LETTERS

A translocation signal for delivery of oomycete effector proteins into host plant cells

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Bacterial[1], oomycete[2] and fungal[3] plant pathogens establish disease by translocation of effector proteins into host cells, where they may directly manipulate host innate immunity. In bacteria, translocation is through the type III secretion system[1], but analogous processes for effector delivery are uncharacterized in fungi and oomycetes. Here we report functional analyses of two motifs, RXLR and EER, present in translocated oomycete effectors[2]. We use the *Phytophthora infestans* RXLR-EER-containing protein Avr3a as a reporter for translocation because it triggers RXLR-EER-independent hypersensitive cell death following recognition within plant cells that contain the R3a resistance protein[4,5]. We show that Avr3a, with or without RXLR-EER motifs, is secreted from *P. infestans* biotrophic structures called haustoria, demonstrating that these motifs are not required for targeting to haustoria or for secretion. However, following replacement of Avr3a RXLR-EER motifs with alanine residues, singly or in combination, or with residues KMIK-DDK—representing a change that conserves physicochemical properties of the protein—*P. infestans* fails to deliver Avr3a or an Avr3a–GUS fusion protein into plant cells, demonstrating that these motifs are required for translocation. We show that RXLR-EER-encoding genes are transcriptionally upregulated during infection. Bioinformatic analysis identifies 425 potential genes encoding secreted RXLR-EER class proteins in the *P. infestans* genome. Identification of this class of proteins provides unparalleled opportunities to determine how oomycetes manipulate hosts to establish infection.

One of the most significant unanswered questions in plant pathology is how microbial eukaryotic pathogens, such as fungi and oomycetes, manipulate host metabolism and defences to establish disease. Identification of bacterial effector proteins has provided unparalleled insights into the evolution of bacterial pathogenesis and host mimicry employed by bacterial proteins to interfere with host signalling and signal transduction processes.

In contrast, we know little about effector function in fungi and oomycetes, and nothing about how such proteins are delivered into host cells. Recognition by intracellular host resistance (R) proteins, triggering localized cell death called the hypersensitive response, is evidence that fungal and oomycete effectors enter plant cells. Such effectors are termed avirulence (AVR) proteins, and several have been identified in fungi[6–8] and oomycetes[4,9–11].

The identification of a common motif, RXLR, in oomycete AVR proteins[2,11] sparked excitement and speculation regarding translocation of effectors from these important filamentous, fungus-like pathogens. The motif resembles, in sequence and relative location, a motif (RXLXE/D/Q) required for translocation of *Plasmodium* (malaria parasite) effectors into host erythrocytes[12,13]. Indeed, the *P. infestans* RXLR motif has been demonstrated to function in the *Plasmodium* pathosystem[14,15]. Three of the four oomycete AVR proteins also possess an EER motif within 25 residues downstream of the RXLR[11]. Two key questions arise regarding the motifs' role: (1) are they required to translocate effector proteins into plant host cells? (2) Are such effector proteins delivered from haustoria, which, following invagination of the host cell membrane, are in intimate contact with host cells? Our findings are summarized schematically in Supplementary Fig. 1.

Phytophthora infestans is the best-studied oomycete as a result of its role in precipitating the Irish potato famines in the mid-19th century, and remains the most economically important potato pathogen. Infection involves two phases: a biotrophic phase up to 36 h post inoculation (h.p.i.) in which *P. infestans* forms haustoria and requires living plant tissue, and an ensuing necrotrophic phase in which infected host tissue becomes necrotic. Gene expression of 38 predicted secreted RXLR-EER class effectors from *P. infestans* was quantified using real-time reverse transcribed polymerase chain reaction (real-time RT–PCR) in pre-infection stages, and throughout infection of potato up to 72 h.p.i. The 38 *P. infestans* genes (see Supplementary Information for bioinformatics analyses) were all induced and could be broadly grouped as: predominantly upregulated in pre-infection only; predominantly in pre-infection and biotrophy; in pre-infection and throughout infection; and, in biotrophy only (Supplementary Fig. 2 and Supplementary Table 1). Upregulation of these genes during infection indicates that the RXLR-EER motifs may have high predictive value in identifying potential secreted pathogenicity determinants. Using a hidden Markov model based on the alignment of the 38 induced genes we queried the preliminary assembly of the *P. infestans* genome sequence (http://www.broad.mit.edu/annotation/genome/phytophthora_infestans), which predicted 284 RXLR-EER class effectors. We also extended existing methodology[14] to predict 310 RXLR-EER class sequences. These combined approaches predicted 425 sequences, representing a proposed upper limit for the number of secreted RXLR-EER class genes in *P. infestans*. One hundred and sixty-nine sequences were identified by both methods, constituting a set of sequences with priority for further investigation (see Supplementary Information for bioinformatics analyses). These approaches also identified 400 and 314 RXLR-EER class effector genes in the recently reported genome sequences[16] of *P. sojae* and *P. ramorum*, respectively.

Resistance protein R3a recognizes Avr3a in the host cytoplasm[4], triggering the hypersensitive response. Intracellular recognition of Avr3a was further investigated here by expression and delivery of Avr3a from the type III secretion system (T3SS) of the bacterial potato pathogen, *Pectobacterium atrosepticum*[17,18]. Secretion of

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Avr3a as a fusion protein by the T3SS was demonstrated by co-infiltration of *Nicotiana benthamiana* leaves with *Agrobacterium tumefaciens* that delivered the *R3a* gene, and yielded a clear hypersensitive response. In contrast, a hypersensitive response was not observed when Avr3a was fused to a bacterial non-secreted protein or a type II secreted protein (Supplementary Fig. 3), or when the T3SS was disabled, indicating that translocation was dependent on the T3SS (Fig. 1). These results confirm that Avr3a must be delivered inside the plant cell for recognition by R3a, implying that Avr3a translocation by *P. infestans* may require a structure and environment—as is created by the haustorium and extrahaustorial matrix—and/or a mechanism functionally analogous to the T3SS.

The ability of Avr3a to trigger hypersensitive-response-mediated resistance was exploited to determine the role of the RXLR-EER motifs in translocation of the effector into host cells. The RXLR and EER motifs were replaced, singly and in combination, by alanine residues, and these forms were stably expressed from a virulent (homozygous for the non-recognized allele *avr3a*; ref. 4) *P. infestans* isolate. As expected, expression of native Avr3a yielded a hypersensitive response on only plants expressing R3a, whereas transformants expressing the alanine replacement forms of Avr3a were virulent (no hypersensitive response) on these plants (Fig. 2). Alanine substitutions may have a more wide-ranging effect than simply masking the side-chains at the motifs, thus more conservative amino acid substitutions KMIK-DDK (see Supplementary Information for bioinformatics analyses) were used to replace the RXLR-EER motifs, and the modified *Avr3a* was stably expressed in a virulent *P. infestans* isolate. As seen for the alanine replacements, the KMIK-DDK-Avr3a transformants were virulent on R3a-expressing potato (Fig. 2). All transformants, irrespective of the level of overexpression of *Avr3a* native, alanine and KMIK-DDK replacement forms (Supplementary Table 2), remained virulent on potato cultivar Bintje, which lacks *R3a*, demonstrating that transformation had not itself affected pathogenicity (transformant K9 shown in Supplementary Fig. 4).

Recognition of Avr3a by R3a occurs within the plant cell[4] independently of the RXLR-EER motifs[5]. Therefore, these results suggest a role for the motifs in translocation of Avr3a into host cells. The oomycete avirulence protein ATR13 from *Hyaloperonospora parasitica*, and numerous secreted effectors from *P. infestans*, have the RXLR motif but lack, or possess a variant of, the EER motif[11]. Transcriptional profiling of seven such genes in *P. infestans* indicated that they were induced during infection (Supplementary Fig. 2 and Supplementary Table 1). These proteins thus either have a function outside plant cells or are able to compensate for the lack of the EER motif with alternative sequences aiding translocation.

Secretion of Avr3a was examined using *P. infestans* transformants expressing Avr3a fused to the monomeric red fluorescent protein (mRFP). Although constitutively overexpressed, localization of Avr3a–mRFP secretion was observed only from haustoria into the extra-haustorial matrix during infection of potato leaves (Fig. 3 and Supplementary Fig. 5). In contrast to similar experiments with *Plasmodium* and erythrocytes[12,13], no fluorescence from

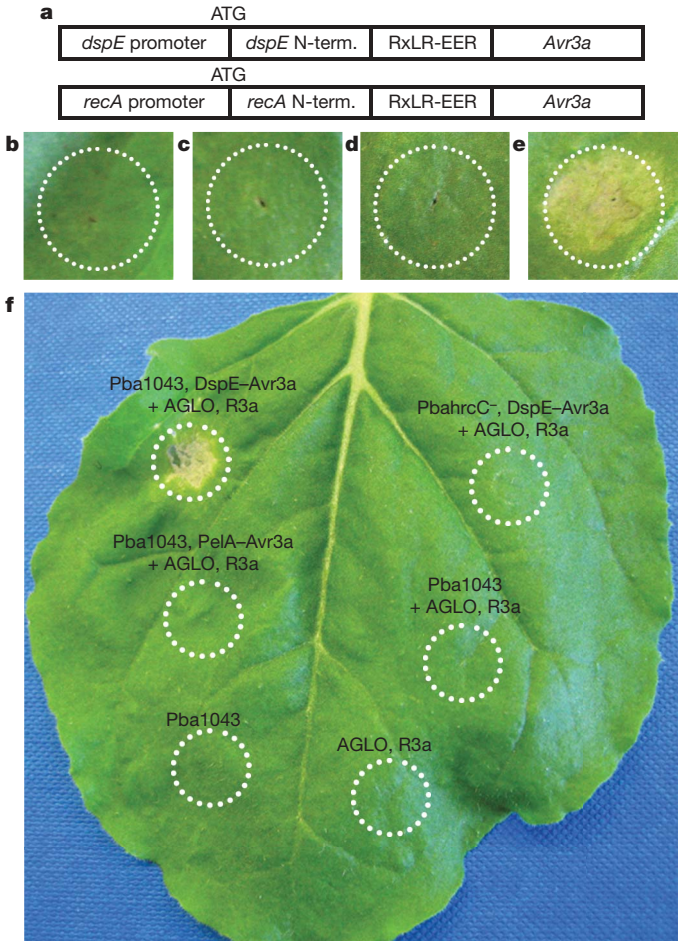

Figure 1 | Recognition of Avr3a by R3a follows delivery into the host cell by the T3SS of *Pectobacterium atrosepticum* (*Pba*). **a**, Constructs for expression of *Avr3a* in *Pba* using promoter regions and N-terminal 100 amino acid (pr100)-coding regions of *dspE* (T3SS-translocated) and *recA* (non-secreted). The N-terminal 50 amino acids of *Pba* DspE (ref. 18) contains the amino acid profile associated with translocation of T3SS effectors in *Pseudomonas syringae*[27]. The translation start site is indicated as ATG. *Avr3a* fusion constructs encode protein lacking native Avr3a signal peptide (amino acids 1–21), but retaining RXLR-EER motifs. **b**, Infiltration of *A. tumefaciens* (AGLO) delivering *R3a*. **c**, Infiltration of *Pba* expressing *dspE(pr100)–Avr3a(22–147)*. **d**, Co-infiltration of *Pba* expressing *recA(pr100)–Avr3a(22–147)* and AGLO delivering *R3a*. **e**, Co-infiltration of *Pba* expressing *dspE(pr100)–Avr3a(22–147)* and AGLO delivering *R3a*, yielding a hypersensitive response, indicating Avr3a fusion protein delivery into the host cell. **f**, Leaf showing (co-) infiltrations as indicated (see Supplementary Fig. 3 for details on *pelA–Avr3a* fusion results). *Pba1043* is the wild-type strain and *PbahrcC*$^{-}$ is T3SS-disabled. Dotted circles indicate zones of infiltration.

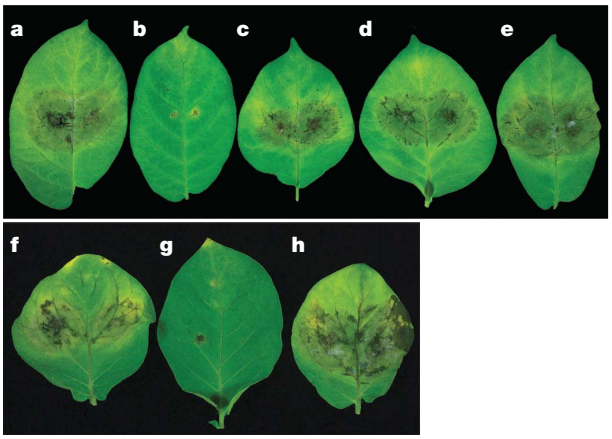

Figure 2 | Replacement of RXLR-EER motifs with alanine residues, singly or in combination, or with amino acids KMIK-DDK, prevents delivery of Avr3a into host cells. Leaves of potato cultivar Pentland Ace (*R3a*), infected with: **a**, **f**, non-transformed *P. infestans* isolate 88069 (*avr3a*); **b**, **g**, transformant K9 expressing *Avr3a* encoding Avr3a with RXLR-EER; **c**, transformant 5A11 expressing *Avr3a* encoding alanine replacement AAAA-EER; **d**, transformant 4A3 expressing *Avr3a* encoding alanine replacement RXLR-AAA; **e**, transformant 7A4 expressing *Avr*3a encoding alanine replacement AAAA-AAA; **h**, transformant KD2 expressing *Avr3a* encoding replacement KMIK-DDK. Transformant K9 was virulent on susceptible potato cultivar Bintje (Supplementary Fig. 4). Expression levels of introduced *Avr3a* variants in all transformants exceeded that of *avr3a* in non-transformed isolate 88069 (Supplementary Table 2).

Avr3a–mRFP was observed within infected plant cells, presumably due to dilution within plant cells, which are many-fold larger than erythrocytes. Transformants expressing Avr3a–mRFP in which the RXLR-EER motifs were replaced by alanine residues also exhibited red fluorescence at haustoria and extra-haustorial matrices (Fig. 3 and Supplementary Fig. 5), demonstrating that these motifs neither function for targeting Avr3a to haustoria, nor act as a secretion signal. Alanine-replacement transformants expressed the fusion gene from at least half the level of that for the transformant expressing the highest level of introduced native *Avr3a–mRFP* fusion (Supplementary Table 3). Nevertheless, fluorescence observed for the alanine replacement form differed in that it was both significantly brighter and was observed surrounding plant cells containing haustoria (Fig. 3, and Supplementary Fig. 5). Our interpretation of these observations is that the alanine replacement fusion protein is secreted but no longer enters host cells, instead accumulating in the extra-haustorial matrix and overspilling into the host apoplast.

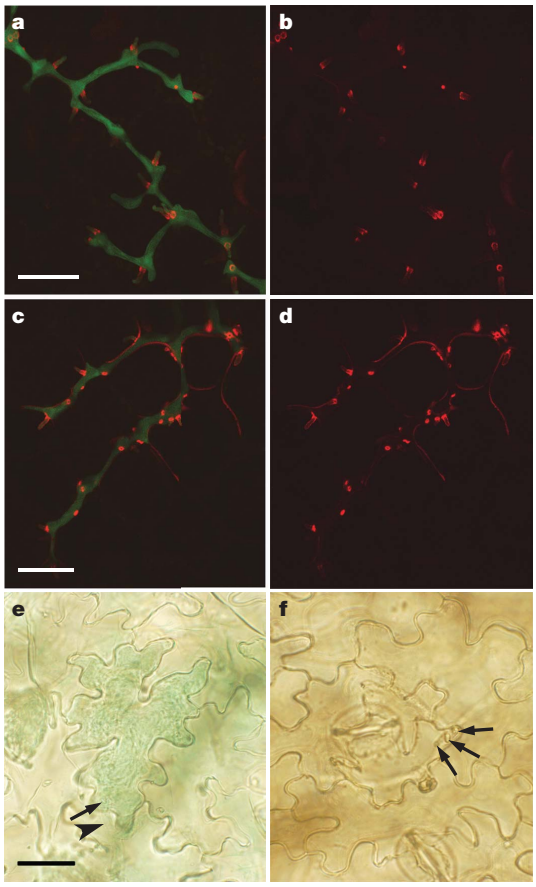

Figure 3 | Avr3a is secreted from haustoria and is translocated into the host cell in an RXLR-EER-dependent manner. Confocal laser scanning microscopy of *Phytophthora infestans* transformants expressing green fluorescent protein to define hyphal cytoplasm, and translational fusions of Avr3a with the monomeric red fluorescent protein. **a**, Overlay projection of signal peptide (SP)-RXLR-EER-Avr3a–mRFP in infected potato leaf tissue showing secretion of Avr3a specifically from finger-like haustoria. **b**, Red channel image of the same field as in **a**. **c**, Overlay projection of SP-AAAA-AAA-Avr3a–mRFP in infected potato, showing red fluorescence in haustoria and the host apoplast adjoining haustoria. **d**, Red channel image of the same field as in **c**. **e**, Light microscopy of *P. infestans* transformant RGUS9 expressing SP-RXLR-EER–GUS fusion (20× objective lens). An intercellular hypha (solid arrowhead) passes underneath the leaf epidermal cell exhibiting GUS activity, and penetrates this cell with a haustorium (long arrow). **f**, *P. infestans* transformant 7AGUS3 expressing SP-AAAA-AAA–GUS fusion (20× objective lens). No GUS activity can be seen in the invaded epidermal cell; at least three haustoria have penetrated this cell (long arrows). Scale bars represent 25 μm for **a** and **b**, 30 μm for **c** and **d**, and 50 μm for **e** and **f**.

To investigate further the translocation of Avr3a from haustoria into the plant cell, the signal peptide (SP)-RXLR-EER-encoding domain of *Avr3a*, and a version in which the motifs were replaced with alanine residues (SP-AAAA-AAA) were fused to the amino terminus of the *Escherichia coli gusA* gene, which encodes β-glucuronidase (GUS), and stably expressed in *P. infestans*. *GusA* was chosen because its product is active within plant cells but inactive in the apoplast[19], and is thus an ideal reporter for translocation of proteins to the inside of plant cells. SP-RXLR-EER–GUS and SP-AAAA-AAA–GUS transformants were selected that expressed and secreted high levels of active GUS into culture medium (Supplementary Fig. 6). However, when infecting potato, GUS activity was observed only with the SP-RXLR-EER–GUS transformants, and only within plant cells in contact with haustoria (Fig. 3 and Supplementary Fig. 7). No GUS activity was observed within plant cells or in the apoplast in the case of the SP-AAAA-AAA–GUS transformants (Fig. 3 and Supplementary Fig. 7), indicating that GUS was not translocated into the plant cell.

Our results show that, following secretion from haustoria into the extra-haustorial matrix, Avr3a is translocated into host cells by an unknown mechanism dependent on the RXLR-EER motifs. This dependence implies that other *P. infestans* RXLR-EER class effectors are transported by a similar mechanism. The similarities of function and positioning between this oomycete translocation signal and the malarial host targeting signal RXLXE/D/Q within effector proteins have been considered to reinforce the phylogenetic relatedness of these organisms[15]. However, haustorium-forming and non-haustorium-forming fungal plant pathogens, which do not possess RXLR-EER class effectors, also secrete avirulence and effector proteins that are perceived by cytoplasmic host resistance proteins, although their mode of translocation is unknown. It is possible that they also contain peptide motifs required for translocation into host cells.

The bacterial T3SS is a well-characterized mechanism for delivery of effectors from bacteria into host cells. However, identification of a protein sequence motif for T3SS-dependent translocation and, consequently, predicting the full range of T3SS-delivered proteins from plant pathogens such as *Pseudomonas syringae*, have provided many challenges[20]. A functionally analogous mechanism for delivery of oomycete effectors into host cells has yet to be elucidated. Indeed, it is possible that, when RXLR-EER class effectors are secreted into the extrahaustorial matrix, and thus in intimate contact with the host cell membrane, that these motifs alone effect translocation, potentially using host endocytic mechanisms. Nevertheless, determination of the RXLR-EER translocation signal permits facile identification of this class of potential translocated virulence proteins. This discovery will facilitate investigations into how this important group of pathogens manipulate host defences and metabolism to establish disease. Moreover, comparisons between the RXLR-EER effector complements of sequenced oomycete genomes, and studies of co-evolution of effectors and their host target proteins, will yield insights into how host range is defined.

METHODS SUMMARY

Gene expression. Plant growth, manipulation of *P. infestans* isolate 88069, plant inoculation, RNA extraction, complementary DNA synthesis, and real-time RT–PCR were carried out as described[21,22] (Supplementary Table 4).

Bioinformatics. Hidden Markov models and an extended heuristic method[14,23] were both used to predict secreted RXLR-EER class effectors in the draft *P. infestans* genome (see Supplementary Information bioinformatics analyses).

***Pectobacterium atrosepticum* delivery of Avr3a.** The native promoter and region encoding approximately the 100 N-terminal amino acids of genes *dspE*, *recA* or *pelA* were fused to *Avr3a* lacking a signal peptide sequence, and transformed into *P. atrosepticum*. Fully expanded leaves of *Nicotiana benthamiana* plants were infiltrated with each *P. atrosepticum* strain.

***P. infestans* transformation.** *Avr*3a, with and without the RXLR and/or EER replaced by alanine or KMIK-DDK residues, was cloned into the oomycete expression vector pTOR[24], incorporating mismatches across the RXLR or EER

motifs. Avr3a and alanine replacement variants were fused in frame to the *mRFP* gene in vector pTORmRFP4. Plasmid pVWII for GFP expression has been described previously[25]. *Avr3a* SP-RXLR-EER- or SP-AAAA-AAA-encoding sequences were fused in frame to the *gusA* gene in pTOR. Transformation of *P. infestans* was as described[26], modified as described in Supplementary Methods.

***P. infestans* potato inoculation.** Sporangia were inoculated onto detached leaflets of potato cultivars Pentland Ace (*R3a*) or Bintje (*R*-gene-free), and incubated at 20 °C for 5 d to enable disease symptoms to develop. Untransformed isolate 88069 was used as a control.

Confocal microscopy. GFP (488 nm excitation; emission 500–530 nm) and mRFP (561 nm excitation; emission 600–630 nm) were imaged on a Leica TCS-SP2 AOBS confocal microscope.

Histochemical GUS assay. Infected leaf tissue of potato cv. Bintje was submerged in assay buffer containing GUS substrate for 24 h at 20 °C. Leaf tissue was cleared with 70:30 ethanol:water before microscopy on an Olympus BH-2 microscope.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature. A summary figure is also included.

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Author Contributions S.C.W., P.R.J.B., P.v.W. and I.K.T developed the concept and designed experiments. S.C.W. and S.G. performed *P. infestans* transformations and plant inoculations. P.C.B. carried out confocal microscopy and advised on cell biology. S.C.W. performed GUS assays and light microscopy. A.O.A. and J.G.M. quantified gene expression. Antibody detection of tagged transformants was performed by I.H. and S.C. L.M., J.G.M., E.M.G. and M.R.A. carried out experiments with *P. atrosepticum.* L.P. conducted all bioinformatics analyses.

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METHODS

RNA extraction and real-time RT–PCR. Growth of potato plants, *P. infestans* isolate 88069, and plant inoculation were carried out as described[21]. Life cycle stages of *P. infestans* (axenic cultured mycelium, sporangia, zoospores, germinated cysts) were also prepared as described[21]. RNA extraction, first strand complementary DNA synthesis and SYBR green real time RT–PCR assays were carried out as described[22]. Primer sequences for real-time RT–PCR are listed in Supplementary Table 4.

Bioinformatic identification of candidate RXLR-EER sequences. A heuristic method to identify sequences containing a signal peptide and the RXLR motif[14,23] was extended to incorporate the presence of an [ED][ED][KR] motif downstream and within 40 amino acids of the RXLR motif. A hidden Markov model was also constructed, based on an alignment of the RXLR-EER-containing regions of 38 proteins from *P. infestans* that are known to be induced during infection. Each model was used to compile a list of candidate open reading frames that potentially encode secreted RXLR-EER class effectors in the draft *P. infestans* genome (described in detail in Supplementary Information bioinformatics analyses).

Expression of Avr3a fusion constructs in *Pectobacterium atrosepticum*. The native promoter and the region encoding approximately 100 amino acids of the N terminus of genes *dspE, recA* or *pelA* were PCR-amplified using primer gene_F and primer gene_Avr3a_R from *P. atrosepticum* (Supplementary Table 5). *Avr3a(67–441)* (nucleotides 67 to 441; GenBank AJ893357; ref. 4) (lacking signal-peptide-encoding sequences) was PCR-amplified from pRTL2-Avr3a (ref. 4) using primer Avr3a_gene_F and Avr3a_R (Supplementary Table 5). N-terminus-encoding PCR products were fused to *Avr3a* purified products by extension followed by overlapping PCR conducted with gene_F and Avr3a_R primers. *Pel*A-*Flag*–*Avr*3a fusions were constructed with sequence encoding a Flag epitope tag located in the PCR overlap between *pelA* and *Avr3a*. Products were purified and cloned into pGEM-T Easy vector (Promega), then electroporated into *Escherichia coli* DH10B cells (Invitrogen). Plasmid preparations were sequenced to confirm the fidelity of the *P. atrosepticum* gene–*Avr3a* fusions. Plasmids were then electroporated into *P. atrosepticum* strain SCRI1043. A full description of transformation constructs and protocol can be found in the Supplementary Methods.

For hypersensitive response assays, fully expanded leaves of *Nicotiana benthamiana* plants (approximately 6 weeks old) were infiltrated on the lower surface by first stabbing lightly with a needle then infiltrating with a blunt syringe. Approximately 100 µl of each strain, *P. atrosepticum* wild-type strain SCRI1043 or *hrcC*$^{-}$ mutants containing plasmids with *P. atrosepticum gene–Avr3a* fusions, with *A. tumefaciens* strain AGLO carrying pBINplus-R3a were infiltrated singly or co-infiltrated as described[4]. All strains were grown overnight in LB broth with an appropriate antibiotic to 2×10^8 colony forming units ml^{-1} then mixed at a ratio of 2:1 *A. tumefaciens*:*P. atrosepticum*. Plants were kept at 18 °C at high humidity overnight (16 h). Images were captured at 120 h using a Nikon digital camera.

***P. infestans* transformation vector construction and transformation.** The *Avr*3a open reading frame was PCR amplified (primers Avr3F and Avr3R; Supplementary Table 5) and cloned into the oomycete constitutive expression vector pTOR (ref. 24). *Avr3a*, with sequence encoding the RXLR or EER to be replaced by alanine residues, was generated by amplification of the *Avr3a* gene in two segments (primer combinations Avr3ClaF2 and 5ANgoM4R or 4ANotR; primers 5ANgoM4F, 4ANotF, or KMIKDDKNgoF and Avr3aStopSacR), incorporating mismatches across the RXLR or EER motifs. The two segments were then ligated together and inserted into the pTOR vector. *Avr3a* with sequence encoding both RXLR and EER motifs to be replaced by alanine residues was generated by starting with the EER alanine replacement sequence and proceeding through the same strategy used to generate the RXLR to AAAA sequence. The *mRFP* gene was PCR amplified (primers mRFPBsiF and mRFPSacR) and incorporated into the pTOR vector to yield pTORmRFP4. *Avr3a* and the RXLR alanine replacement variants described previously were then cloned in frame without a stop codon (primers Avr3ClaF2 and Avr3BsiR), ahead of the mRFP gene. Insert integrity and correct reading frame orientation of the cloned inserts were verified by sequencing. Plasmid pVWII for enhanced green fluorescent protein expression has been described previously[25].

RXLR-EER–GUS and AAAA-AAA–GUS fusions were constructed by amplification of the *Avr3a* SP-RXLR-EER-encoding sequence (primers Avr3ClaF2 and RXLRnotR), SP-AAAA-AAA-encoding sequence (primers Avr3ClaF2 and 7AnotR), and *gusA* (primers GUSnotF and GUSsacR), followed by ligation of the SP-RXLR-EER- or SP-AAAA-AAA-encoding sequence to the *gusA* gene in pTOR. Transformation of *E. coli* and determination of construct integrity were as described.

Stable transformation of *P. infestans* was achieved using a PEG-$CaCl_2$-Lipofectin protocol[26] (http://138.23.152.128/protocols/protocols.html), modified by substituting Novozym 234 with a mixture of 5 mg ml^{-1} lysing enzymes (from *Trichoderma harzianum*; Sigma L1412) and 2 mg ml^{-1} cellulase (from *Trichoderma reesei*; Sigma C8546) to release protoplasts from germinating sporangia. A full description of transformation constructs and protocol can be found in Supplementary Methods.

Inoculation of *P. infestans* transformants onto potato leaves. Sporangia (5×10^4 ml^{-1}) of stable transformants were inoculated in 10 µl droplets onto detached leaflets of potato cultivars Pentland Ace (*R3a*) or Bintje (*R*-gene-free). Inoculated leaflets were incubated at 20 °C for 5 d to enable disease symptoms to develop. For each transformant, six leaflets were inoculated on three independent occasions. Samples were taken at 120 h.p.i. for RNA extraction and real-time RT–PCR analysis, as described previously. As a control, untransformed isolate 88069 was used. Infected leaves were photographed under polarized light to avoid reflection from the leaf surfaces.

Imaging and quantification of fluorescent proteins. All GFP and mRFP imaging was conducted on a Leica TCS-SP2 AOBS confocal microscope using HC PL FLUOTAR ×63 0.9 (numerical aperture) (as for Fig. 3a–d), HCX APO L U-V-I ×40 0.8 or HCX APO L U-V-I ×20 0.5 water-dipping lenses. For all images shown in this paper, the 561 nm line from the 'lime' diode laser was set at 100% on the AOBS. PMT2 was set at either 670 V (Fig. 3a, b) or 603 V (Fig. 3c, d). The total mRFP fluorescence of each haustorium with a circular profile was measured within a region-of-interest with an area of approximately 13 µm^2, using the Leica confocal software quantification package. Fluorescence measurements were exported to Microsoft Excel and standard errors of the mean calculated.

Histochemical GUS assay. GUS substrate (0.3 mg ml^{-1} 5-bromo-4-chloro-3-indolyl β-D-glucuronide) was allowed to infiltrate into infected leaf tissue of potato cv. Bintje submerged in assay buffer (50 mM $NaPO_4$, pH 7.0, 0.5% Triton X-100) for 24 h at 20 °C. This gentle procedure was adopted because vacuum infiltration of substrate and 37 °C incubation resulted in disruption of host and pathogen cells, giving non-specific localization of GUS activity. Leaf tissue was cleared with 70:30 ethanol:water before microscopy on an Olympus BH-2 microscope fitted with 6.3×, 10×, 20× or 40× objective lenses, and an Olympus DP70 digital camera for image capture.