Plasmodium falciparum and *Hyaloperonospora parasitica* effector translocation motifs are functional in *Phytophthora infestans*

Severine Grouffaud,^{1,2} Pieter van West,² Anna O. Avrova,¹
Paul R. J. Birch³ and Stephen C. Whisson¹

Correspondence
Stephen C. Whisson
Steve.Whisson@scri.ac.uk

¹Plant Pathology Programme, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

²Aberdeen Oomycete Group, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, UK

³University of Dundee, Division of Plant Sciences, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

Received 8 July 2008
Revised 15 August 2008
Accepted 25 August 2008

The oomycete potato late blight pathogen, *Phytophthora infestans*, and the apicomplexan malaria parasite *Plasmodium falciparum* translocate effector proteins inside host cells, presumably to the benefit of the pathogen or parasite. Many oomycete candidate secreted effector proteins possess a peptide domain with the core conserved motif, RxLR, located near the N-terminal secretion signal peptide. In the *Ph. infestans* effector Avr3a, RxLR and an additional EER motif are essential for translocation into host cells during infection. Avr3a is recognized in the host cytoplasm by the R3a resistance protein. We have exploited this cytoplasmic recognition to report on replacement of the RxLR-EER of Avr3a with the equivalent sequences from the intracellular effectors ATR1NdWsB and ATR13 from the related oomycete pathogen, *Hyaloperonospora parasitica*, and the host targeting signal from the *Pl. falciparum* virulence protein PfHRP2. Introduction of these chimeric transgenes into *Ph. infestans* and subsequent virulence testing on potato plants expressing R3a demonstrated the alternative motifs to be functional in translocating Avr3a inside plant cells. These results suggest common mechanisms for protein translocation in both malaria and oomycete pathosystems.

INTRODUCTION

An emerging view of the interaction mechanisms of pathogenic microbes and parasites with their hosts involves the movement, or translocation, of pathogen proteins inside infected host cells (Haldar *et al.*, 2006; Ellis *et al.*, 2007; He *et al.*, 2007; Birch *et al.*, 2008). These proteins are termed effectors and may perform a variety of functions in the establishment of host infection, but are generally considered to reprogramme the host cell to the benefit of the pathogen. This may be through redirection of cellular resources, cell structural reorganization, or suppression of defence responses. The mechanisms by which effector proteins are translocated into host cells may include direct injection, as for plant-pathogenic nematodes (Davis *et al.*, 2008), delivery via a specialized type III secretion system (T3SS), as for bacteria (Gálan & Wolf-Watz, 2006), or by as yet undetermined mechanisms mediated through host-targeting (HT) peptide motifs, as for malaria parasites (Hiller *et al.*, 2004; Marti *et al.*, 2004), fungi (Dodds *et al.*,

2004; Ellis *et al.*, 2006; Catanzariti *et al.*, 2007) and oomycetes (Rehmany *et al.*, 2005; Armstrong *et al.*, 2005; Whisson *et al.*, 2007; Birch *et al.*, 2008).

Evolutionarily distinct from true fungi, oomycetes encompass a wide range of destructive filamentous plant pathogens that include the genera *Phytophthora* and *Pythium*, and the downy mildews (genera *Hyaloperonospora*, *Bremia*, *Plasmopara*). Oomycetes belong within the stramenopiles (heterokonts) and are phylogenetically rooted with the apicomplexan genera such as *Plasmodium* (malaria parasites), *Cryptosporidium* and *Toxoplasma* (Baldauf *et al.*, 2000; Burki *et al.*, 2007). *Phytophthora infestans* is the best-studied oomycete, following its role in precipitating the Irish potato famines in the mid-19th century. It remains the most economically important potato pathogen worldwide.

Ph. infestans, like many other oomycete and fungal pathogens, produces specialized intracellular infection structures called haustoria through invagination of the host cell membrane, allowing the pathogen to form an intimate association with the host cell. Haustoria are thus

Abbreviations: HR, hypersensitive response; HT, host targeting; T3SS, type III secretion system.

encased in the host plasma membrane and this situation is reminiscent of the parasitophorous vacuole produced by *Pl. falciparum* in infected animal erythrocytes. The pathogen/parasite exports proteins into the extrahaustorial matrix/lumen of the parasitophorous vacuole by means of a conventional hydrophobic signal sequence. Transport of effector/virulence proteins through the host-derived membrane requires an additional HT signal, located near the N-terminal signal peptide of many secreted proteins, the core of which is RxLxE/D/Q (*Plasmodium*) or RxLR (oomycetes) (Hiller *et al.*, 2004; Marti *et al.*, 2004; Bhattacharjee *et al.*, 2006, 2008; Whisson *et al.*, 2007; Birch *et al.*, 2008; MacKenzie *et al.*, 2008). A subset of RxLR effectors also possess a region enriched for acidic residues following the RxLR motif, and terminating in EER. Peptide sequences surrounding the core RxLxE/D/Q or RxLR signal also have a role in translocation (Lopez-Estraño *et al.*, 2003; Bhattacharjee *et al.*, 2006; Whisson *et al.*, 2007) and have additional properties such as hydrophobicity profile relevant to subcellular location (Hiss *et al.*, 2008).

The avirulence gene *Avr3a* from the oomycete *Ph. infestans* encodes the RxLR-EER effector protein Avr3a, which is recognized by the R3a resistance protein in the host cytoplasm, triggering the hypersensitive response (HR) (Armstrong *et al.*, 2005; Whisson *et al.*, 2007). The HR is a form of programmed cell death in the resistant plant that limits the spread of pathogen growth, as the pathogen requires living host tissue for survival. The C-terminal region of Avr3a is sufficient for recognition (Bos *et al.*, 2006), and both of the RxLR and EER motifs are essential for translocation of Avr3a into the host cell (Whisson *et al.*, 2007). Furthermore, the RxLR-EER domain was capable of translocating other fused proteins, such as the β -glucosidase (*gusA*) gene, without the involvement of any additional peptide sequences from the C-terminal region of Avr3a (Whisson *et al.*, 2007). The validated recognition of Avr3a only within host cells yields a useful bioassay to study the specificity and mechanisms of effector translocation in oomycetes. That is, transformation of a virulent *Ph. infestans* isolate (carrying the non-recognized *avr3a* allele) with various *Avr3a* constructs, in which the endogenous translocation motif has been replaced with alternative sequences, should generate avirulent transformants if the alternative sequence is functionally similar.

Previously, alanine (AAAA-EER and RxLR-AAA) and physico-chemically conservative (KMIK-DDK) substitutions of the RxLR-EER motifs in Avr3a were shown to abolish translocation of the effector following its secretion from haustoria (Whisson *et al.*, 2007). However, some RxLR effectors from *Ph. infestans*, *Phytophthora sojae* and *Hyaloperonospora parasitica* exhibit potential amino acid variation in the 'EER' motif of candidate effectors (Rehmany *et al.*, 2005; Win *et al.*, 2007; Whisson *et al.*, 2007). Examples are the Avr1b protein from *Ph. sojae*, which contains the variant GER sequence instead of EER (Rehmany *et al.*, 2005), and the ATR13 protein from *H. parasitica*, which lacks a recognizable EER motif (Allen

et al., 2004). Although the RxLR-AAA and KMIK-DDK variants of Avr3a were not translocated, investigation of the translocation potential of ATR13-like RxLR sequences lacking an EER motif was not addressed (Whisson *et al.*, 2007). ATR13 contains heptad leucine/isoleucine repeats commencing within 10 residues of the RxLR motif (Fig. 1; Allen *et al.*, 2004) where the EER motif is located in ATR1NdWsB and Avr3a, and it is possible that these repeats could also be involved in the translocation process.

It has been demonstrated that the RxLR-EER domain from Avr3a was sufficient to export the green fluorescent protein (GFP) from *Pl. falciparum* to the erythrocyte (Bhattacharjee *et al.*, 2006), implying that plant and animal eukaryotic pathogens may share some conserved mechanism to deliver effector/virulence proteins into the host cell. Here we have performed the reciprocal experiments with *Ph. infestans* transformants to demonstrate function of the *Pl. falciparum* host targeting signal in translocation. Additionally, we have demonstrated that the RxLR and RxLR-EER motifs found in avirulence proteins from a distantly related oomycete can function to deliver the Avr3a effector into host cells.

METHODS

Cultures and growth conditions. *Ph. infestans* isolate 88069, homozygous for the R3a-non-recognized SEM allele *avr3a*, virulent on the R3a-expressing potato 'Pentland Ace', was maintained at 20 °C on RyeA agar amended with the antibiotics pimarcin (10 µg ml⁻¹; Sigma) and rifampicin (30 µg ml⁻¹; Sigma). 'Bintje' and 'Craig's Royal' (fully susceptible to *Ph. infestans*), and 'Pentland Ace' (*Ph. infestans* resistance allele R3a) potato plants were grown at 22 °C under a minimum of 16 h light per day.

Transformation vector construction. The National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) accession numbers for the genes used in this study are: AJ893356 (*Ph. infestans* Avr3a), AY842877 (*H. parasitica* ATR1NdWsB), AY785301 (*H. parasitica* ATR13) and AAD31511 (*Pl. falciparum* PfHRP11). ATR1NdWsB (Rehmany *et al.*, 2005) and ATR13 (Allen *et al.*, 2004) are cloned avirulence genes from the oomycete *H. parasitica*, the causal agent of downy mildew in the model plant *Arabidopsis thaliana*. The primary positions of the RxLR-EER motifs relative to each other and relative to the signal peptide are similar between Avr3a and ATR1NdWsB (Fig. 1). To conserve the primary physical location between the signal peptide and the 33 aa functional core of the *Pl. falciparum* histidine-rich protein II (PfHRP11) HT sequence (Bhattacharjee *et al.*, 2006), 10 residues downstream of the signal peptide of Avr3a were also retained, as this physical spacing might be required for function (Fig. 1).

For all transformation vectors, the C-terminal, elicitor-active region of the *Ph. infestans* Avr3a gene encoding amino acids 61–147 was amplified by PCR from an existing plasmid clone (Whisson *et al.*, 2007) using the following conditions: each 50 µl PCR contained 0.4 U Phusion Hot Start polymerase (New England BioLabs), 10 µl of 5 × reaction buffer (New England BioLabs), 15 mM deoxynucleotide triphosphates (Promega), 30 µM forward and reverse primers (Table 1), and 10 ng Avr3a plasmid DNA. All primer pairs for PCR of Avr3a sequence encoding the C-terminal 86 aa used the Avr3aStopSacR reverse primer. Forward primers were Avr3aPspF (fusion to ATR1NdWsB SP-RxLR-EER), Avr3aNotF (fusion to

A

Pi AVR3a: MRLAIMLSATAVAINFATCSAIDQTKVLVYGTGAHYIHDSAGR**RLLR**KNEENEETS**EER**-----

Hp ATR1: MRVCYFVLVPSVALAVIATESSETSGTIVHVPLRDVADHRNDALIN**RALR**AQTALDD**EER**-----

Hp ATR13: MRLVHAVLLPGIIVFVSNGLLHAHALHEDETGVTAG**RQLR**AAASEVFGLSRASFGLGKAQDPLDKFFRKIINSRKPIETSYSA

Pf HRPII: MVSFSKNKVLAAVFAVSVLLLDNNNSAFNNNL**CSKNAKGLNLNKRL****LHETQ**AHVDDAHHAHHVAD-----

B

Fig. 1. (A) Similarities in sequence and position between the conserved translocation motifs from oomycetes and *Pl. falciparum*. Amino acid sequences of N-terminal regions are shown, with the oomycete translocation motif and the malarial host-targeting (HT) signal (in red), for three oomycete avirulence proteins [Avr3a from *Ph. infestans* (*Pi*), ATR1NdWsB (ATR1) and ATR13 from *Hyaloperonospora parasitica* (*Hp*)], and the PfHRPII virulence protein from *Pl. falciparum* (*Pf*). Signal peptides for secretion are shown in blue. ATR13 heptad repeats are underlined. The defined 33-residue HT domain from *Pl. falciparum* PfHRPII is shown in bold italics. (B) Schematic representations of chimeric Avr3a constructs used in this study. Avr3a sequences are shaded blue, *H. parasitica* ATR1 sequences are shaded orange or yellow, ATR13 sequences are shaded green (SP and RxLR) or purple (heptad repeats), and the defined 33-residue HT domain from *Pl. falciparum* PfHRPII is shaded pink. ATR1NdWsB and ATR13 chimeric constructs used the signal peptides from these genes. ATR13(SP-RxLR)::Avr3a included the three alanine residues following the RxLR. Avr3a¹⁻³¹-PfHRPII(RxLxE)::Avr3a⁶¹⁻¹⁴⁷ used the signal peptide and following 10 residues from Avr3a. Numbers above each construct show final amino acid positions of motifs, or fusion junctions to Avr3a sequences. The elicitor-active C-terminal region of Avr3a (Avr3a⁶¹⁻¹⁴⁷) is present in each construct, although the overall length (final number of each construct) of the chimeric proteins may be longer or shorter due to different lengths of the N-terminal signal peptide-translocation motif region.

ATR13 SP-RxLR only), Avr3aBssF (fusion to ATR13 SP-RxLR-5 × Heptad), and AVRPFHTSalF2 (fusion to PfHRPII RxLxE). Thermocycling conditions were as follows: 96 °C for 5 min, followed by 35 cycles of 96 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s. A final extension step of 72 °C for 10 min was included. Plasmid clones of *H. parasitica* avirulence genes *ATR1NdWsB* and *ATR13* were obtained from J. Beynon, University of Warwick, UK. Sequences encoding the N-terminal 31 or 42 aa of *Ph. infestans* Avr3a, 62 aa of *H. parasitica* ATR1NdWsB, and 43 or 85 aa of *H. parasitica* ATR13 were amplified by PCR as described above using the same thermocycling conditions. Primer pairs were as follows: ATR1ClaF and ATR1PspR (ATR1NdWsB SP-RxLR-EER), ATR13claF and ATR13NotR (ATR13 SP-RxLR), ATR13claF and ATR13BssR (ATR13 SP-RxLR-5 × Heptad), Avr3aClaF2 and AVRPFHTSalR2 (Avr3a 31 aa including PfHRPII RxLxE). All PCR products were purified with the Qiagen Minelute kit using the supplied protocol. The two sections of the chimeric Avr3a effector genes were digested with the following restriction endonucleases: *PspOMI* (ATR1NdWsB),

NotI or *BssHII* (ATR13), and *SalI* (PfHRPII). Ligations of the two sections of gene used T4 DNA ligase (Promega) and were carried out at 4 °C for 16 h. Ligated fragments were separated by agarose gel electrophoresis and DNA fragments of the expected size purified from the gel (Qiagen Minelute kit) using the supplied protocol. Purified ligation products were digested with *ClaI* and *SacI*, purified again, and ligated (T4 DNA ligase, 4 °C, 16 h) into the pTOR oomycete expression vector (Blanco & Judelson, 2005; GenBank accession no. EU257520) digested with the same enzymes.

Plasmids were electroporated into *Escherichia coli* DH10B Electromax electrocompetent cells (Invitrogen). Insert integrity and correct reading frame orientation of the cloned inserts were verified by sequencing.

Transformation of *Ph. infestans*. Stable transformation of *Ph. infestans* was achieved as described by Whisson *et al.* (2007), using a modified PEG-CaCl₂-Lipofectin protocol (Judelson *et al.*, 1991). A

Table 1. Oligonucleotide primers used in plasmid construction and RT-PCR

Primer name	Sequence (5'–3')	Amplicon
Avr3ClaF2	GGAAATCGATACCATGCGTCTGGCAATTATGCTG	<i>Avr3a</i> from start, <i>ClaI</i> site
Avr3aStopSacR	GGAACCGCGGCTAATATCCAGTGAGCCCCAGGT	<i>Avr3a</i> stop codon reverse, <i>SacI</i> site
ATR1claF	GGAAATCGATACCATGCGCGTCTGCTACTTCGT	<i>ATR1</i> from start, <i>ClaI</i> site
ATR1PspR	GGAAGGGCCCTCTCTTCATCGTCATCGA	<i>ATR1</i> N-term. reverse, <i>PspOMI</i> site
Avr3aPspF	GGAAGGGCCCAATTTCAATTTGGCGAATCTA	<i>Avr3a</i> C-term. after EER, <i>PspOMI</i> site
AVRPfHTSalF2	GGAAGTCGACGACGCCCATCACGCTCATCACGTAGC- AGATGCCCCAAATTTCAATTTGGCGA	<i>PfHRP11-Avr3a</i> C-term., <i>Sall</i> site
AVRSPPfHTSalR2	GGAAGTCGACATGTGCTTGAGTTTCGTGTAATAATC- TTTTATTAAATTTAATCCTTTAGCATTTTTGCTACAA- TACACCAGGACCTTGGTT	<i>PfHRP11-Avr3a</i> N-term. reverse, <i>Sall</i> site
ATR13claF	GGAAATCGATACAATGCGCCTTGTTACGCG	<i>ATR13</i> from start, <i>ClaI</i> site
ATR13NotR	GGAAGCGGCCGCTCGGAGCTGGCGACCAG	<i>ATR13</i> RxLR reverse, <i>NotI</i> site
ATR13BssR	GGAAGCGCGCCGAATAACTGGTTTCGATG	<i>ATR13</i> heptad repeats reverse, <i>BssHII</i> site
Avr3aBssF	GGAAGCGCGCGCCCCAAATTTCAATTTGG	<i>Avr3a</i> C-term. after EER, <i>BssHII</i> site
Avr3aNotF	GGAAGCGGCCGCCCCAAATTTCAATTTGGCGAAT	<i>Avr3a</i> C-term. after EER, <i>NotI</i> site
RTATR1Avr3aF	ATCGCAACGACGCTCTAATCA	RT-PCR of <i>ATR1NdWsB-Avr3a</i>
RTATR1Avr3aR	GCCCTCTCTCTTCATCGTCATC	RT-PCR of <i>ATR1NdWsB-Avr3a</i>
RTPfAvr3F	GCTGTGCGCCATAAACTTTGCA	RT-PCR of <i>PfHRP11-Avr3a</i>
RTPfAvr3R	CGTCGACATGTGCTTGAGTTTC	RT-PCR of <i>PfHRP11-Avr3a</i>
RTATR13-Avr3F	CGTATCCAACGGGAATCTGC	RT-PCR of <i>ATR13-Avr3a</i>
RTATR13-Avr3R	CCTCATTTAGATTCGCCAAATTG	RT-PCR of <i>ATR13-Avr3a</i>
RTAvr3KIEM-F	TCGCCATAAACTTTGCAACC	RT-PCR of <i>Avr3a</i>
RTAvr3KIEM-R	TGCCGGCTGAATCGTGTAT	RT-PCR of <i>Avr3a</i>
ActAF2	CATCAAGGAGAAGCTGACGTACA	RT-PCR of <i>ActA</i>
ActAR2	GACGACTCGGCGGCAG	RT-PCR of <i>ActA</i>

version of this protocol can be found at <http://138.23.152.128/protocols/protocols.html>. The protocol was 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 digest cell walls of germinating sporangia for 30 min to release protoplasts. The protocol was scaled up to use 1 ml of prepared protoplasts per transformation. For each transformation, 50 µg of plasmid DNA, purified with the Qiagen Plasmid Maxi kit, using the supplied protocol, was mixed with 86 µl Lipofectin reagent (Invitrogen) and water to a final volume of 142 µl. Protoplasts suspended in 1 ml mannitol/Tris (MT) osmoticum were mixed with the 142 µl of DNA/Lipofectin mixture. After transformation, protoplasts were regenerated for 48–72 h in pea broth (Whisson *et al.*, 2005) containing 20 g sucrose l⁻¹ and 1 M mannitol, prior to selection of transformants on RyeA agar amended with 5 µg geneticin ml⁻¹. Transformants were then transferred to, and maintained on, RyeA agar amended with 20 µg geneticin ml⁻¹ and 10 µg pimaricin ml⁻¹.

Inoculation of potato leaves with *Ph. infestans* transformants. Sporangia (5 × 10⁴ ml⁻¹) of stable transformants were inoculated in 10 µl droplets on either side of the midvein of detached leaflets of 'Pentland Ace' (*R3a*), 'Bintje' or 'Craig's Royal' (no *R* genes) potato plants. Inoculated leaflets were incubated at 20 °C for 5–7 days in high relative humidity in sealed clear plastic boxes to enable disease symptoms to develop. For each transformant, six leaflets were inoculated. Samples were taken at 5 days for RNA extraction and RT-PCR analysis. Non-transformed isolate 88069 was used as a control for virulence on *R3a* 'Pentland Ace'. Transformant K-7 (Whisson *et al.*, 2007), overexpressing *Avr3a*, was used as an avirulent control for *Avr3a* recognition in *R3a* 'Pentland Ace'. Transformants were classified as virulent on *R3a* 'Pentland Ace' if an expanding necrotic lesion was formed, and aerial sporulation was visible at the lesion centre at 5 days post-inoculation. Transformants were classified as

avirulent on *R3a* 'Pentland Ace' if necrotic flecking or spotting, or restricted lesion with no sporulation, was formed at the inoculation sites. Infected leaves were photographed under polarized light to minimize reflection from the leaf surface.

Total RNA isolation, first-strand cDNA synthesis, and RT-PCR.

Total RNA was isolated from frozen or fresh infected leaf tissue with the Qiagen RNeasy Plant Mini kit, using the protocol for fungi supplied by the manufacturer. First-strand cDNA was synthesized by oligo dT priming with the First Strand cDNA Synthesis kit (GE Healthcare), using the supplied protocol. PCR from first-strand cDNA used the following conditions: each 20 µl PCR contained 0.5 U Taq polymerase (Molzym), 2 µl of 10 × reaction buffer (New England BioLabs), 15 mM deoxynucleotide triphosphates (Promega), 30 µM forward and reverse primers (Table 1), and 10 ng first-strand cDNA. Thermocycling conditions were as follows: 96 °C for 5 min, followed by 40 cycles of 96 °C for 30 s, and 60 °C for 60 s. Amplification of the *Ph. infestans ActA* gene was used as a constitutively expressed positive control for pathogen presence in infected leaf tissue. PCR products were separated on 2 % agarose gels in 1 × Tris/borate/EDTA (TBE) buffer (Sambrook *et al.*, 1989) and visualized on a UV transilluminator after staining with SYBRsafe (Invitrogen).

RESULTS

RxLR and RxLR-EER domains from *H. parasitica* effectors function in *Ph. infestans*

To test the cross-species function of the RxLR and RxLR-EER motifs, the N-terminal region, including the

RxLR-EER motifs, in AVR3a was replaced by the N-terminal region of *H. parasitica* ATR1NdWsB and ATR13, with and without five copies of the ATR13 heptad repeat (Fig. 1): constructs *ATR1NdWsB::Avr3a*, *ATR13(SP-RxLR-5×Heptad)::Avr3a* and *ATR13(SP-RxLR)::Avr3a*, respectively. Construct *ATR13(SP-RxLR)::Avr3a* encoded a protein that included only the three alanine residues normally following the ATR13 RxLR.

Inoculation of 'Bintje' or 'Craigs Royal' (no *R* genes) potatoes with stable *Ph. infestans* *ATR1NdWsB::Avr3a*, *ATR13(SP-RxLR-5×Heptad)::Avr3a* and *ATR13(SP-RxLR)::Avr3a* transformants showed the majority to have wild-type or near wild-type levels of aggressiveness on susceptible 'Craigs Royal' or 'Bintje' potatoes. The exceptions were transformants ATR1Avr3a-16 and 22, which exhibited reduced aggressiveness but were still capable of infection. In contrast, on 'Pentland Ace' potatoes (expressing the *R3a* resistance gene), transformants ATR1Avr3a-1, 2, 8, 14, 16 and 22 exhibited an avirulent phenotype (restricted necrotic flecking or pitting), indicating

recognition of Avr3a inside the host plant cells. The positive control transformant K-7, overexpressing intact Avr3a (from Whisson *et al.*, 2007), also exhibited an avirulent phenotype (Fig. 2). Occasionally, a small, restricted lesion developed on *R3a* leaves for transformants (including positive control K-7) in individual virulence tests that was characteristic of a 'trailing' HR, in that lesion size was greatly reduced compared to the lesion on 'Bintje' or 'Craigs Royal', and no sporulation was observed (results not shown). A virulent reaction on *R3a* 'Pentland Ace' leaves was observed for transformants ATR1Avr3a-9, 15, and 17 (Table 2).

When tested on 'Pentland Ace' (*R3a*), the RxLR motif alone in the *ATR13(SP-RxLR)::Avr3a* construct was found to be sufficient to translocate the Avr3a elicitor for transformants ATR13Avr3a-1, 2, 4, 5 and 11 (Table 2; Fig. 2 for example). Transformants HEPT-1, 3, 4, 5 and 6 expressing the *ATR13(SP-RxLR-5×Heptad)::Avr3a* fusion were also avirulent on *R3a* leaves, whereas transformants HEPT-2, 7 and 8 failed to trigger *R3a*-mediated recognition (Table 2).

Fig. 2. Leaves of 'Pentland Ace' and 'Bintje' potatoes infected with *Ph. infestans* transformants expressing Avr3a with the RxLR-EER motifs replaced with the malarial host-targeting (HT) signal or with translocation motifs from *H. parasitica*. The interaction between the products of avirulence gene *Avr3a* and cognate resistance gene *R3a* was used as a reporter for translocation. (A) Virulent *Ph. infestans* isolate 88069 (*avr3a*) is not recognized by *R3a* 'Pentland Ace' and disease symptoms develop. (B) Transformant K-7, overexpressing *Avr3a*, is recognized by *R3a* 'Pentland Ace' and is avirulent, causing a hypersensitive response (HR) seen as brown spotting, pitting, or restricted non-sporulating lesion. Transformants expressing *ATR1NdWsB(SP-RxLR-EER)::Avr3a*^{61–147} (C), *Avr3a*^{1–31}-PfHRPII(RxLxE)::*Avr3a*^{61–147} (D) or *ATR13(SP-RxLR)::Avr3a*^{61–147} (E) are avirulent on *R3a* 'Pentland Ace', triggering the HR. All transformants and untransformed 88069 were virulent on potato 'Bintje', containing no *R* genes (F to J; same order as for A to E).

Table 2. Interaction phenotypes of *Ph. infestans* transformants expressing *Avr3a* and chimeric *Avr3a* constructs, on potato 'Pentland Ace' expressing the *R3a* resistance gene

Transformants confirmed as expressing transgenic *Avr3a* constructs are shown in the right-hand column.

Construct	Transformant phenotypes on potato 'Pentland Ace' (<i>R3a</i>)		Transgene expression (RT-PCR)
	Avirulent	Virulent	
<i>Avr3a</i> *	K-1, 2, 4, 5, 6-, 7, 9	K-3, 8	K-1, 2, 3, 4, 5, 6, 7, 8, 9
<i>ATR1NdWsB(SP-RxLR-EER)::Avr3a</i> ⁶¹⁻¹⁴⁷	ATR1Avr3a-1, 2, 8, 14, 16, 22	ATR1Avr3a-9, 15, 17	ATR1Avr3a-1, 2, 8, 9, 14, 15, 16, 17, 22
<i>ATR13(SP-RxLR)::Avr3a</i> ⁶¹⁻¹⁴⁷	ATR13Avr3a-1, 2, 4, 5, 11	None	ATR13Avr3a-1, 2, 4, 5, 11
<i>ATR13(SP-RxLR-5 × Heptad)::Avr3a</i> ⁶¹⁻¹⁴⁷	HEPT-1, 3, 4, 5, 6	HEPT-2, 7, 8	HEPT-1, 2, 3, 4, 5, 6, 7, 8
<i>Avr3a</i> ¹⁻³¹ - <i>PfHRPII(RxLxE)::Avr3a</i> ⁶¹⁻¹⁴⁷	PfHT-1, 6, 9, 14, 15, 17, 20, 22	PfHT-5, 7, 8, 18, 19, 23	PfHT-1, 6, 7, 9, 14, 15, 17, 20, 22, 23

**Avr3a* transformant data from Whisson *et al.* (2007) included for comparison.

RT-PCR assays using primers specific to the sequences encoding the RxLR-EER or RxLR domains of *ATR1NdWsB* or *ATR13*, respectively, for the transformants revealed expression of the transgene fusion in all cases (Table 2). Additionally, RT-PCR assays using primers specific to the endogenous *avr3a* sequence demonstrated that the endogenous *avr3a* expression had not been transcriptionally silenced in transformants exhibiting a virulent phenotype.

The host-targeting (HT) signal from the apicomplexan *Pl. falciparum* is functional in *Ph. infestans*

The region encoding RxLR and EER motifs from *Avr3a* was replaced with the HT signal from the *Pl. falciparum* PfHRPII protein, a virulence protein known to be translocated into erythrocytes during infection (Bhattacharjee *et al.*, 2006). Inoculation of potato leaves with *PfHRPII(HT)::Avr3a* transformants, as for *ATR1NdWsB::Avr3a* and *ATR13::Avr3a* fusions (Figs 1 and 2), revealed the majority (PfHT-1, 6, 9, 14, 15, 17, 20, and 22) to be avirulent on potato expressing the resistance gene *R3a* (Table 2; Fig. 2 for example), implying that the product of the transgene had been translocated into the host cell. Transformants PfHT-5, 7, 8, 18, 19 and 23 were virulent on *R3a* 'Pentland Ace' potato leaves (Table 2). With the exceptions of transformants PfHT-6 and 14, all transformants exhibited wild-type aggressiveness. Transformants PfHT-6 and 14 exhibited reduced aggressiveness on the susceptible 'Bintje' and 'Craigs Royal' potatoes (results not shown).

RNA was extracted from the infected leaves, and the expression of the *PfHRPII::Avr3a* transgene assayed by RT-PCR using primers (Table 1) specific to the *PfHRPII* HT sequence (Table 2). All transformants eliciting an avirulent phenotype also expressed the transgenic *Avr3a* sequence. Virulent transformants (on 'Pentland Ace') PfHT-5, 8, 18, and 19 exhibited no transgene expression, while expression was detected in PfHT-7 and 23.

DISCUSSION

Here we have demonstrated that the effector translocation (host targeting) signals from related oomycetes and an apicomplexan parasite can function in the *Ph. infestans*-potato pathosystem. Our results provide the reciprocal evidence to experiments showing that the RxLR-EER domain of *Avr3a* could translocate fused GFP from *Pl. falciparum* to the inside of erythrocytes (Bhattacharjee *et al.*, 2006). Along with this previous demonstration (Bhattacharjee *et al.*, 2006), our findings confirm that the host specificity of the originating organisms has little bearing on the generic function of these motifs in translocating effector proteins to the inside of host animal and plant cells.

The majority of, but not all, *Ph. infestans* transformants containing *H. parasitica* or *Pl. falciparum* translocation sequences fused to *Avr3a* triggered the HR on *R3a* 'Pentland Ace' potato leaves. For some of these virulent transformants, no expression of the transgenic sequences could be detected by RT-PCR. Detection by RT-PCR of expression for the endogenous *avr3a* allele indicated that transcriptional silencing of both transgene and endogenous *avr3a* had not occurred. These transformants were not considered further in this study. There remained some transformants for which transgene expression could be detected, but which were consistently virulent on *R3a* 'Pentland Ace' leaves. The proportion of virulent transformants with detectable transgene expression in the present study is similar to that observed previously (two out of nine transformants; data from Whisson *et al.*, 2007 shown in Table 2) for *Avr3a* transformants. There are at least two explanations as to how this may occur. Firstly, the level of the chimeric protein may be below a threshold to either translocate the effector protein, or be detected by the *R3a* protein. Alternatively, disruption of the fusion transgene during genomic integration may have led to detection of expression by the primers targeted to the unique 5' region

of the chimeric transgene, while the sequence encoding the recognized Avr3a elicitor may have been disrupted. Transformation of *Ph. infestans* uses intact plasmids, which presumably integrate into the genome through random breaks in the plasmid sequence; disruption of the promoter-transgene-terminator will therefore occur in a proportion of transformants. This possibility was not investigated further here. However, that all avirulent transformants exhibited transgene expression provides evidence of Avr3a translocation by the alternative RxLR, RxLR-EER or RxLxE/D/Q domains, since R3a recognition of Avr3a has been shown to occur inside host cells (Armstrong *et al.*, 2005; Whisson *et al.*, 2007).

Our results support the hypothesis that some plant and animal eukaryotic pathogens share a conserved mechanism to deliver effector/virulence proteins into the host cell, inform on the flexibility of these protein translocation motifs, and raise the question of whether RxLxE/D/Q and RxLR (including RxLR-EER) domains are evolutionarily convergent solutions to the common requirement of effector delivery to the inside of host cells (Birch *et al.*, 2008). The motif RxL is clearly shared between *Plasmodium* and oomycetes, as is the position relative to the signal peptide, and the requirement of surrounding sequences (Fig. 1). These properties have been proposed as signifying involvement of secondary or tertiary peptide structures (Bhattacharjee *et al.*, 2006). Sequencing of either genomes or expressed sequence tags (ESTs), and functional characterization of motifs from a broader phylogenetic spectrum across the stramenopile and apicomplexan lineages, may reveal if both translocation motifs arose from a common ancestor or independently. Amongst the oomycetes, ESTs encoding candidate RxLR proteins have been identified from *Aphanomyces euteiches* and *Saprolegnia parastica*, both of which are more distantly related to *Ph. infestans* than is *H. parasitica* (van West, 2006; Gaulin *et al.*, 2008; Phillips *et al.*, 2008).

It was intriguing that the ATR13(RxLR)::Avr3a construct was sufficient to translocate the Avr3a elicitor. This suggests that additional downstream sequences, such as an aspartic/glutamic acid-enriched stretch ending in EER (Avr3a, ATR1NdWsB), or heptad leucine repeats (ATR13), are not always required for translocation and that the peptide sequence upstream of the RxLR motif may also function in effector delivery. The predicted cytoplasmic location of the RPP13 resistance protein (Bittner-Eddy *et al.*, 2000), and recent experimental evidence with delivery of ATR13 from the T3SS of the bacterium *Pseudomonas syringae* pv. *tomato* DC3000, have demonstrated that translocation is required for recognition to occur (Sohn *et al.*, 2007; Rentel *et al.*, 2008). The implications of our results with ATR13 is that the genomic pool of potentially translocated effectors in *Ph. infestans*, and other oomycetes, may be far greater than existing predictions based on the dual RxLR-EER motif. A regular expression prediction from signal-peptide-encoding ORFs in the *Ph. infestans* genome revealed 1960 SP-RxLR-

containing sequences (Whisson *et al.*, 2007). Whilst this prediction is likely to contain many false positives, real-time RT-PCR of seven RxLR-only genes selected from ESTs demonstrated expression profiles for these genes similar to those of RxLR-EER genes in being upregulated during plant infection (Whisson *et al.*, 2007).

The RxLR-EER domain from the *Ph. sojae* avirulence protein Avr1b has recently been demonstrated to translocate a fusion protein into plant cells in the absence of the oomycete pathogen (Dou *et al.*, 2008). Various arginine-rich short peptides have been reported to translocate through the cell plasma membrane. These cell-penetrating peptides (CPPs) include Tat derived from the HIV-1 Tat protein (Frankel & Pabo, 1988; Mann & Frankel, 1991), penetratin derived from the Antennapedia homeodomain from *Drosophila melanogaster* (Derossi *et al.*, 1994, 1996), and oligoarginine peptides (Futaki *et al.*, 2001). Despite an absence of obvious sequence or structural homology, common internalization mechanisms have been suggested for these CPPs on the basis of their abundance of arginine residues (Suzuki *et al.*, 2002). Thus, by virtue of arginine content and in function, peptide domains with core RxLR and RxLxE/D/Q motifs may also be considered as CPPs.

Two models for oomycete effector translocation have been proposed. The first involves interactions with pathogen-derived chaperones in a 'translocon' to mediate effector translocation (Morgan & Kamoun, 2007). The second model is based on pathogen mimicry of RxLR-EER motifs in host proteins to gain entry to host cells via interactions with the phospholipid membrane and endocytosis (Birch *et al.*, 2008). Although both *Pl. falciparum* and oomycete motifs appear to be functionally analogous, the mechanisms mediating the delivery of effectors into the host cell are presently unknown and will be the focus of future research.

ACKNOWLEDGEMENTS

The authors are grateful to Leighton Pritchard for bioinformatics. This work was supported by the Scottish Government Rural and Environment Research and Analysis Directorate (RERAD) (S. C. W., A. O. A., P. R. J. B.), The Royal Society and the Biotechnology and Biological Sciences Research Council (BBSRC) (P. v. W.). Financial support for S. G. was jointly provided by SCRI, UK and the University of Aberdeen, UK. Genetic modification of *Ph. infestans* was carried out under plant health licence PH/11/2008.

REFERENCES

- Allen, R. L., Bittner-Eddy, P. D., Grenville-Briggs, L. J., Meitz, J. C., Rehmany, A. P., Rose, L. E. & Beynon, J. L. (2004). Host-parasite coevolutionary conflict between *Arabidopsis* and downy mildew. *Science* **306**, 1957–1960.
- Armstrong, M. R., Whisson, S. C., Pritchard, L., Bos, J. I. B., Venter, E., Avrova, A. O., Rehmany, A. P., Böhme, U., Brooks, K. & other authors (2005). An ancestral oomycete locus contains late blight avirulence gene *Avr3a*, encoding a protein that is recognized in the host cytoplasm. *Proc Natl Acad Sci U S A* **102**, 7766–7771.

- Baldauf, S. L., Roger, A. J., Wenk-Siefert, I. & Doolittle, W. F. (2000). A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science* **290**, 972–977.
- Bhattacharjee, S., Hiller, N. L., Liolios, K., Win, J., Kanneganti, T., Young, C., Kamoun, S. & Haldar, K. (2006). The malarial host-targeting signal is conserved in the Irish potato famine pathogen. *PLoS Pathog* **2**, e50.
- Bhattacharjee, S., van Ooij, C., Balu, B., Adams, J. H. & Haldar, K. (2008). Maurer's clefts of *Plasmodium falciparum* are secretory organelles that concentrate virulence protein reporters for delivery to the host erythrocyte. *Blood* **111**, 2418–2426.
- Birch, P. R., Boevink, P. C., Gilroy, E. M., Hein, I., Pritchard, L. & Whisson, S. C. (2008). Oomycete RXLR effectors: delivery, functional redundancy and durable disease resistance. *Curr Opin Plant Biol* **11**, 373–379.
- Bittner-Eddy, P. D., Crute, I. R., Holub, E. B. & Beynon, J. L. (2000). RPP13 is a simple locus in *Arabidopsis thaliana* for alleles that specify downy mildew resistance to different avirulence determinants in *Peronospora parasitica*. *Plant J* **21**, 177–188.
- Blanco, F. A. & Judelson, H. S. (2005). A bZIP transcription factor from *Phytophthora* interacts with a protein kinase and is required for zoospore motility and plant infection. *Mol Microbiol* **56**, 638–648.
- Bos, J. I. B., Kanneganti, T., Young, C., Cakir, C., Huitema, E., Win, J., Armstrong, M. R., Birch, P. R. J. & Kamoun, S. (2006). The C-terminal half of *Phytophthora infestans* RXLR effector AVR3a is sufficient to trigger R3a-mediated hypersensitivity and suppress INF1-induced cell death in *Nicotiana benthamiana*. *Plant J* **48**, 165–176.
- Burki, F., Shalchian-Tabrizi, K., Minge, M., Skjaeveland, A., Nikolaev, S. I., Jakobsen, K. S. & Pawlowski, J. (2007). Phylogenomics reshuffles the eukaryotic supergroups. *PLoS ONE*, **2**, e790. doi:10.1371/journal.pone.0000790
- Catanzariti, A. M., Dodds, P. N. & Ellis, J. G. (2007). Avirulence proteins from haustoria-forming pathogens. *FEMS Microbiol Lett* **269**, 181–188.
- Davis, E. L., Hussey, R. S., Mitchum, M. G. & Baum, T. J. (2008). Parasitism proteins in nematode–plant interactions. *Curr Opin Plant Biol* **11**, 360–366.
- Derossi, D., Joliot, A. H., Chassaing, G. & Prochiantz, A. (1994). The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem* **269**, 10444–10450.
- Derossi, D., Calvet, S., Trembleau, A., Brunissen, A., Chassaing, G. & Prochiantz, A. (1996). Cell internalization of the third helix of the antennapedia homeodomain is receptor-independent. *J Biol Chem* **271**, 18188–18193.
- Dodds, P. N., Lawrence, G. J., Catanzariti, A., Ayliffe, M. A. & Ellis, J. G. (2004). The *Melampsora lini* AvrL567 avirulence genes are expressed in haustoria and their products are recognized inside plant cells. *Plant Cell* **16**, 755–768.
- Dou, D., Kale, S. D., Wang, X., Jiang, R. H., Bruce, N. A., Arredondo, F. D., Zhang, X. & Tyler, B. M. (2008). RXLR-mediated entry of *Phytophthora sojae* effector Avr1b into soybean cells does not require pathogen-encoded machinery. *Plant Cell* **20**, 1930–1947.
- Ellis, J., Catanzariti, A. & Dodds, P. (2006). The problem of how fungal and oomycete avirulence proteins enter plant cells. *Trends Plant Sci* **11**, 61–63.
- Ellis, J. G., Dodds, P. N. & Lawrence, G. J. (2007). The role of secreted proteins in diseases of plants caused by rust, powdery mildew and smut fungi. *Curr Opin Microbiol* **10**, 326–331.
- Frankel, A. D. & Pabo, C. O. (1988). Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* **55**, 1189–1193.
- Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K. & Sugiura, Y. (2001). Arginine-rich peptides: an abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J Biol Chem* **276**, 5836–5840.
- Gálan, J. E. & Wolf-Watz, H. (2006). Protein delivery into eukaryotic cells by type III secretion machines. *Nature* **444**, 567–573.
- Gaulin, E., Madoui, M. A., Bottin, A., Jacquet, C., Mathé, C., Couloux, A., Wincker, P. & Dumas, B. (2008). Transcriptome of *Aphanomyces euteiches*: new oomycete putative pathogenicity factors and metabolic pathways. *PLoS ONE*, **3**, e1723. doi:10.1371/journal.pone.0001723
- Haldar, K., Kamoun, S., Hiller, N. L., Bhattacharjee, S. & van Ooij, C. (2006). Common infection strategies of pathogenic eukaryotes. *Nat Rev Microbiol* **4**, 922–931.
- He, P., Shan, L. & Sheen, J. (2007). Elicitation and suppression of microbe-associated molecular pattern-triggered immunity in plant–microbe interactions. *Cell Microbiol* **9**, 1385–1396.
- Hiller, N. L., Bhattacharjee, S., Van Ooij, C., Liolios, K., Harrison, T., Lopez-Estraño, C. & Haldar, K. (2004). A host-targeting signal in virulence proteins reveals a secretome in malarial infection. *Science* **306**, 1934–1937.
- Hiss, J. A., Przyborski, J. M., Schwarte, F., Lingelbach, K. & Schneider, G. (2008). The *Plasmodium* export element revisited. *PLoS ONE* **3**, e1560. doi:10.1371/journal.pone.0001560
- Judelson, H. S., Tyler, B. M. & Michelmores, R. W. (1991). Transformation of the oomycete pathogen, *Phytophthora infestans*. *Mol Plant Microbe Interact* **4**, 602–607.
- Lopez-Estraño, C., Bhattacharjee, S., Harrison, T. & Haldar, K. (2003). Cooperative domains define a unique host cell-targeting signal in *Plasmodium falciparum*-infected erythrocytes. *Proc Natl Acad Sci U S A* **100**, 12402–12407.
- MacKenzie, J. J., Gomez, N. D., Bhattacharjee, S., Mann, S. & Haldar, K. (2008). A *Plasmodium falciparum* host-targeting motif functions in export during blood stage infection of the rodent malarial parasite *Plasmodium berghei*. *PLoS ONE* **3**, e2405. doi:10.1371/journal.pone.0002405
- Mann, D. A. & Frankel, A. D. (1991). Endocytosis and targeting of exogenous HIV-1 Tat protein. *EMBO J* **10**, 1733–1739.
- Marti, M., Good, R. T., Rug, M., Knuepfer, E. & Cowman, A. F. (2004). Targeting malaria virulence and remodeling proteins to the host erythrocyte. *Science* **306**, 1930–1933.
- Morgan, W. & Kamoun, S. (2007). RXLR effectors of plant pathogenic oomycetes. *Curr Opin Microbiol* **10**, 332–338.
- Phillips, A. J., Anderson, V. L., Robertson, E. J., Secombes, C. J. & van West, P. (2008). New insights into animal pathogenic oomycetes. *Trends Microbiol* **16**, 13–19.
- Rehmany, A. P., Gordon, A., Rose, L. E., Allen, R. L., Armstrong, M. R., Whisson, S. C., Kamoun, S., Tyler, B. M., Birch, P. R. J. & Beynon, J. L. (2005). Differential recognition of highly divergent downy mildew avirulence gene alleles by RPP1 resistance genes from two *Arabidopsis* lines. *Plant Cell* **17**, 1839–1850.
- Rentel, M. C., Leonelli, L., Dahlbeck, D., Zhao, B. & Staskawicz, B. J. (2008). Recognition of the *Hyaloperonospora parasitica* effector ATR13 triggers resistance against oomycete, bacterial, and viral pathogens. *Proc Natl Acad Sci U S A* **105**, 1091–1096.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sohn, K. H., Lei, R., Nemri, A. & Jones, J. D. (2007). The downy mildew effector proteins ATR1 and ATR13 promote disease susceptibility in *Arabidopsis thaliana*. *Plant Cell* **19**, 4077–4090.

Suzuki, T., Futaki, S., Niwa, M., Tanaka, S., Ueda, K. & Sugiura, Y. (2002). Possible existence of common internalization mechanisms among arginine-rich peptides. *J Biol Chem* **277**, 2437–2443.

van West, P. (2006). *Saprolegnia parasitica*, an oomycete pathogen with a fishy appetite; new challenges for an old problem. *Mycologist* **20**, 99–104.

Whisson, S. C., Avrova, A. O., Van West, P. & Jones, J. T. (2005). A method for double-stranded RNA-mediated transient gene silencing in *Phytophthora infestans*. *Mol Plant Pathol* **6**, 153–163.

Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., Armstrong, M. R., Grouffaud, S., Van West, P. & other authors (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature* **450**, 115–118.

Win, J., Morgan, W., Bos, J., Krasileva, K. V., Cano, L. M., Chaparro-Garcia, A., Ammar, R., Staskawicz, B. J. & Kamoun, S. (2007). Adaptive evolution has targeted the C-terminal domain of the RXLR effectors of plant pathogenic oomycetes. *Plant Cell* **19**, 2349–2369.

Edited by: C. Boucher