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

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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 PfHRPII. 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 Hyaloperonopsora, 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...
encased in the host plasma membrane and this situation is reminiscent of the parasitophorous vacuole produced by \textit{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 \textit{RxLx}E/D/Q (\textit{Plasmodium}) or \textit{RxLR} (oomycetes) (Hiller \textit{et al.}, 2004; Marti \textit{et al.}, 2004; Bhattacharjee \textit{et al.}, 2006, 2008; Whisson \textit{et al.}, 2007; Birch \textit{et al.}, 2008; MacKenzie \textit{et al.}, 2008). A subset of \textit{RxLR} effectors also possess a region enriched for acidic residues following the \textit{RxLR} motif, and terminating in EER. Peptide sequences surrounding the core \textit{RxLx}E/D/Q or \textit{RxLR} signal also have a role in translocation (Lopez-Estraño \textit{et al.}, 2003; Bhattacharjee \textit{et al.}, 2006; Whisson \textit{et al.}, 2007) and have additional properties such as hydrophobicity profile relevant to subcellular location (Hiss \textit{et al.}, 2008).

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

Previously, alanine (AAA-\textit{EER} and \textit{RxLR-AAA}) and physico-chemically conservative (KMIK-\textit{DDK}) substitutions of the \textit{RxLR-EER} motifs in \textit{Avr3a} were shown to abolish translocation of the effector following its secretion from haustoria (Whisson \textit{et al.}, 2007). However, some \textit{RxLR} effectors from \textit{Ph. infestans}, \textit{Phytophthora sojae} and \textit{Hyaloperonospora parasitica} exhibit potential amino acid variation in the ‘EER’ motif of candidate effectors (Rehmaney \textit{et al.}, 2005; Win \textit{et al.}, 2007; Whisson \textit{et al.}, 2007). Examples are the \textit{Avr1b} protein from \textit{Ph. sojae}, which contains the variant GER sequence instead of EER (Rehmany \textit{et al.}, 2005), and the \textit{ATR13} protein from \textit{H. parasitica}, which lacks a recognizable EER motif (Allen \textit{et al.}, 2004). Although the \textit{RxLR-AAA} and KMIK-\textit{DDK} variants of \textit{Avr3a} were not translocated, investigation of the translocation potential of ATR13-like \textit{RxLR} sequences lacking an EER motif was not addressed (Whisson \textit{et al.}, 2007). ATR13 contains heptad leucine/isoleucine repeats commencing within 10 residues of the \textit{RxLR} motif (Fig. 1; Allen \textit{et al.}, 2004) where the EER motif is located in \textit{ATR1NdWsb} and \textit{Avr3a}, and it is possible that these repeats could also be involved in the translocation process.

It has been demonstrated that the \textit{RxLR-EER} domain from \textit{Avr3a} was sufficient to export the green fluorescent protein (GFP) from \textit{Pl. falciparum} to the erythrocyte (Bhattacharjee \textit{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 \textit{Ph. infestans} transformants to demonstrate function of the \textit{Pl. falciparum} host targeting signal in translocation. Additionally, we have demonstrated that the \textit{RxLR} and \textit{RxLR-EER} motifs found in avirulence proteins from a distantly related oomycete can function to deliver the \textit{Avr3a} effector into host cells.

**METHODS**

**Cultures and growth conditions.** \textit{Ph. infestans} isolate 88069, homozygous for the R3a-non-recognized SEM allele \textit{avr3a}, virulent on the \textit{R3a}-expressing potato ‘Pentland Ace’, was maintained at 20 °C on RyeA agar amended with the antibiotics pimaricin (10 μg ml\textsuperscript{-1}; Sigma) and rifampicin (30 μg ml\textsuperscript{-1}; Sigma), ‘Bintje’ and ‘Craigs Royal’ (fully susceptible to \textit{Ph. infestans}), and ‘Pentland Ace’ (\textit{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: AY893356 (\textit{Ph. infestans} \textit{Avr3a}), AY842877 (\textit{H. parasitica} \textit{ATR1NdWsB}), AY785301 (\textit{H. parasitica} \textit{ATR13}) and AAD31511 (\textit{Pl. falciparum} \textit{PfHRPII}), \textit{ATR1NdWsB} (Rehmany \textit{et al.}, 2005) and \textit{ATR13} (Allen \textit{et al.}, 2004) are cloned avirulence genes from the oomycete \textit{H. parasitica}, the causal agent of downy mildew in the model plant \textit{Arabidopsis thaliana}. The primary positions of the \textit{RxLR-EER} motifs relative to each other and relative to the signal peptide are similar between \textit{Avr3a} and \textit{ATR1NdWsB} (Fig. 1). To conserve the physical primary location between the signal peptide and the 33 aa functional core of the \textit{Pl. falciparum} histidine-rich protein II (\textit{PfHRPII}) HT sequence (Bhattacharjee \textit{et al.}, 2006), 10 residues downstream of the signal peptide of \textit{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 \textit{Ph. infestans} \textit{Avr3a} gene encoding amino acids 61–147 was amplified by PCR from an existing plasmid clone (Whisson \textit{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 x reaction buffer (New England BioLabs), 15 mM deoxynucleotide triphosphates (Promega), 30 μM forward and reverse primers (Table 1), and 10 ng \textit{Avr3a} plasmid DNA. All primer pairs for PCR of \textit{Avr3a} sequence encoding the C-terminal 86 aa used the \textit{Avr3aStopSacR} reverse primer. Forward primers were \textit{Avr3aPapE} (fusion to \textit{ATR1NdWsB} SP-RxLR-EER), \textit{Avr3aNotF} (fusion to...
ATR13 SP-RxLR only), Avr3aBssF (fusion to ATR13 SP-RxLR-5\times 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 ATR13BssR (ATR13 SP-RxLR-5\times 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 BsaHI (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 Sall, ligated and purified (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

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’–3’)</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avr3ClaF2</td>
<td>GGAAAATCGATACCATCTGGCTGGCAAATTATATGCTG</td>
<td>Avr3a from start, ClaI site</td>
</tr>
<tr>
<td>Avr3aStopSacR</td>
<td>GGAAACCGGGCTCTATCATGCAGGCGCGCGAGGT</td>
<td>Avr3a stop codon reverse, SacII site</td>
</tr>
<tr>
<td>ATR1claF</td>
<td>GGAAATGCACTACCTGGCCCTGGCTCAGTGCAGT</td>
<td>ATR1 from start, ClaI site</td>
</tr>
<tr>
<td>ATR1PspR</td>
<td>GGAAAGGGCCCTCCCTCTCATGGTCTATGCA</td>
<td>ATR1 N-term. reverse, PspOMI site</td>
</tr>
<tr>
<td>Avr3aPspF</td>
<td>GGAAAGGGCCCTCCCTCTCATGGTCTATGCA</td>
<td>Avr3a N-term. reverse, PspOMI site</td>
</tr>
<tr>
<td>AVRPpHTSAlF2</td>
<td>GGAAAGTGCAAGACGCCATCCGCTCGTACGTCGAGTCGAGTCG</td>
<td>PfHRPII-Avr3a C-term., SalI site</td>
</tr>
<tr>
<td>AVRSPpHTSAlR2</td>
<td>GGAAAGTGCAAGACGCCATCCGCTCGTACGTCGAGTCG</td>
<td>PfHRPII-Avr3a N-term. reverse, SalI site</td>
</tr>
<tr>
<td>ATR13claF</td>
<td>GGAAAGTCGATACAATTGCTGGTTTATGCTGCGCGGCGG</td>
<td>ATR13 from start, ClaI site</td>
</tr>
<tr>
<td>ATR13NotR</td>
<td>GGAAAGCGGCAGCGGAGATGGCTGGACCAG</td>
<td>ATR13 RLR reverse, NotI site</td>
</tr>
<tr>
<td>ATR13BssR</td>
<td>GGAAAGCGGCAGCGGAGATGGCTGGACCAG</td>
<td>ATR13 heptad repeats reverse, BssHI site</td>
</tr>
<tr>
<td>Avr3aBss</td>
<td>GGAAAGCGGCAGCGGAGATGGCTGGACCAG</td>
<td>Avr3a C-term. after EER, BssHI site</td>
</tr>
<tr>
<td>Avr3aNotF</td>
<td>GGAAAGCGGCAGCGGAGATGGCTGGACCAG</td>
<td>Avr3a C-term. after EER, NotI site</td>
</tr>
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<td>RTATR1Avr3aF</td>
<td>ATCCGAAGCAGCGCCTCCTAGA</td>
<td>RT-PCR of ATR1NdWsB-Avr3a</td>
</tr>
<tr>
<td>RTATR1Avr3aR</td>
<td>GCCCTCTCTCCTCTCATGCTCATC</td>
<td>RT-PCR of ATR1NdWsB-Avr3a</td>
</tr>
<tr>
<td>RTPfAvr3R</td>
<td>GCTGCTGCAATTAACCTTGGCA</td>
<td>RT-PCR of PHRP-II-Avr3a</td>
</tr>
<tr>
<td>RTPfAvr3F</td>
<td>GCTGCTGCAATTAACCTTGGCA</td>
<td>RT-PCR of PHRP-II-Avr3a</td>
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<tr>
<td>RTATR13-Avr3F</td>
<td>GCTATCCAAACGGGAAATCTG</td>
<td>RT-PCR of ATR13-Avr3a</td>
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<tr>
<td>RTATR13-Avr3R</td>
<td>CCTCTATTGATAGTCGACACAG</td>
<td>RT-PCR of ATR13-Avr3a</td>
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<tr>
<td>RTAvr3KlE3F</td>
<td>TGCCTATCAGATTGTCGACAGG</td>
<td>RT-PCR of Avr3a</td>
</tr>
<tr>
<td>RTAvr3KlE3R</td>
<td>TGCCTATCAGATTGTCGACAGG</td>
<td>RT-PCR of Avr3a</td>
</tr>
<tr>
<td>ActAR2</td>
<td>CATCAAGAGAAGCATGTCAGC</td>
<td>RT-PCR of ActA</td>
</tr>
<tr>
<td>ActAR2</td>
<td>GACGACGGCCGCGG</td>
<td>RT-PCR of ActA</td>
</tr>
</tbody>
</table>

Inoculation of potato leaves with Ph. infestans transformants. Sporangia (5 × 10^6 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 ‘Craigs 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 X reaction buffer (New England BioLabs), 15 mM dNTPs, 0.5 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 X 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 ATR1Avr3a-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)::Avr3a61–147 (C), Avr3a1–31-PHHRPII(RxLxE)::Avr3a61–147 (D) or ATR13(SP-RxLR)::Avr3a61–147 (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).
**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* ‘Pentland Ace’ 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 transgenic 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.

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.

**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.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Transformant phenotypes on potato ‘Pentland Ace’ (R3a)</th>
<th>Transgene expression (RT-PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avr3a*</td>
<td>Virulent: K-1, 2, 4, 5, 6, 7, 9, 16, 22</td>
<td>Avr3a: K-1, 2, 3, 4, 5, 6, 7, 8, 9</td>
</tr>
<tr>
<td>ATR1NdWsB(SP-RxLR-EER)::Avr3a61–147</td>
<td>Avr3a: K-3, 8, 15, 17</td>
<td>ATR1Avr3a: K-1, 2, 3, 4, 5, 6, 7, 8, 9</td>
</tr>
<tr>
<td>ATR13(SP-RxLR)::Avr3a61–147</td>
<td>Avr3a61–147: None</td>
<td>ATR13Avr3a: K-1, 2, 4, 5, 11</td>
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<tr>
<td>ATR13(SP-RxLR-5×Heptad)::Avr3a61–147</td>
<td>Avr3a61–147: HEPT-2, 7, 8</td>
<td>ATR13Avr3a: K-1, 2, 4, 5, 11</td>
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<tr>
<td>Avr3a1–31-PfHRPII(RxLxE)::Avr3a61–147</td>
<td>Avr3a61–147: PfHT-5, 7, 8, 19, 23</td>
<td>PfHT-1, 6, 7, 9, 14, 15, 17, 20, 22, 23</td>
</tr>
</tbody>
</table>

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

**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* PfHRPIII protein, a virulence protein known to be translocated into erythrocytes during infection (Bhattacharjee et al., 2006). Inoculation of potato leaves with PfHRPIII(HT)::Avr3a transformants, as for ATR1NdWsB::Avr3a and ATR13::Avr3a fusions (Figs 1 and 2), revealed the majority of transformants PfHT-5, 6, 9, 14, 15, 17, 20, and 22) to be avirulent on potato expressing the resistance gene (*PfHT-1, 6, 7, 9, 14, 15, 17, 20, and 22*) to be avirulent on potato leaves. For some of these virulent transformants for which transgene expression could be 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.

RNA was extracted from the infected leaves, and the expression of the PfHRPIII::Avr3a transgene assayed by RT-PCR using primers (Table 1) specific to the PfHRPIII HT sequence (Table 2). All transformants eliciting an avirulent phenotype also expressed the transgenic Avr3a sequence. Virulent transformants (on ‘Pentland Ace’) PfHT-5, 6, 9, 14, 15, 17, 20, and 19 exhibited no transgene expression, while expression was detected in PfHT-7 and 23.

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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 eutiches and Saprolegnia parastica, both of which are more distantly related to Ph. infestans than is H. parasitica (van West, 2006; Gaulin et al., 2008; Phillipps 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.

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