PTI can attenuate Hpa growth during a compatible interaction, we pre-treated young Col-0 plants with flg22 (100 nM), an inactive flg22 (from Agrobacterium tumefaciens), or chitin (200 µg/ml) 24 h before the plants were sprayed with spores of Hpa isolate Noco2. Reduced hyphal growth was observed in the areas where the PAMPs were applied, as assessed by trypan blue staining of the pathogen in the leaves (Figure 4 A). We also noticed a decrease in the rate of Hpa sporulation (Figure 4 B). These phenotypes were not observed with inactive flg22 (Figure 4 A, B), or when the treatment was applied on plants mutant for these PAMP receptors (flo2-1 and cerc1-1, data not shown). These data suggest that the phenomenon is specific for PTI. We also noticed that the “protection” that flg22 and chitin conferred to the plants was higher near the infiltrated site, was dose-dependent, and diminished with the time of pre-infiltration relative to Hpa infection (24 hs>48 hs>72 hs) (Figure 4 A lower panel and data not shown), consistent with the transient nature of the local PTI response [61]. We did not observe extensive local micro-HR in flg22-treated leaves [62]. The “local” effect of flg22 and chitin in restricting Hpa hyphal growth (Figure 4 A) might indicate that either we did not induce systemic acquired resistance (SAR) [63], or we applied Hpa before SAR was established.

One of the earliest PTI responses is the generation of reactive oxygen species (ROS burst) [64]. To determine if Hpa can suppress ROS, we measured flg22-induced ROS in infected leaf tissues. We observed a highly reproducible reduction by ~50% in ROS accumulation induced by flg22 if leaves were pre-infected with Hpa isolate Noco2 (Figure 4C). Oy-0 plants infected with Emoy2 showed the same pattern (data not shown). Thus, Hpa infection can dampen PTI responses.

**Most HaRxLs that increase bacterial growth in Col-0 can suppress Pst-CEL-induced callose deposition**

PTI results in callose deposition in the cell wall [65] and microbial effectors that impair PTI also suppress callose deposition [66,67,68,69]. Pst-CEL is unable to suppress callose deposition due to lack of HopM1 and Aavr [70]. We introduced pEDV-HaRxL constructs into Pst-CEL and evaluated if they could restore callose suppression when infiltrated in Col-0.

Sixty-two pEDV-HaRxLs and 2 control proteins were delivered through this system. Due to variability between leaf reactions in the same plant and among experiments, we established a threshold to define significant reductions in callose deposition (see Materials and Methods). Taking into account the maximum levels of random callose suppression observed after delivery of the negative controls (NC1 and NC2), we set up the threshold to 40% of callose suppression because negative controls could reach up to 29% or 34% of the callose dots found when Pst-CEL delivered the additional controls (EDV5:HA-AvrRPS41–136, EDV6:HA-YFP or EDV6:HA-AvrRps4A). Using this stringent criterion we found that 33 HaRxLs were able to suppress callose deposition by more than 40%, while 27 HaRxLs did not. Those effectors complementing the phenotype of Pst-CEL are indicated with asterisks (*) in Figure 3.

We noticed that most of the HaRxLs able to complement Pst-CEL were also able to enhance Pst-LUX growth in four or more host accessions (Figure 3). To establish the degree of correlation between both phenotypes, the extent of callose suppression was compared with the changes in Pst-LUX growth produced by each effector in the accession Col-0 (Figure 4 D). For this, we classified the effector’s conferred phenotype in the host as follows: enhanced susceptibility = 27 HaRxLs (right side of the graph), decreased susceptibility = 10 HaRxLs (left side of the graph), no change = 25 HaRxLs (axes intersection). When we plotted the percentage of callose suppression of each of the effectors in these groups, we found 77.7% of HaRxLs candidate effectors with a positive effect on Pst-LUX growth in Col-0 were able to suppress callose deposition, while only 32.0% of those decreasing Pst-LUX growth could reduce callose levels (Figure 4 D). These percentages deviate significantly from those expected for a random distribution (Z-test p<0.05). The HaRxLs located at the top right side of Figure 4 D were those that strongly suppressed callose deposition, and are indicated with plus (+) signs in Figure 3. The HaRxLs with no effect on Pst-LUX growth showed no clear trend in ability to suppress callose deposition.

We conclude that most HaRxLs that enhance Pst growth also suppress PTI, increasing host-susceptibility. Based on the data presented in Figure 3, Tables S2 and S3, Figure S3 and Figure 4D, we prioritized HaRxL14, 21, 44, 45/45b, 57, 62, 106, HaRxL160, 464, 492 and 495 for further detailed studies. HaRxL14, 21, 44, 57 and 62 were chosen because they increased Pst-LUX growth in more than 6 accessions and strongly suppressed callose deposition in Col-0 (>60%) (Figure 3 and top right side on Figure 4D). HaRxLL164 and HaRxL45/45b strongly enhanced Pst-LUX growth in 9 or more accessions, but showed a mild reduction in callose deposits (Figure 3 and Table S2); HaRxL106 conferred enhanced susceptibility to Pst-LUX in several ecotypes, except Col-0, where it nevertheless reduced

---

**Testing the Arabidopsis Downy Mildew Effectorome**

PLoS Pathogens | www.plospathogens.org 7 November 2011 | Volume 7 | Issue 11 | e1002348
callose deposits (Figure 3). HaRxLL60 and HaRxLL492 conferred enhanced growth of Pst-LUX in 5–6 accessions, but were unable to complement the Pst-DCel phenotype. Arabidopsis transgenic lines constitutively expressing HaRxLs show PTI suppression and enhanced susceptibility

To investigate if the phenotypes observed with the Pst-EDV-delivery screenings were also conferred by stably expressing the corresponding HaRxLs directly in the host plant, transgenic Arabidopsis plants were generated initially in the Col-0 background. The following HaRxLs were expressed from the constitutive (CaMV 35S) promoter: HaRxL14, 21, 44, 45/45b, 57, 62, 106, HaRxLL60, 464, 492 and 495. Of these, for three candidates (HaRxL62 and HaRxL45/45b) either we did not obtain transgenic lines or the ones generated showed segregation of pleiotropic effects, and in consequence are not described here. Some plants (lines 35S-HaRxLL60 and 35S-HaRxL44) showed a 20–30% increase in fresh weight and others (line 35S-HaRxL106) a 30–40% decrease in fresh weight. In some cases (35S-HaRxL106 and 35S-HaRxLL60) the shape of the leaves changed, becoming either elongated and darker, or serrated and smaller, respectively (data not shown). The level of expression of the transgene in each line was verified by semi-quantitative RT-PCR (Figure S5).

Figure 4. Suppression of PTI as a virulence tool for Hpa. (A) Pre-treatment of Col-0 leaves with flg22 or Chitin reduces Hpa isolate Noco2 hyphal colonization. Leaves of four-week-old Col-0 plants were pre-infiltrated with 100 nM flg22, inactive flg22 (from A. tumefasciens) or Chitin (200 μg/ml) 24 h before inoculation of Hpa Noco 2 (5 × 10⁴ sp/ml). Pictures show trypan blue stained leaves at 5 days post-Hpa spraying (dps). This experiment was repeated three times with similar results and also for Emoy2 on Oy-0 plants. Panels i, ii and iii: the whole area shown was pre infiltrated. Panels iv, v and vi: only the right side of the picture was infiltrated. Dotted vertical line indicates approximated infiltration boundaries. Bar is 500 μm. (B) Pre-Induced PTI responses reduce Hpa asexual reproduction. Leaves of three-week old Col-0 plants were infiltrated with the indicated solutions 24 h before infection with Noco2 (5 × 10⁴ sp/ml). Conidiophores per leaf were counted on trypan blue stained leaves excised at 5 dps. Bars represent the average ±2 × SE of 40 leaves. This experiment was repeated three times with similar results. (b) p value <0.01, T-test. (C) Hpa infected tissues show reduced ROS response to flg22. Leaf discs from uninfected and infected Col-0 plants were treated with 100 nM flg22, and the level of ROS generated measured with a Luminometer. Values indicated are average of Relative Luminescence Units (RLUs) ± SE of 24 leaf discs. (D) HaRxLs delivered by Pst-DCel in Col-0 plants suppress callose deposition. Effector’s impact on the level of Pst-DCel-triggered callose deposition is presented in the Y-axis. The average reduction (in percentage) of callose deposits observed when a given candidate effector was delivered, compared to the number of callose deposits observed when control proteins were delivered by Pst-DCel, is represented by the shapes in the body of the graph. HaRxLs were also categorized according to their phenotype on Pst-LUX bioluminescence in Col-0 (X-axis). The arrow indicates the threshold set up to consider callose deposition as significantly suppressed. The numbers in the body of the graph correspond to the percentage of HaRxLs able to suppress callose deposition among each bioluminescence category. (*) Indicates p<0.05 of Z-test versus random distribution expected for the number (n) of HaRxLs on each group. doi:10.1371/journal.ppat.1002348.g004
Using three independent lines for the remaining nine candidate effectors, that did not showed perturbed growth, we assessed whether in planta over-expression altered pathogen development, PTI or ETI. We characterized the responses of these transgenic lines to both bacterial (Pst-LUX, Pst ΔavrPto/ΔavrPtoB) and oomycete pathogens (Hpa isolates Noco2 and Emoy2) (Figure 5 and Figure S6). Transgenic lines expressing different HaRXLs showed increased susceptibility to Pst-LUX when spray-inoculated (8 lines, Figure S6), or to Pst ΔavrPto/ΔavrPtoB when infiltrated (7 lines, Figure 5 A). Also, seven lines showed enhanced susceptibility to Hpa isolate Noco2 (Figure 5 B). These phenotypes were observed in at least two out of the three transgenic lines recovered for each effector.

We investigated if the transgenic lines were compromised in ROS burst and callose deposition in response to flg22 (Figure 6). Eight 35S-HaRxLs were able to reduce flg22-triggered ROS accumulation by 22 to 65% compared to controls (Figure 6 A). Also, callose deposition was diminished by an average of 40% compared to controls (Figure 6 B). The ROS and callose suppression in transgenic lines expressing HaRxL 14, 21, 44, 57, 106, HaRXLL 464 was comparable to that observed in plants that express the bacterial effectors HopU1 and HopAO1 (Figure 6 A, B). In summary, six different Hpa HaRXLs, when stably-expressed in planta, displayed a positive correlation between increased susceptibility to Pst and/or Hpa and reduced levels of ROS and callose deposition elicited by flg22 (Figure 7).

To establish if any of the nine HaRXLs could also compromise ETI, we tested transgenic lines for altered resistance to Hpa Emoy2 which is recognized in Col-0 [71]. Two-week-old seedlings were sprayed with Emoy2 conidiospores and trypan blue-stained at 5 dpi. While some restricted hyphal growth was detected, we did not observe asexual or sexual reproduction -in true leaves- in any line (summarized in Figure 7 and data not shown). We then studied the ETI response to AvrRpm1 from P. syringae pv. maculicola [72]. AvrRpm1 was delivered via Pf0-1 in leaves of 4-week-old plants and a macroscopic HR recorded. The onset of HR was delayed but not completely suppressed in four different lines (data not shown). We therefore performed a more sensitive

Figure 5. Arabidopsis Col-0 plants expressing constitutively HaRXLs support enhanced growth of P. syringae ΔavrPto/ΔavrPtoB and Hpa isolate Noco2. (A) Four leaves of three five-week-old plants of two independent transgenic lines per HaRxL were infiltrated with Pst-ΔavrPto/ΔavrPtoB at OD600 = 0.0005. Bacterial growth was determined at 3 dpi by traditional growth curve assays. Bacterial populations immediately after inoculation (3 h; 0 dpi) were averaged among plants and are represented by the solid black horizontal line, with 2 × SE represented by the dashed horizontal lines. (a) T-test p value < 0.05, (b) T-test p value < 0.01. This experiment was repeated two times with similar results. (B) Two-week-old seedlings were spray inoculated with a suspension of 1 × 104 conidiospores per ml of Hpa isolate Noco2. At 6 dps, whole seedlings were cut and stained with Trypan blue. The number of conidiophores per leaf was counted in 4 leaves per seedling. Ten seedlings were analyzed per transgenic line per HaRxL. The horizontal black and dashed lines represent the average ± 2 × SE of the number of conidiophores per leaf found in the hyper-susceptible mutant Col-0 eds1-2. (a) T-test p value < 0.01, (b) T-test p value < 0.05. This experiment was repeated three times with similar results. doi:10.1371/journal.ppat.1002348.g005
assay by quantifying the levels of ion leakage upon AvrRpm1 detection (Figure S7). Notably, the same four sets of transgenic lines (35S-HaRxL106, 35S-HaRxLL492, 35S-HaRxLL464 and 35S-HaRxLL60), slightly but significantly reduced the levels of ion leakage caused by recognition of AvrRpm1 compared to the control lines (Figure S7 and Figure 7).

Our results suggest that the majority (six out of nine) of Hpa Emoy2 HaRxLs constitutively expressed in planta gave phenotypes (enhanced susceptibility to Pst-LUX and suppression of PAMP-triggered callose deposition) consistent with the results obtained from the Pst-LUX EDV-screening in the Col-0 accession (Figure 7). Two transgenic lines (35S-HaRxL106 and 35S-HaRxLL60) showed phenotypes that contrasted with those observed in the EDV screen on Col-0, and for another line (35S-HaRxL70) no effect was detected with the EDV system in Col-0, while enhanced Pst-LUX growth was observed in the over-expressing plants (Figure 7). When HaRxL106 is delivered by EDV from Pst DC3000 strains, it appears to be recognized by Col-0 and Ksk-1 accessions, but this HR-like cell death is not evident when it is delivered by Pf0-1 (Table S4). Expression of 35S-HaRxLL60 caused the plants to be smaller, accumulate callose constitutively and become resistant to the pathogens tested (Figure 5, Figure 6B, Figure S6). Nevertheless, 35S-HaRxLL60 reduced flg22 ROS production (Figure 6A). The set of six lines with enhanced susceptibility to Pst and/or Hpa also displayed a reduced ROS burst and callose deposition after PAMP treatment (Figure 7). Overall, our analysis points to suppression of PTI-related responses as a predominant mode of action of Hpa candidate effectors in planta.

Emoy2 HaRxL candidate effectors are mostly ineffective or reduce pathogen growth in the Hpa non-host Brassica rapa

Most plants are resistant to most pathogens, and this so called “non-host resistance” (NHR) could be caused by either ineffectiveness of effectors, resulting in failure to suppress PTI, or recognition of effectors, resulting in resistance via ETI [8]. To test these hypotheses we delivered HaRxLs via Pst-LUX in the non-host Brassica rapa cv. Just Right (turnip).
**Figure 7. Summary of phenotypes observed upon expression of HaRxL effectors in Arabidopsis.** Graphical comparison of the results obtained using the transient EDV assays and stable constitutive expression for nine different HaRxLs. The phenotypes analyzed include bioluminescence of Pst-LUX, suppression of callose deposition triggered by Pst-ACEL, growth of Pst-aavrPrto/aavrPrtoB, suppression of leaf necrosis triggered by delivering AvrRPM1 via Pto-1, growth (conidiation) of Hpa compatible (Noco2) and incompatible (Emo2) isolates, and suppression of the levels of ROS and callose deposition triggered by fgl2 treatments. doi:10.1371/journal.ppat.1002348.g007

_Pst_-LUX is virulent in turnip and causes disease symptoms at 3 dpi when inoculated at low dose. We tested our collection of HaRxL-carrying _Pst_-LUX strains in turnip and monitored symptoms and growth. After three rounds of screening we found that 20 effectors can alter _Pst_-LUX growth in turnip (13 increase, 7 decrease), while the remaining 44 did not cause any changes (Figure S8 and Table S2, column U). We compared these data with the number of “effective” HaRxLs in one given _Arabidopsis_ accession (39 in Col-0) and the average for the 12 accessions we tested (35) (green plus magenta bars vs. black bars in Figure S4). In contrast, only thirteen HaRxLs increased _Pst_-LUX growth in turnip, while in _Arabidopsis_ accessions an average of 27, and minimum of 18 (in Br-0) increased _Pst_-LUX growth (see Figure S4 and S8). Those candidates enhancing _Pst_-LUX growth in turnip also did so in >3 _Arabidopsis_ accessions, implying that their effect on plant immunity is not species-specific and some plant targets might be conserved. Conversely, while similar numbers of HaRxLs decrease _Pst_-LUX growth in _Arabidopsis_ (8 in average) and turnip (7), we noticed three HaRxLs (HaRxL17, HaRxL47 and HaRxL63) that reduced _Pst_-LUX growth and disease symptoms in turnip that did not show this phenotype in the 12 _Arabidopsis_ accessions. To assess if these HaRxLs might be specifically recognized in turnip and contributing to NHR against _Hpa_, we used higher dose inocula to deliver them using _Pst_-ACEL. We did not observe HR-like cell death, but we confirmed the reductions in growth and disease symptoms (data not shown). It remains possible that these HaRxLs might be triggering weak ETI in turnip that does not involve HR-like cell death but still contributes to NHR (see Discussion).

**Discussion**

Genome sequences of plant pathogens have enabled searches for effectors that might manipulate host cells ([9,28,73,74,75], this work). Verified effectors provide molecular probes to investigate plant defense mechanisms and better understand pathogen adaptation to hosts. In _Hpa_, ~134–140 HaRxLs have been identified [9,28], this work) and the challenge is to identify those that are functional and then investigate their biological mechanisms.

For effector discovery, we combined the use of a heterologous system for _Hpa_ candidate effector delivery from _Pst_ DC3000, with a rapid and sensitive assay for bacterial growth in _in planta_, and for suppression of callose synthesis. To independently assess the efficacy of the most promising candidates, we also tested the consequences of constitutively expressing selected HaRxLs in _in planta_.

EDV vectors and _Pst_-LUX strains provide a sensitive assay for effects of HaRxLs on plant immunity

We found that of 64 expressed HaRxLs that could be secreted by the EDV delivery system, 43 (~67%) could increase the growth of _Pst_-LUX in several (>4) _host_ accessions. We were surprised that so many candidate effectors can enhance growth of an already virulent pathogen. The false positive rate is likely to be low, while the false negative rate could be high, because we set a stringent threshold to judge a putative effector “effective” compared to four internal controls. Despite the intrinsic variability of _Pst_-LUX spraying assays, the phenotypes were reproducible, even when the measurable differences were small (Table S2 and S3). We found that detection of LUX activity is more sensitive and less laborious than growth curve assays and noticed that 2-fold differences in LUX emission usually corresponded to differences between 0.3 and 0.6 log in growth. These might be considered small contributions to virulence, but are consistent with previously reported observations for bacterial effectors such as AvrRpm1, AvrRpt2, AvrPtoB, HopF2, HopAO1 and HopU1, where their individual contribution to _Pst_ DC3000 growth is of the same order of magnitude (around 0.4 log) or only detectable using low virulence _Pst_ mutants [47,69,76,77,78].

A strong advantage of the EDV approach is that one _Pst_ DC3000 strain can be tested on many different _Arabidopsis_ accessions to reveal accession-specific differences in HaRxL efficacy. This would be extremely laborious by generating stable transgenic lines in multiple accessions. Furthermore, some HaRxLs confer severe pleiotropic defects when expressed directly _in planta_, hampering efforts to test whether such lines are immuno-compromised.

_Hpa_ virulence likely involves multiple effectors with weak accession-specific effects

Since not all HaRxLs are effective in all accessions, it seems likely that each _Hpa_ isolate expresses a repertoire of effectors, each of which may be functional on some but not all host genotypes. The level of infection in Col-0 by compatible _Hpa_ isolates is quite variable, with Waco9 more virulent than Noco2, which is more virulent than Emco5. Such observations are usually interpreted in terms of variation in avirulence gene content. However, variation in host targets as well as in _Hpa_ effector complement may also underpin quantitative differences in host susceptibility. _Hpa_ isolate Emo2 was reported as having the highest likelihood of producing high levels of sporulation in a study involving 96 _Arabidopsis_ accessions, and isolate Emco5 the lowest [79]. Although _Hpa_ virulence appears to depend on multiple virulence genes with weak
effects, rather than a few genes with strong effects, some effectors, such as HaRxLs 122, 14, 44, 57 and 106, are particularly effective and will repay detailed mechanistic investigation in the future.

**HaRxLs that enhance Pst growth usually suppress callose deposition**

Significantly, we found that most of the HaRxLs (77%) that increase Pst-LUX growth in Col-0 were also able to suppress Pst-ΔCEL-induced callose deposition. Conversely, those HaRxLs reducing Pst-LUX growth in Col-0 were generally unable to suppress callose deposition. Callose deposition is a late PTI response (though also associated with ETI). We speculate that HaRxLs may enhance Pst-LUX growth via additional PTI suppression, either alone or in conjunction with Pst effectors. The increased susceptibility to Pst-LUX observed using the EDV system was usually consistent with phenotypes of plant lines that constitutively express the corresponding HaRxLs (Figure 7). Moreover, seven of the transgenic lines also showed increased susceptibility to Hpa isolate Noco2. We infer that enhanced susceptibility results from suppression of host mechanisms that are active against diverse pathogens. The fact that they further elevate virulence conditioned by Pst-DC3000 effectors may reflect HaRxLs interference with targets that are not identical to those of Pst-DC3000, resulting in an additive effect.

**Infection with Hpa, and many Hpa effectors, suppress PTI**

Assays using flg22-induced ROS or callose deposition on stable transgenic lines indicate that the main target of HaRxLs is PTI. All of the transgenic lines tested showed either reduced levels of flg22-induced ROS or callose deposition or both. The PAMP complement in Hpa is unknown, as are their receptors and downstream signal transduction pathways in Arabidopsis. Several molecules have been reported as oomycete PAMPs [17,80] but their existence in Hpa is not known [9,81]. We show that pre-elicitation of PTI by bacterial and fungal PAMPs impairs Hpa growth and reproduction, indicating that to infect, Hpa must counteract these host responses. Moreover, in host tissues where high numbers of haustoria are established, PTI responses are attenuated.

PTI involves multiple processes that can be attenuated by diverse pathogen effectors [76,77,78,82]. Our data support the idea that the function of the majority of the effector proteins is to inhibit plant immunity [4,83,84]. For Pst, 13 out of 28 active effectors (12 belonging to Pst-DC3000) have been reported to suppress PTI [84,85,86]. Thus, ~50% of this bacterial pathogen’s effector repertoire targets PTI in one host. Importantly, this hemibiotroph can infect Solanaceae as well as Brassicaceae, so more effectors or effectors might emerge as PTI suppressors when other host species are studied. It has also been observed that 91% of Pst-DC3000 effectors, when delivered at high titers from Pf0-1, are able to suppress the HR induced by the bacterial effector HopA1 (from P. syringae syringae) in tobacco [36]. Since tobacco is a non-host for Pst-DC3000, this study again points to a high functional redundancy between effectors in suppressing HR. In oomycetes, experimental characterization of several RxLR effector genes suggests that many function to suppress host defenses [37,30,86]. Also, out of 32 P. infestans RXLR candidate effectors were able to suppress PAMP-triggered programmed cell death (PCD) in N. benthamiana, while another 13 induced either non-specific or R-mediated PCD [57].

HaRxLs are rarely avirulence determinants

We also identified HaRxLs that reduced Pst-LUX growth in the interaction with Arabidopsis accessions, and investigated whether they are new avirulence determinants (ATRs). Surprisingly, we observed that strong incompatibility caused by HaRxLs is rare. None was able to trigger macroscopic ETI when delivered in planta at high titer, as ATR13-∆melano did in Ndl-0. Instead, several were identified that can reduce Pst-LUX growth in specific Arabidopsis accessions and four triggered micro HR-like lesions when delivered via Pst-ΔCEL. Since we tested only 64 HaRxLs from just one isolate, on only 12 host accessions, our survey was not exhaustive, and the anticipated ATRs might not have been in our repertoire. Alternatively, the EDV assay may not be sensitive enough to detect new Hpa ATRs because these ATR-RPP recognitions are weaker than those already described with this system (ATR13-RRP13 or ATR1-RRP1). Conceivably, some ATRs might not carry an RxLR motif and therefore were not identified as candidate effectors in our bioinformatic analysis, as with the recently cloned ATR5 [58]. It also remains possible that either the sub-cellular localization or post-translational modifications of the EDV-delivered HaRxLs are not similar enough to their native form to be able to elicit ETI, although this has not been the case for ATR1 and ATR13 alleles [37,50].

**Non-host resistance could involve a combination of recognized HaRxLs and ineffective HaRxLs**

We also tested if the recognition or non-functionality of HaRxLs could be involved in non-host resistance to Hpa in Brassica rapa (turnip). We found that HaRxLs were “less effective” in turnip, but those HaRxLs that enhanced Pst-LUX growth in B. rapa also did so in Arabidopsis, suggesting conservation in their targets. Notably, three HaRxLs conferred reduced Pst-LUX and Pst-CEL growth in B. rapa, but did not reduce growth in any Arabidopsis accession. Therefore, the inability of Hpa to grow in turnip might result not only from reduced “effectiveness” of the effector complement, but also from recognition in the “non-host” of a subset of effectors that are not recognized by most Arabidopsis accessions.

**Concluding observations**

As with any screening protocol, this heterologous system has some limitations. For example, HaRxLs that require extensive post-translational modifications will not be correctly produced by a prokaryotic system. Also, the co-delivery of an HaRxLs with c.a 30 effectors from Pst DC3000 might alter the outcome of the assay if positive or negative interactions exist between them. This might explain some of the discrepancies we observed between results obtained with the EDV system and those generated by expressing the candidate effectors directly in the plant. A further potential limitation of the EDV system is that effectors required to elaborate a haustorium inside the host cell might not be revealed as promoting Pst growth by the assays we developed. Despite such limitations, most of the phenotypes observed with the EDV system were confirmed in the transgenic lines.

In conclusion, the EDV-based system has enabled the systematic analysis of the biological relevance of effector candidate proteins. The Pst-LUX and Pst-ΔCEL screens allowed the generation of a “ranking of effectors” that permitted the selection of highly interesting candidates as targets for subsequent mechanistic studies. Further detailed investigations of Hpa effectors will help reveal how Hpa alters host cellular processes to promote its growth and reproduction.

**Materials and Methods**

**Bioinformatic identification of HaRxLs**

Different versions of the genome of Hpa isolate Emoy2 (http://vmd.vbi.vt.edu; v3.0, v6.0 and v8.3.2) were translated in all 6
reading frames. ORFs from ATG to Stop codon were identified using FgenesH (www.softberry.com) and GETORF (http://emboss.sourceforge.net). Only sequences that encoding ≥100 aminoacids were considered. Secreted proteins were identified using SignalP v3.0 (score cut-off >0.9), TargetP and PSort (www.psort.org) [89]. Proteins were considered as secreted if two out of three programs called the Signal Peptide as significant. HaRxLs were selected as fulfilling the following criteria: i) Signal Peptide (SP) length <30 amino acids, ii) RxLR-like motif (RxLR/Q RxL) between 4 and 60 amino acids from the SP cleavage site, iii) predicted protein had >40 amino acids after the RxLR like motif. Redundancy in the ORF dataset was corrected using BlastP. Sequences with 100% identity and E<10^-5 were clustered and simplified to the one with highest SP score. A sub-set of RxLR-EE proteins was identified containing an acidic motif (EE, EER/G/D) [28] between 4 and 30 aminoacids from the RxLR like motif. The expression of HaRxLs was verified using the following resources: i) ESTs generated by Sanger sequencing and 454 sequencing from cDNA extracted from Emoy2 conidia suspensions [6], ii) Illumina sequence tags (SAGE using 3’ tags from 7 dpi infected tissue), iii) Illumina normalized/concatamized cDNA (from 3 and 7 dpi infected tissue) [Ishaque et al., unpublished] and iv) RT-PCR with primers designed at 100 bp flanking the ORF sequence (Baxter et al., unpublished). The presence of the predicted and alternative ATGs and Stops Codons, as well as introns, was verified. Nucleotidic sequence polymorphisms on the HaRxLs across seven Hpa isolates was assessed using either PCR products or in silico assemblies of Illumina short reads [Ishaque et al., unpublished]. The HaRxLs were roughly classified as: No polymorphic (0 SNPs), Low (≥1 SNP ≤5), Medium (≥6 SNPS ≤15) and High Polymorphic (>16 SNPs). To complete the characterization of the Hpa Emoy2 HaRxLs set, its sub-cellular localization (PSORTII) and known controlled at 12, 24, 32 and 44 hs post inoculation of 10 ml Kings B media with a dilution corresponding to 0.00001 OD of an overnight culture of each of the Pst-LUX clones harbouring a different HaRxL or control proteins (GFP, AvrRPS4 [43]). Three colonies per clone were assayed in different experiments. Growth was measured assessing turbidity at OD600 (for liquid cultures) or counting colonies of plated dilutions (in solid media). No significant differences in growth kinetics were observed for the Pst-LUX carrying HaRxLs regarding the clones carrying control proteins or the empty vector pEDV5.

**Bacterial strains**

Bacterial strains used in this study include *E. coli* DH5α, *Pseudomonas syringae pv tomato* DC3000 carrying the luxCDABE operon from *Photobacterium luminescens* (Pst-LUX) [41], *Pseudomonas syringae pv tomato* DC3000 mutant ΔCEL [90], *Pseudomonas syringae pv tomato* DC3000 double mutant ΔAvrPto/ΔAvrPtoB [91], *Pseudomonas fluorescens* P0-1 carrying a functional TTSS [57] and Agrobacterium tumefaciens GV3101 (pMP90 RK). *E. coli*, and Agrobacterium were grown in low salt Luria-Bertani broth at 37°C (E. coli) or 28°C (Agro) using either liquid media or petri dishes. *Pseudomonas* strains were grown in either LB or King’s B medium at 28°C in liquid media or petri dishes. Antibiotics concentrations (µg/ml) were as follows: Rifampicin 100, Kanamycin 50, Gentamycin 25, Spectinomycin 50, Chloramphenicol 50, Tetra-cycline 10, Carbenicilllin 50.

**Plant materials and growth**

*A. thaliana* accessions used in this study were obtained from NASC. The bfer-1 mutant was obtained from Cyril Zipfel and the cerk1-1 mutant was a kind gift of JP Rathjen. Transgenic lines constitutively expressing HopU1 and HopAO1 were kindly provided by Jim Alfano. Turnip seeds (*Brassica napus cv Just Right*) were purchased from Gurney’s seeds (http://gurneys.com). Tobacco (*Nicotiana tabacum cv petit Havana*) and tomato (*Solanum lycopersicum cv Moneymaker*) seeds were obtained from John Innes Horticultural services. *Arabidopsis* plants were grown in Scotts and Levinton F1 modular compost in controlled environment rooms under short day cycles (10 h/14 h day/night and 150–200 µE/ m²s) at 22°C and 60% relative humidity and slightly watered every day from below. Tobacco, tomato and turnip plants were grown under similar conditions as *Arabidopsis* for 5 weeks post-germination. Plants expressing constitutively HaRxLs were generated by recombining the corresponding ORFs cloned in pDONR221 in the Gateway destination binary vector pB2GW7 [92] under the control of the CaMV 35S promoter. Constructs were transferred to *A. tumefaciens* strain GV3101 (pMP90 RK) [93] and transformed into *Arabidopsis* accession Col-0 by the floral dipping method. Primary transformants (T₀) were selected on soil containing BASTA (Bayer CropScience, Wolfenbüttel, Germany) and self-pollinated. The progeny of the T₂ generation was observed and 3:1 (BASTA-resistant/BASTA-susceptible) segregating lines were taken further. Homozygous lines were selected by examining the BASTA resistance of T₃ seedlings. Three independent transgenic lines per HaRxL (T₄s) were analysed and for simplicity we present results for two.

**Pathogen growth and inoculations**

Primary streaks of Pst-LUX complemented with the controls or HaRxLs were made from isolated colonies onto selective King’s B plates and grown overnight at room temperature. Selected individual colonies were then spread with a sterile loop in solid KB plates and incubated overnight at room temperature to produce even bacterial lawns. Cells were scraped from plates with a sterile loop and suspended in 50 to 100 ml of 10 mM MgCl₂ to a

---

**Testing the Arabidopsis Downy Mildew Effectorome**
isolates Emoy2 and Noco2 were maintained in compatible host legends or other sections of M and M. The fourth most recently expanded leaves were used. Concentrations with controls on the same leaf. For tomato, leaflets of the third and turnip, leaf panels of the third- to fifth-oldest leaves were used. Concentrations of E. coli solutions (or water as control) were kept uncovered in low humidity conditions. At three days post-spraying, sets of five plants sprayed were covered with a transparent lid to keep high humidity conditions. At three days post-spraying, sets of whole five plants were placed in an ultra low light CCD camera (Photek, www.photek.com). Photos emitted per second were scored per plant and referred to the whole plant fresh weight to account for the foliar area. The average level of Photon Counts per Second per gram of Fresh Weight was obtained for each Pst-LUX EDV-delivered protein and the ratio versus the control was scored on each accession (Figure 2). To verify the correspondence between increase on photon emission and bacterial population growth, leaf discs were sampled from the five plants sprayed to generate at least 6 technical replicates. The leaves were surface sterilized (30 s in 70% ethanol, then 30 s sterile distilled water). Traditional growth curve assays were performed as described [94].

Gene expression analysis
Total RNA was extracted from three-week-old HaRxL overexpressing lines (T4 generation) using the RNasey Plant Mini Kit (Qiagen, Hilden, Germany). 1 µg DNAse-treated total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). For semi-quantitative RT-PCR 1 µl of cDNA was used per reaction and amplified with an initial denaturation step at 95°C for 3 minutes followed by 23 cycles with the following conditions: 45 s at 94°C, 30 s at 55°C and 30 s at 72°C. In a last cycle a final elongation step at 72°C for 5 min was added. PCR products were separated on 1.5% TAE agarose gels. To control the equal amount of cDNA in every reaction the Actin2 gene (At3g18780) was used. The specificity of the primers for amplification of the HaRxLs transcripts was tested on pDONR221 clones harboring the corresponding HaRxLs. In case of control RT-PCRs on Arabidopsis Col-0 wild type plants cDNA, no unspecific amplification using the HaRxLs’ primers was observed. The sequences of primers used in this study are available on request.

Measurement of Reactive Oxygen Species (ROS) generation
ROS released by the leaf tissue in response to flg22 was measured using a chemiluminescent assay [95]. Briefly, leaf discs (0.38 cm²) from Col-0, fls2-1 mutant, transgenic lines expressing a given 35S-HaRxL lines, and control lines were sampled. At least 24 leaf discs from four five-week-old plants per accession line were sampled and floated for 14–16 h in sterile distilled water in a 96 flat-bottom white multiwell plate (Greiner Bio-One) kept in the dark. ROS production in response to flg22 was measured by replacing the water by 100 µl of a working solution of Luminol (34 µg/ml final concentration, Sigma), Horseradish Peroxidase (20 µg/ml final concentration, Sigma) and flg22 (100 nM final concentration, Peptron, South Korea). The plate was immediately introduced in a Luminometer (Varioskan, Thermo Scientific) and photon counts were recorded every 40 seconds for at least 40 minutes.

Callose and Trypan Blue Stainings and microscopic analysis
Leaves of five-week-old plants were hand-infiltrated at the bottom of the leaf area with 5 × 10⁸ cfu/ml (OD₆₀₀ = 0.05) Pst-ACEL suspensions. Bacteria complemented with control or HaRxL proteins were infiltrated in different plants of the same set. Twenty leaf samples were taken 12 to 14 h after infiltration at the top of the infiltrated area to avoid visualization of mechanical damage induced callose. Leaf discs were cleared 2 times (1 h) in 96% ethanol and then re-hydrated in ethanol/water series (70%, 50%, 30%) per 30 min each. Leaf discs were floated in water 1 h and then transferred to a solution of 0.01% (w/v) Aniline Blue (Gurr, BHD, England) in K₂HPO₄ Buffer (150 mM pH 9.5) for 1 h. The samples were then transferred to Glycerol 60% (v/v) and mounted for observation with a Leica DM R fluorescence microscope using UV light and E3 filter (A4-UV). Pictures were taken with a Leica DFC 300 FX Digital Camera. Images were analyzed and callose dots quantified using Image J software and an in house written Macro. The same imaging system was used to visualize Hpa infection structures after staining with lactophenol trypan blue. The number of conidiophores per leaf was scored by manually scanning the abaxial and adaxial surfaces of each leaf on at least four true leaves of five ten plants per accession/transgenic line analyzed.

Ion leakage measurements
Five-week-old plants were infiltrated with 1 × 10⁸ cfu/ml (OD₆₀₀ = 0.1) P. fluorescens Pf0-1 carrying pVSP61-AvrRPM1. To be able to detect HR symptoms the bacteria had to be grown in plates. Leaf discs were collected right after infiltration using a cork borer number 4. Twenty-four leaf discs were collected per transgenic line and shacked in falcon tubes with 45 ml distilled water for 1 h at room temperature. Four leaf discs were transferred multi-well plates with 2 ml distilled water. The level of ion leakage caused by AvrRPM1 recognition by RPM1 in the Col-0 background was detected as an increment in conductivity. This was measured with a Conductivity meter (Horiba Twin B-173) every 60 min over 14 h on at least 6 technical replicates per transgenic line.

Secretion assay and protein analysis
To verify the accumulation proteins corresponding to the AvrRPS4-effector fusions in Pseudomonas and its secretion by the TTSS, the strains complemented with Hpa HaRxLs were grown overnight at 28°C in 25 ml liquid LB media to a final
OD<sub>600</sub> = 0.2, centrifuged, washed twice with 10 mM MgCl<sub>2</sub> and re-suspended in 20 ml minimal media (N<sub>2</sub>-inducing minimal medium, MM) [50 mM potassium phosphate buffer, 7.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.7 mM MgCl<sub>2</sub>, 1.7 mM NaCl, pH 6.0, with 20 mM glucose added]. The cultures were incubated at 22 °C at 200 rpm on a rotator shaker to a final OD<sub>600</sub> of 0.5. The pellet and supernatant fractions were separated by centrifugation at 5,200 × g for 15 min at 4°C. The pellets were re-suspended in 300 ul of bacterial protein extraction buffer [100 mM NaCl, 25 mM Tris-HCl pH 8, 10% glycerol, 10 mM DTT and 1 μl of protease inhibitors cocktail (Complete EDTA-free tablets, Roche)], sonicated 3 times (10 s), and centrifuged at 12,000 rpm for 5 minutes. Then 100 μl of supernatant were taken and 25 μl of SDS loading buffer were added. Samples were boiled for 5 minutes at 96°C before loading. The supernatant of the 20 ml MM culture was filtered through a 0.2 μm pore filter and concentrated using centricron YM-10 columns (Amicon Bioseparation) by sequential centrifugations of 30 min at 5000 g at 4°C. The process was repeated several times. Proteins in the final 2 ml of concentrated supernatant were precipitated using Stratagene beads (Stratagene). Five to ten μl of beads were used per ml of supernatant; incubated 10 min at 4°C inverting gently, centrifugated at 2000 g for 2 min and re-suspended in 25 ul Laemmli buffer containing 0.1 M NaOH. Samples were boiled for 5 minutes at 96°C and centrifuged at 12,000 rpm for 2 minutes before loading. The pellet fraction (15 μl) and the culture fluid fraction (25 μl) were analyzed by SDS–PAGE, electro blotted onto PVDF membrane (Bio-Rad), and probed with horseradish peroxidase- conjugated high affinity anti–HA antibody (Roche) and re-probed with anti-NPTII antibody (Upstate). Bands were visualized using PICO kit (Thermo Scientific) and imaged with Kodak scientific imaging film.

Supporting Information

Figure S1 Verification of the functionality of the TTSS of Pst-LUX clones expressing HaRxLs. (A) Immunoblot showing the accumulation of AvrRPS4<sub>1-136</sub>-HA-HaRxL fusions on the bacterial pellet (left panel) and its secretion into TTSS-inducing minimal media (right panel, supernatant). Approximated molecular weights in KDa are shown on the left of each panel. (B) Four-week old tobacco (N. tabacum cv. petit havane) leaves were infiltrated with Pst-LUX clones carrying either control proteins (YFP, AvrRPS4<sub>1-136</sub>, or HaRxLs at OD<sub>600</sub> = 0.01. Pst-hrcC and Pst-hrcC-LUX strains were included as positive controls for TTSS impairment. Symptoms of HR cell death were screened at 2,3 and 4 dpi. Picture was taken at 2 dpi. (C) Five-week old tomato (Solanum lycopersicum cv. maymone) leaflets syringe infiltrated with OD<sub>600</sub> = 0.001 of different Pst-LUX clones expressing HaRxLs or controls (YFP). Leaflets were detached and imaged at 3 dpi with a Photek camera to detect bioluminescence.

Figure S2 Correlation between Pst-LUX bioluminescence and its growth in planta. (A), (C) Five-week-old plants of the indicated Arabidopsis accessions were sprayed-inoculated with Pst-LUX delivering the indicated Hpa effector or control proteins. At 3 dpi, five whole plants per treatment were imaged using a Photek camera to record photons counts per second. Bars illustrate the average photon counts per gram of plant fresh ± SD. (a) p value of T-test assuming unequal variances<0.05, (b) p<0.01. (B), (D) Twenty-four leaf discs obtained from the above mentioned plants were excised and used to determine the number of bacteria per leaf area, showed in Log<sub>10</sub> scale. Bars indicate the average ± SD of six technical replicates. One-way ANOVA test was applied with (a) p<0.05, (b) p<0.01. (TIF)

Figure S3 Behavior of Pst-LUX delivering HaRxLs assessed via growth curves. Histograms illustrate the changes in growth levels (measured as colony forming units –CFU-) of Pst-LUX strains delivering the indicated HaRxLs, compared to control strains, on different Arabidopsis accessions.

Figure S4 Pattern of HaRxLs induced changes in Pst-LUX virulence plotted per Arabidopsis accession. Bars indicate the number of HaRxLs that enhanced (green), decreased (red) or did not changed (black) the growth of Pst-LUX on each Arabidopsis accession tested. The outcome of the interaction of the Hpa isolate Emo2 with each accession is indicated as Susceptible (S) or Resistant (R). Known and predicted ATR/RPP interactions are described. (?) indicate putative/unknown ATR/RPP genes.

Figure S5 Semi-quantitative RT-PCR applied to RNA extracted from the stable transgenic lines generated and tested in this paper. Expression levels of each HaRxL were tested in two independent homozygous transgenic lines (1,2). For comparison, the expression of the Arabidopsis housekeeping gene actin (Act2) is shown.

Figure S6 Arabidopsis Col-0 plants expressing constitutively HaRxLs support enhanced growth of Pst-LUX. Five-four week-old plants of two independent transgenic lines expressing the corresponding Hpa candidate effector and one line per control protein, were sprayed at OD<sub>600</sub> = 0.2 with Pst-LUX. At 3 dpi, photon counts per plant were measured, as well as plant’s fresh weight. Bars represent means of 5 replicates ± 2 × Standard Errors (SE). (a) p value of T-test assuming unequal variances <0.05; (b) p<0.01. This experiment was repeated three times with similar results.

Figure S7 Constitutive expression of four different HaRxLs caused a mild reduction on the levels of ion leakage triggered by AvrRPM1 recognition. Five-week-old plants were hand infiltrated with Pf0-1 delivering P. maculicolla AvrRPM1 at OD<sub>600</sub> = 0.1. Twenty-four leaf discs were sampled from four infiltrated transgenic plants per line per HaRxL in each of five different experiments. At least six technical replicates were done per line. Conductivity was measured in the water were discs were floating, as an indication of ion leakage into the media. Measurements were taken every hour until the peak of ion leakage was detected in the wild type and control lines (around 14 hours post-infiltration). Bars correspond to the average ± 2 × SE of the maximum level of ion leakage observed for each line expressed as a percentage of the value displayed by Col-0 wild type (100%). Values take into account averaged results of 5 different experiments. (a) p value<0.05 of two tailed t-test.

Figure S8 Fewer HaRxLs can alter the growth of Pst-LUX in the Hpa-non-host Brassica rapa compared to Arabidopsis. Columns show the distribution of the number of candidate effectors that enhance or decrease Pst-LUX virulence in the non-host B. rapa compared to one accession (Col-0) and the average results of the screening in the set of twelve accessions of the host Arabidopsis.
Table S1 Names and sequences of *Hyaloperonospora arabidopsidis* isolate Emoy2 HaRxLs candidates cloned. (a) Nucleotide sequence predicted in genome versions of the Hpa Emoy2 race (v3.0 to v6.0) generated previously to the published one (v8.3.2: Baxter and Tripathy et al., 2010). (b) Translation of the predicted nucleotide sequence in Hpa Emoy Genome v8.3.2. (c) Nucleotide sequence cloned in pENTRY/pDONR vectors. The clones were amplified from the Signal Peptide cleavage until the stop codon, adding a Methionine (M) at the N-terminal, unless stated otherwise. (d) Sequence cloned in pEDV vectors. When pEDV6 was used, sequences were identical to the pENTRY donors. For clones in pEDV3/5 (non-Gateway versions) no pENTRY clones were generated. (e) Comparison of sequence similarity of the pEDV clone with the predicted nucleotide sequence on the Emoy2 genome (Column G vs Column C). (f) Comparison of sequence similarity of the pEDV clone translated aminocacid sequence with the predicted one the Emoy2 genome (Column H vs Column D). (g) Level of Polymorphism of candidate RxLR effectors predicted by “in silico” comparison of the Hpa Emoy2 race genome (v8.3.2, v6.0, v3.0) with the genomes of seven Hpa races sequenced via illumina short reads. The number of SNPs predicted is indicated between brackets. We classified them as No polymorphic (0 SNPs), Low (≥1 SNPs ≤5), Medium (≥6 SNPs ≤15) and High Polymorphic (>16 SNPs). (h) Level of Expression of candidate RxLRs was tested using different expression libraries, and categorized as: 1-Sanger ESTs obtained from Spores, 2- 454 ESTs generated from infected tissue at 3 days post-inoculation (dpi), 3- Illumina short reads generated from infected tissue at 3 and 7 dpi. (i) Predicted subcellular localization using Wolf-PSORT1 (of sequences after signal peptide cleavage site). The localization stated corresponds to the ones consistently found using both Plant and Fungi databases. The score numbers representing the number of closest neighbours in each alignment are represented in brackets. (+) Cloned from RxLR onwards, without M. (*) Cloned from RxLR onwards, X or L replaced by M. (−) Cloned from SP cleavage site onwards, with M introduced. ND Not determined. (1) There are two sequences highly similar in Emoy2 genome. The paralog cloned here is not assembled in the published Emoy2 genome (v8.3.2). They differ only in three aminocacids (E→MW×K×MIR). (2) Putative gene family. The paralog cloned is not the one assembled in V8.3.2 of the genome. (3) The cloned sequence was not assembled in V8.3.2. It is highly similar except for the C-terminal end. Putative paralogs. (4) Putative gene family. The paralog cloned is not assembled in V8.3.2 of the genome. (5) There are two paralog sequences in Emoy2 genome. Only HaRxL45 is assembled in v8.3.2. (6) These RxLR candidates are not assembled in all of the V8.3.2 of the genome, but they were predicted in previous versions of it (v3.0 and v6.0). (7) The sequence of this clone has an internal deletion of 20 aminoacids regarding the sequence predicted in v8.3.2 of the genome. Putative paralog. (0) NC1:2,3,4: Negative controls.

Table S2 Effect of 64 HaRxLs on *Pst-LUX* bioluminescence tested across 12 *Arabidopsis* accessions. (a) *Pst-LUX* complemented with the corresponding candidate HaRxL or control was infiltrated in leaves of *Nicotiana tabacum* cv. *Petit Havana*. Development of hypersensitive response (HR) cell death was evaluated at 2 days post-infiltration. Representative picture on Figure S1B. (b) *Pst-LUX* complemented with the corresponding candidate HaRxL, or control was infiltrated in leaflets of *Lycopersicum esculentum* cv. *Moneymaker*. Disease symptoms were determined between 3 and 5 dpi. Presence of the bacteria on the lesions was confirmed imaging *Pst-LUX* bioluminescence.

Table S3 Comparison of photon counts versus colony forming units for *Pst-LUX*. Results obtained delivering 13 Hpa candidate effectors via EDV by *Pst-LUX* in different *Arabidopsis* accessions. Numbers in the body of the table correspond to the ratio between *Pst*-LUX delivering the indicated effector and the control *Pst*-LUX clone delivering YFP. For the spray inoculation, the quantization of the bacterial bioluminescence (Photon Counts) and colony forming units (CFUs) is displayed for two to 3 replicate experiments. Experiments displaying the results obtained when the bacteria are introduced in the plant by syringe infiltration are shown for comparison. For the spray inoculation experiments, bacterial growth was scored at 3 dpi using five plants to record *Pst*-LUX bioluminescence. The same plants were ground to determine bacterial growth by plating in selective media and CFUs. Experiments in parallel were done inoculating the bacteria at OD600 0.001 by syringe infiltration. Numbers highlighted in bold indicate T-test p value <0.05. (*) Result differs from the one obtained with the EDV screen (see Table S2). (a) Values of Photon counts correspond to the ratio of counts per second (CPS)/fresh weight (FW) in grams of *Pst*-LUX clones delivering via EDV the stated *Hpa* candidate effectors versus CPS/FW of the YFP or AvrRPS4 control. (b) Values of CFUs correspond to the ratio colony forming units (CFUs/FW of *Pst*-LUX clones delivering via EDV the stated *Hpa* candidate effectors versus CFUs/FW of the YFP control or AvrRPS4 control. (c) The concordance between the Photon counts and CFUs ratios is indicated.

Table S4 Putatively recognized *Hpa* candidate effectors and HR-like cell death. The table displays the number of leaves showing HR-like micro-lesions when *Trypan blue*-stained 24 h post-infiltration with *Pst*-ACEL (OD<sub>600</sub> = 0.01) or P00-1 (OD<sub>600</sub> = 0.1) delivering the indicated *Hpa* RXLR effector candidates in the corresponding *Arabidopsis* accessions. Numbers in parenthesis correspond to P00-1 data. Twenty-four leaves were infiltrated per candidate per bacterium. This experiment was repeated twice for each vehicle bacterium, with similar results. Values significantly different from negative controls (NC1 or NC2) across replicate experiments are indicated in bold (p value T-test <0.05).
Acknowledgments

We thank the JIC horticulturist team, Matthew Smoker, Avril Soards, Tina Payne and Jaqueline Bautor for technical assistance. We thank Craig Brooks-Roomey, Nicolas Battich, Belen Tejada-Romero, Sharon Hall, Peter Bittner-Eddy and Astrid Vauldaine for contributing with the cloning of HaRxLs. We thank Cyril Zipfel, Sophien Kamoun, Ruslan Yatusevich and Ana Maria Cruz-Cabral for helpful comments on the manuscript. This paper is dedicated to Triziano and Francesco Lunello-Falvo and to Federico Lunello.

Author Contributions

Conceived and designed the experiments: JDGJ JB JEP GvA GF. Performed the experiments: JS PS JW KA EK RLA SJMP ARC DG RL JB MCC. Analyzed the data: GB LI NJ DS JDGJ. Contributed reagents/materials/analysis tools: GF JS MC NJ EK RLA SJMP ARC DG RL JB MCC KHS LB NI DS. Wrote the paper: JDGJ. Edit the paper: JB JS GvA MCC GF JDGJ.

References

1. Oliver R, Ipeko S (2004) Arabidopsis pathology breathes new life into the necrotoxins-ribostoxin classification of fungal pathogens. Mol Plant Pathol 5: 347–352.
2. Jones JD, Dangl JL (2006) The plant immune system. Nature 444: 325–329.
3. Zipfel C (2009) Early molecular events in PAMP-triggered immunity.Curr Opin Plant Biol 12: 414–420.
4. Boller T, He SY (2009) Immune induction in plants: an arms race between pattern recognition receptors in effectors and effectors in microbial pathogens. Science 324: 742–744.
5. van der Hoorn RA, Kamoun S (2008) From Guard to Decoy: a new model for perception of plant pathogen effectors. Plant Cell 20: 2009–2017.
6. Raffaele S, Farrer RA, Cano LM, Studholme DJ, MacLean D, et al. (2010) Genome evolution following host jumps in the Irish potato famine pathogen. Science 330: 1540–1545.
7. Hajri A, Brin C, Hunaoui L, Lardeux F, Lemaire C, et al. (2009) A “repertoire for repertoire” hypothesis: repertoires of type three effectors are candidate determinants of host specificity in Xanthomonas. PLoS One 4: e6632.
8. Lipka U, Fuchs R, Lipka V (2008) Arabidopsis non-host resistance to powdery mildew. Curr Opin Plant Biol 11: 404–411.
9. Baxter L, Tripathy S, Ishaque N, Boot N, Cabral A, et al. (2010) Signatures of adaptation to obligate biotrophy in the Hyaloperonospora arabidopsidis genome. Science 330: 1549–1551.
10. Spanu PD, Albert JC, Anselme J, Burgis TA, Soanes DM, et al. (2010) Genome expansion and gene loss in powdery mildew fungal rewire tradeoffs in extreme parasitism. Science 330: 1543–1546.
11. Schirawski J, Mannhaupt G, Munch K, Brefort T, Schipper K, et al. (2010) Pathogenicity determinants in attaccine fungi revealed by genome comparison. Science 330: 1546–1548.
12. Judelson HS, Tyler BM, Michelmore RW (1991) Transformation of the model organism Arabidopsis thaliana. Mol Plant Cell Biol 12: 1015.
13. van der Henst RA, Kamoun S (2008) From Guard to Decoy: a new model for perception of plant pathogen effectors. Plant Cell 20: 2009–2017.
14. Holub EB (2006) Natural history of Arabidopsis thaliana and oomycete pathogens. Annu Rev Phytopathol 48: 329–345.
15. Torto TA, Li SS, Stryer L, Huitema E, Tena A, et al. (2003) EST Mining and Functional Expression Assay Identify Extracellular Effector Proteins From the Plant Pathogen Phytophthora. Genomics Res 13: 1675–1685.
16. O’lick A, Win J, Raffaele S, Morgan W, Bos J, Krasileva KV, et al. (2010) Recent developments in effector biology of filamentous plant pathogens. Cell Microbiol 12: 1015.
17. Schornack S, van Damme M, Bockrot TO, Cano LM, Smoker M, et al. (2010) Ancient class of translocated oomycete effectors targets the host nucleus. Proc Natl Acad Sci U S A 107: 17421–17426.
18. Birch PR, Boevink PC, Gilroy EM, Hein I, Pritchard L, et al. (2008) Oomycete RXLR effectors: delivery, functional redundancy and durable disease resistance. Curr Opin Plant Biol 11: 373–379.
19. Campo C, Kamoun S, Anagnostakis S (2008) Translocation signal for delivery of oomycete effector proteins into host plant cells. Nature 450: 115.
20. Dou D, Kaile SD, Wang X, Jiang RH, Bruce NA, et al. (2008) RXLR-mediated entry of Phytophthora sojae effector AvrLb into soybean cells does not require pathogen-encoded machinery. Plant Cell 20: 1930–1947.
21. Hoyle SJ, Gb, Capelutto DG, Dou D, Feldman E, et al. (2010) External lipid PIP2 mediates entry of eukaryotic pathogen effectors into plant and animal host cells. Cell 142: 284–295.
22. Schornack S, van Damme M, Bockrot TO, Cano LM, Smoker M, et al. (2010) Ancient class of translocated oomycete effectors targets the host nucleus. Proc Natl Acad Sci U S A 107: 17421–17426.
23. Birch PR, Boevink PC, Gilroy EM, Hein I, Pritchard L, et al. (2008) Oomycete RXLR effectors: delivery, functional redundancy and durable disease resistance. Curr Opin Plant Biol 11: 373–379.
24. Panstruga R, Dodds PN (2009) Terrific protein activity: the mystery of effector protein delivery by filamentous plant pathogens. Science 324: 748–750.
25. Settembre HS, Tyler BM, Michelmore RW (1991) Transformation of the oomycete pathogen, Phytophthora infestans. Mol Plant Microbe Interact 4: 602–607.
26. Huitema E, Smoker M, Kamoun S (2010) A straightforward protocol for electro-transformation of Phytophthora capsici zoospores. Methods Mol Biol 712: 129–135.
27. Sohn KH, Lei R, Nenni A, Jones JDG (2007) The downy mildew effectors proteins ATR1 and ATR19 promote disease susceptibility in Arabidopsis thaliana. Plant Cell 19: 4077–4090.
28. Guo M, Tian F, Wamboldt Alfano J, Jones JDG (2009) The majority of the type III effector inventory of Pseudomonas syringae pv. tomato DC3000 can suppress Arabidopsis immunity. Mol Plant Microbe Interact 22: 1069–1080.
29. Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7: 1513–1523.
30. Ganev IA, Debi J, Viles J, Smokor C, Apel R, et al. (2008) Bacterial type III effector delivery system: a complex machinary for intracellular delivery. Mol Plant Microbe Interact 23: 375–384.
31. Andreason SR, Campbell K, Theodore S, Legall B, Jones JDG (2008) Macromolecular signals required for type III secretion and translocation of the Xanthomonas campestris AvrB2 protein to pepper plants. Proc Natl Acad Sci USA 97: 13324–13329.
32. Fan J, Crooks C, Lamb C (2008) High-throughput quantitative luminescence assay of the growth in planta of Pseudomonas syringae chronomically tagged with Photobacterium luminescens luxCDABE. Plant J 53: 393–399.
33.Zipfel C, Roubatzek S, Navarro L, Oakeley EJ, Jones JDG, et al. (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. Nature 428: 764–767.
34. Girard J, Bano J, Alfano J, Smokor C, Apel R, et al. (2008) Bacterial type III effector inventory of Pseudomonas syringae pv. tomato DC3000 can suppress Arabidopsis immunity. Mol Plant Microbe Interact 22: 1069–1080.
35. Cornelis GR (2010) The type III secretion injectionosome, a complex nanomachinary for intracellular delivery. Biol Chem 391: 745–751.
36. Mudgett MB, Chenkova O, Dahlbeck D, Clark ET, Rossier O, et al. (2000) Molecular signals required for type III secretion and translocation of the Xanthomonas campestris AvrB2 protein to pepper plants. Proc Natl Acad Sci USA 97: 15324–15329.
37. Fan J, Crooks C, Lamb C (2008) High-throughput quantitative luminescence assay of the growth in planta of Pseudomonas syringae chronomically tagged with Photobacterium luminescens luxCDABE. Plant J 53: 393–399.
38. Zipfel C, Roubatzek S, Navarro L, Oakeley EJ, Jones JDG, et al. (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. Nature 428: 764–767.
72. Rohmer L (2003) Nucleotide sequence, functional characterization and
71. Tor M, Holub EB, Brose E, Musker R, Gunn N, et al. (2004) Map positions of
70. DebRoy S, Thilmony R, Kwack YB, Nomura K, He SY (2004) A family of
69. Underwood W, Zhang S, He SY (2007) The Pseudomonas syringae type III
67. Nomura K, Debroy S, Lee YH, Pumplin N, Jones J, et al. (2006) A bacterial
62. Naito K, Taguchi F, Suzuki T, Inagaki Y, Toyoda K, et al. (2008) Amino Acid
58. Donofrio NM, Delaney TP (2001) Abnormal callose response phenotype and
56. Yu IC, Parker J, Bent AF (1998) Gene-for-gene disease resistance without the
53. Nordborg M, Hu TT, Ishino Y, Jhaveri J, Toomajian C, et al. (2005) The
49. Gassmann W, Hinsch ME, Staskawicz BJ (1999) The Arabidopsis RPS4
31. Horsch RB, Fraley RT, Horsch DR, Eichholtz D, Symons M, et al. (1985) A novel
29. Friesen JD, Collmer A (2001) Plant resistance genes encode bacterial
28. Gubler D, Jeffers W (1992) A bacterial Art gene determines avirulence on a
27. Kvarnes E, Stabell M (1994) Virulence expression of the bacterial disease
26. Rask U, Hovander EM, Jorgensen HS, et al. (2003) A virulence-related protein
25. Shewry PR, Troughton C, Wilks K, et al. (1981) Transformation of oat
24. He SY, Underwood W, Li J, et al. (2003) The Pseudomonas syringae pv. syringae type III effector gene hopQ1 is required for virulence in Arabidopsis.
23. Hatfull GF, Pugsley AP, Egan RA, et al. (1989) Bacteriophage P22 and its
22. Glick B, Tcherkezov A, Catvjr C, et al. (1999) Role of the LRR domain of
21. Frank A, Manners J, Ecker J, et al. (2003) Analysis of the Arabidopsis mosaic
20. Lee Y, Sun J, Lee Y, et al. (2003) A genome-wide screen for Arabidopsis mutants
19. Grossberg EJ, Yu IC, Parker J (1996) The Arabidopsis RPS5 gene encodes a
18. Gutterson JN, Eiserling FA, Dangl JL, et al. (1998) The avirulence gene avrB of
17. Gelfand D, Kramer R, Hubert H, et al. (1987) Rapid amplification of cDNA
16. Froux P (2002) Receptor-like kinases and their role in systemic acquired
15. Farris D, Pan H, Tang J, et al. (2005) A bacterial type III effector gene hopQ1
14. Eshed Y, Ecker J (1994) Arabidopsis RPS2 encodes a putative leucine-rich repeat protein.
13. Eisen JA, Bisht S, Liu L, et al. (2003) Functional annotation of the Arabidopsis genome using the whole-genome expression pattern database.
12. Currah K, Chua NH, Chang J, et al. (1994) Expression and function of two genes encoding plant cell wall chitinases.
11. Chen S, Franklin JL, O’Connell JO, et al. (2003) The Arabidopsis phytoalexin NRT1.1 is a component of the natural resistance to Powdery mildew.
10. Chang J, Larkindale J, Dimmelmeier H, et al. (2003) Rapid and sensitive detection of DNA and RNA by AgI/Peroxidase colorimetric assay.
9. Chang J, Larkindale J, Dimmelmeier H, et al. (2003) Rapid and sensitive detection of DNA and RNA by AgI/Peroxidase colorimetric assay.
8. Chang J, Larkindale J, Dimmelmeier H, et al. (2003) Rapid and sensitive detection of DNA and RNA by AgI/Peroxidase colorimetric assay.
7. Chang J, Larkindale J, Dimmelmeier H, et al. (2003) Rapid and sensitive detection of DNA and RNA by AgI/Peroxidase colorimetric assay.
6. Chang J, Larkindale J, Dimmelmeier H, et al. (2003) Rapid and sensitive detection of DNA and RNA by AgI/Peroxidase colorimetric assay.
5. Chang J, Larkindale J, Dimmelmeier H, et al. (2003) Rapid and sensitive detection of DNA and RNA by AgI/Peroxidase colorimetric assay.
4. Chang J, Larkindale J, Dimmelmeier H, et al. (2003) Rapid and sensitive detection of DNA and RNA by AgI/Peroxidase colorimetric assay.
3. Chang J, Larkindale J, Dimmelmeier H, et al. (2003) Rapid and sensitive detection of DNA and RNA by AgI/Peroxidase colorimetric assay.
2. Chang J, Larkindale J, Dimmelmeier H, et al. (2003) Rapid and sensitive detection of DNA and RNA by AgI/Peroxidase colorimetric assay.
1. Chang J, Larkindale J, Dimmelmeier H, et al. (2003) Rapid and sensitive detection of DNA and RNA by AgI/Peroxidase colorimetric assay.