Presence/absence, differential expression and sequence polymorphisms between *PiAVR2* and *PiAVR2-like* in *Phytophthora infestans* determine virulence on *R2* plants

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Summary

- A detailed molecular understanding of how oomycete plant pathogens evade disease resistance is essential to inform the deployment of durable resistance (*R*) genes.
- Map-based cloning, transient expression *in planta*, pathogen transformation and DNA sequence variation across diverse isolates were used to identify and characterize *PiAVR2* from potato late blight pathogen *Phytophthora infestans*.
- PiAVR2 is an RXLR-EER effector that is up-regulated during infection, accumulates at the site of haustoria formation, and is recognized inside host cells by potato protein R2. Expression of *PiAVR2* in a virulent *P. infestans* isolate conveys a gain-of-avirulence phenotype, indicating that this is a dominant gene triggering *R2*-dependent disease resistance. *PiAVR2* presence/absence polymorphisms and differential transcription explain virulence on *R2* plants. Isolates infecting *R2* plants express *PiAVR2-like*, which evades recognition by *R2*. PiAVR2 and PiAVR2-like differ in 13 amino acids, eight of which are in the C-terminal effector domain; one or more of these determines recognition by R2. Nevertheless, few polymorphisms were observed within each gene in pathogen isolates, suggesting limited selection pressure for change within *PiAVR2* and *PiAVR2-like*.
- Our results direct a search for *R* genes recognizing *PiAVR2-like*, which, deployed with *R2*, may exert strong selection pressure against the *P. infestans* population.

Introduction

Oomycetes comprise a major group of eukaryotic microbial pathogens that cause devastating diseases on dicotyledonous plants (Kamoun, 2003). One notorious representative is *Phytophthora infestans*, the cause of late blight, the most significant global disease of potato. The genetic flexibility of *Phytophthora* (Brasier, 1992) and the coevolution of *P. infestans* populations in Central and South America with wild *Solanum* species have yielded a remarkable source of

genetic diversity in populations of this pathogen. *P. infestans* thus possesses an alarming, and demonstrated, adaptibility, able to respond rapidly to selection pressures within agricultural systems, resulting in global late blight epidemics (Fry, 2008). Breeding efforts to control this disease by introgression of resistance from wild *Solanum* species have had limited success, probably because of the genetic diversity within pathogen populations (Hein *et al.*, 2009a). Indeed, the genome sequence of *P. infestans* reveals striking potential for genetic change (Haas *et al.*, 2009). A major scientific goal is thus to develop a detailed understanding of how disease resistance to *P. infestans* has been overcome to date. Such knowledge is critical to combating this and other economically important oomycete plant pathogens.

Inducible disease resistance in plants is based on detection of two distinct classes of pathogen molecules. Recognition of secreted or surface-exposed pathogen/microbe-associated molecular patterns (PAMP/MAMPs) by pattern recognition receptors (PRRs) in the host leads to broadly effective PAMP (or, more generally, Pattern)-triggered immunity (PTI). Pathogens deploy effector proteins that suppress this response (effector-triggered susceptibility; ETS). Effectors are a second class of molecules that can be detected by plants, often by nucleotide-binding leucine-rich repeat (NB-LRR) resistance (R) proteins. When detected, effectors are termed avirulence (AVR) proteins, and the consequent disease resistance is referred to as effector-triggered immunity (ETI), or the hypersensitive response (HR) (Jones & Dangl, 2006; Chisholm *et al.*, 2006).

In recent years, a number of *AVR* genes have been identified from oomycete plant pathogens. These include *AVR3a* (Armstrong *et al.*, 2005), *AVR4* (Van Poppel *et al.*, 2008), *AVR-blb1* (Vleeshouwers *et al.*, 2008; Champouret *et al.*, 2009) and *AVR-blb2* (Oh *et al.*, 2009) from *P. infestans*; *Avr1b* (Shan *et al.*, 2004), *Avr3c* (Dong *et al.*, 2010), *Avr3a*, *Avr1a* (Qutob *et al.*, 2009) and *Avr4/6* (Dou *et al.*, 2008) from the soybean pathogen *P. sojae*; and *ATR13* (Allen *et al.*, 2004) and *ATR1* (Rehmany *et al.*, 2005) from the *Arabidopsis thaliana* pathogen *Hyaloperonospora arabidopsidis.* All are members of the extensive, diverse RXLR class of effectors (Birch *et al.*, 2006, 2008, 2009; Kamoun, 2006, 2007; Hein *et al.*, 2009b; Schornack *et al.*, 2009). These effectors are so named for the amino acid motif, RXLR (Arg-any amino acid-Leu-Arg), often closely followed by the motif EER (Glu-Glu-Arg), which is required for their entry into living plant cells (Whisson *et al.*, 2007; Dou *et al.*, 2008).

Each of these effectors has enhanced our understanding of how oomycete plant pathogens can evade ETI and thus overcome disease resistance. Single nucleotide polymorphisms (SNPs) within allelic forms may give rise to proteins with amino acid changes that evade recognition. This has been well documented for *AVR3a* from *P. infestans*; only two alleles reported within the pathogen population encode proteins differing in two amino acids (K80E and I103M) which dictate recognition by R3a (Armstrong *et al.*, 2005; Bos *et al.*, 2006). As AVR3a is an essential pathogenicity determinant (Bos *et al.*, 2010), deployment of an *R* gene that targets the virulent form, $AVR3a^{EM}$, in combination with *R3a*, which targets the avirulent form, $AVR3a^{KI}$, would potentially impose strong selection pressure on the pathogen population. In addition to *AVR3a*, SNPs that encode alternative, virulent alleles have been reported for *ATR1* and *ATR13* from *H. arabidopsidis* (Allen *et al.*, 2004; Rehmany *et al.*, 2005) and *Avr1b* and *Avr3c* from *P. sojae* (Shan *et al.*, 2004; Dong *et al.*, 2009). In addition to amino acid polymorphisms, which can retain the virulence function of the effector (Bos *et al.*, 2010), virulence on plants containing some *R* genes has been achieved by loss of a functional *AVR* gene. Frame-shift mutations, resulting in truncated versions of *AVR4*, have been reported in *P. infestans* isolates that infect potato expressing *R4* (Van Poppel *et al.*, 2008). Moreover, differential gene expression, sometimes associated with gene deletion or gene copy number variation, has been reported for *Avr1b* (Shan *et al.*, 2004), *Avr1a* and *Avr3a* (Qutob *et al.*, 2009) from *P. sojae*. Presumably, loss of an effector gene, or of its expression, may be compensated for by functional redundancy in the effector complement (Birch *et al.*, 2008).

In theory, durable disease resistance may be achieved with an R protein, or a judicious combination of R proteins, that target all of the effectors (or effector forms deriving from alleles) contributing to an essential pathogenicity function, as hypothesized earlier for *AVR3a* from *P. infestans*. This is apparently the case for the resistance protein Rpi-blb2 from *Solanum bulbocastanum*, which recognizes multiple members of a closely related *P. infestans* RXLR effector family, of which there are seven paralogues within the genome sequence of clone T30-4 (Oh *et al.*, 2009). *Rpi-blb2* has so far proven durable.

Here, we report the map-based cloning of *PiAVR2* from *P. infestans*. This gene (PITG_22870) from *P. infestans* clone T30-4 was previously shown to trigger *R2*-dependent cell death (Lokossou *et al.*, 2009). Transformation of a virulent isolate with *PiAVR2* conferred a gain-of-avirulence phenotype when inoculated on to the *R2* potato differential, indicating that it is a dominant gene specifying *R2*-mediated recognition and disease resistance. We show that, whereas all avirulent isolates possess *PiAVR2*, virulent isolates lack either *PiAVR2* or its expression. By contrast, virulent isolates express a divergent form, *PiAVR2-like*, which is not recognized by *R2* or *R2*-like orthologues from wild *Solanum* species. Remarkably, while there is little sequence diversity within either *PiAVR2* or *PiAVR2-like* in the sampled *P. infestans* populations, the predicted PiAVR2 and PiAVR2-like polypeptides differ from each other by 13 amino acids in the mature protein. Given that each form, though markedly divergent, nevertheless appears to be

highly conserved in diverse *P. infestans* isolates, and that all isolates tested possess one or other, or both forms, we propose a strategy for durable late blight disease resistance.

Materials and Methods

Plant and microbial strains and growth conditions

Potato plants and *P. infestans* isolates and transgenic strains were maintained, and infection assays performed, as described in Whisson *et al.* (2007). *Nicotiana benthamiana* and potato genotypes were grown as in Bos *et al.* (2010). *Escherichia coli* strain DH10B and *Agrobacterium tumefaciens* strain AGL1 were used for cloning. Agrobacterium strains for transient expression of potato *R2* and its orthologues *R2-like*, *BLB3* and *ABPT* are described in Lokossou *et al.* (2009). All *A. tumefaciens* cultures were grown at 27°C at 200 rpm for 2–3 d in LB (Luria Bertani broth), spun at 4000 rpm and the pellet resuspended in sterile 10 mM 2-(N-morpholine)-ethanesulphonic acid (MES) and 10 mM $MgCl_2$ buffer with 200 μM acetosyringone, to OD_{600} = 0.5 for each construct.

Map-based cloning of *PiAVR2*

Amplified fragment length polymorphism (AFLP) markers E + GA/M + ATs513 and E + TG/M + CTs338, which span the *Avr2* locus of *P. infestans* (Van der Lee *et al.*, 2001; Fig. 1a), were used to screen the pooled bacterial artificial chromosome (BAC) library of *P. infestans* T30-4 as described previously (Whisson *et al.*, 2001). BAC clones identified with E + GA/M + ATs513 (23O22 and 39L24) and with E + TG/M + CTs338 (8C12 and 5D19) were end-sequenced using SP6 and T7 primers as described (Whisson *et al.*, 2001), and these sequences were positioned on the genome sequence of T30-4 (Haas *et al.*, 2009), in supercontig 1.16 using BlastN. The region delimited by the outer BAC sequences was screened for annotated RXLR-EER coding sequences, revealing PITG_22870, PITG_08943 and PITG_08949.

Transient expression of *P. infestans* genes in plants to assess *R* gene responses

PITG_08949 and PITG_22870 (*PiAVR2*) sequences were initially amplified from genomic DNA of *P. infestans* isolate 88069 using *Asc*1-For and *Bam*H1-Rev primers (Supporting Information, Table S1) and cloned into vector pGRAB using these restriction sites and transformed into *A. tumefaciens* strain AGL1 cells by electroporation as described in Bos *et al.* (2010). Positive transformants were subsequently grown in LB supplemented with rifampicin and kanamycin for transient expression in potato and *N. benthamiana*. For gateway cloning® (Invitrogen), PITG_22870 and PITG_08949 were amplified from sequences encoding from the cleavage site of the signal peptide (SP) to the stop codon from genomic DNA of *P. infestans* isolate T30-4 with gene-specific primers (Table S1). The N-terminus-encoding region of $PiAVR2^{N31}$ was cloned from the SP cleavage site to the last amino acid in the EER motif. The C-terminus-encoding region of *PiAVR2* was cloned from the first amino acid after the EER motif to the stop codon (see Table S1 for PCR primers). AttB recombination sites were added by a second PCR using the AttB1/AttB2 to all sequences and recombined into pDNR221 using BP clonase® (Invitrogen). *Piavr2* DNA encoding from the SP

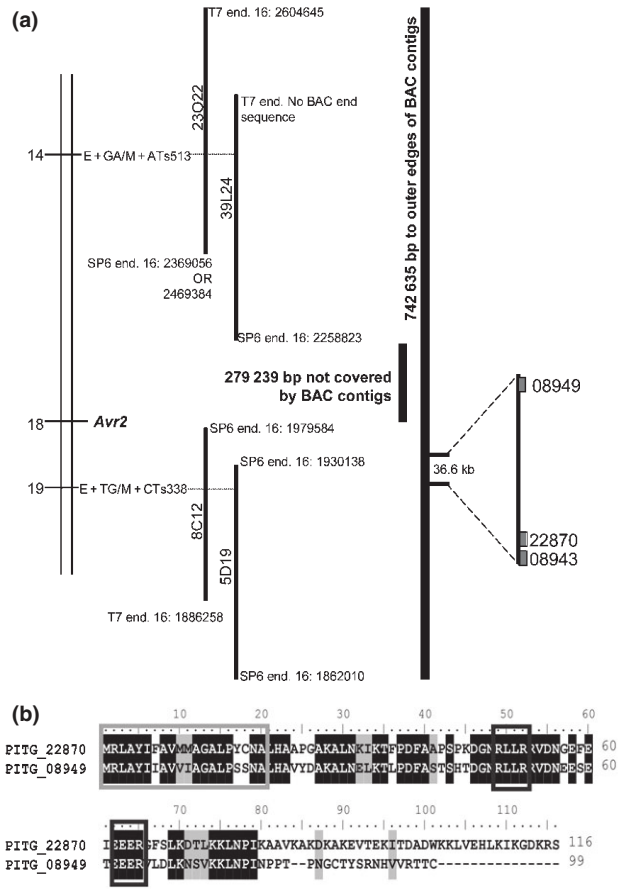

Fig. 1 Identification of *PiAVR2* candidates by map-based cloning. (a) Genetic interval (left) showing amplified fragment length polymorphism markers flanking the *AVR2* locus. Bacterial artificial chromosome (BAC) clones containing these markers were end-sequenced and the DNA sequences positioned in supercontig 1.16 of the *Phytophthora infestans* T30-4 genome sequence, spanning a region of 742.6 kb. Within a 36.6 kb region of this (right), three candidate RXLR-dEER effector genes were identified: PITG_08949, PITG_08943 and PITG_22870. This diagram is not drawn to scale. (b) Alignment of the predicted protein sequences of PITG_22870 and PITG_08949 revealed considerable similarity over the first 79 amino acids, and subsequent divergence, as a result of a likely DNA recombination event (Supporting Information, Fig. S2). The pale box represents the signal peptide and the dark boxes indicate the RLLR and EER motifs.

cleavage site to the stop codon (357 bp), and the C-terminus-encoding region from the first amino acid after the EER motif until the stop codon, or without the stop (222 bp), was synthesized and delivered in pUC57 (Genscript, Piscataway, USA).

LR clonase® (Invitrogen) was used to recombine DNA sequences into pMDC32 plant expression vector (Curtis & Grossniklaus, 2003), transformed into *E. coli* by electroporation, sequenced, and plasmids with confirmed inserts were transformed into *A. tumefaciens* strain AGL1, pSoup, pVirG cells by electroporation. Positive transformants were subsequently grown for 2 d at 27°C in LB supplemented with rifampicin, chloroamphenicol, tetracycline and kanamycin for transient expression in potato and *N. benthamiana*. For plant inoculations, all *A. tumefaciens* cultures were resuspended to OD_{600} = 0.5. Cultures carrying RXLRs were mixed 1 : 1 with one of the cultures carrying *R2* or an *R2* orthologue so the final OD_{600} of each is 0.25. Cultures not mixed with either an RXLR or an *R* gene construct were diluted with an equal volume of buffer to a final OD_{600} = 0.25. Cultures were infiltrated with a 1 ml syringe without a needle through the abaxial leaf surface superficially wounded with a needle. Three to four leaves on at least four plants were used for each biological replicate. HRs were recorded and photographed between 2 and 5 d postinfiltration depending on the expression vector and plant species. An individual inoculation was counted as positive if > 50% of the inoculated area developed a clear HR. Data graphs present the mean percentage of total inoculations per plant developing a clear HR with error bars representing ± standard error (SE) of combined data from at least three biological replicates. Co-bombardment assays with *GUS* were performed and assayed as described in Armstrong *et al.* (2005) using pGRAB::*PiAVR2*.

Gene expression analyses

Standard RT-PCR to examine gene expression in a number of isolates at 48 h postinoculation (hpi) on potato was performed using primers diagnostic for expression of *PiAVR2* (AVR2F4 and AVR2R4) and *PiAVR2-like* (avr2diagF1 and avr2diagR1) (Fig. S1) at an annealing temperature of 61°C for 30 cycles. Quantitative gene expression analyses of RXLR genes were performed as described in Whisson *et al.* (2007), with Power SYBR® Green (Applied Biosystems, Warrington, UK), using a Chromo4® Real-Time Detector (Bio-Rad). *ActA* was used as an endogenous control gene as described previously (Bos *et al.*, 2010). General *PiAVR2* and *PiAVR2-like* expression was quantified using qRT-PCRfwd and qRT-PCRrev primers (Fig. S1). PITG_08949 expression was quantified using For 5′-AGGAATCTGAG-ACCGAGGAA-3′ and Rev 5′-GGGGGTTAATGGGAT-TGAG-3′. Data are presented as fold change relative to normalized expression in sporangia growth stage of each individual *P. infestans* isolate, calculated by the ΔΔCt method with error bars representing ± SE.

Presence/absence and sequence diversity in *P. infestans* isolates

PiAVR2 and *PiAVR2-like* were PCR-amplified from *P. infestans* isolates detailed in Table 1. Primers AVR2F1 and AVR2R1 (Fig. S1) were designed against PITG_22870 and amplified the predicted size product (541 bp) from *PiAVR2*. However, they repeatedly failed to generate PCR products from nine isolates (Fig. 2c; Table 1). The reverse primer AVR2R1 also proved unsuitable for initiating DNA sequencing of the PCR products as a result of a 1 bp indel adjacent to the primer (Fig. S1). Additional primer pairs AVR2F2/AVR2R2 and AVR2F4/AVR2R4 were designed (Fig. S1) to amplify this region from the nine isolates that failed previously and to provide improved sequencing primers. Both pairs amplified the expected product sizes (480 and 340 bp, respectively) from isolates that yielded products with the primer pair AVR2F1/R1 but neither amplified products from the nine isolates that failed previously. The primer pair AVR2F2/R2 proved optimal for PCR and sequencing of *PiAVR2* and was used in all subsequent work. The use of the primer pair AVR2F2/R2 at a lower annealing temperature of 58°C generated weak PCR products from three isolates (01/29, MP618 and 06_3928A; Table 1). The sequences of these products differed from PITG_22870 and PITG_08943 at 25 bases, revealing *PiAVR2-like* (Fig. S1). Primer pairs specific for the *PiAVR2-like* sequence (avr2F6/avr2R6 and avr2F7/avr2R7 (Fig. S1) amplified PCR products of the predicted size (392 and 472 bp, respectively) from the nine isolates that had failed to amplify *PiAVR2* but also from 12 isolates from which *PiAVR2* was also amplified. Primers NitRedF (5′-GGACCGCTGGGCCACTTCAC-3′)/NitRedR (5′-CGCTGGCTTGCAGGCGTACT-3′) were used as control reactions to amplify a 435 bp region from the nitrate reductase gene (GenBank accession U14405; PITG_13012).

All PCR reactions were carried out with the same reagents in a 20 μl reaction volume using a Primus 96^{plus} Thermalcycler (MWG-Biotech, Ebersberg, Germany). Each reaction contained 1 × GoTaq® Flexi buffer, 20 μg BSA, 1.5 mM $MgCl_2$, 100 μM dNTPs, 0.8 unit of Taq polymerase (GoTaq® DNA polymerase; Promega), 0.2 μM of primers and *c.* 20 ng of template DNA. Amplification conditions consisted of one cycle of 94°C for 1 min, 30 cycles of 95°C for 30 s, 60°C for primer pairs AVR2F1/R2, F2/R2 and F4/R4, 58°C for primer pairs avr2F6/R6 and F7/R7, and 55°C for the primer pair NitRedF/R for 30 s, 72°C for 30 s and a final cycle of 72°C for 5 min.

Isolate 06_3928A genome sequence and alignment to T30-4 genome

For the genomic DNA extraction, *Phytophthora infestans* strain 06_3928A was cultured in rye sucrose agar (RSA) plates at 18°C for 12 d. Plugs with mycelium of *P. infestans* strain 06_3928A were transferred to modified plich medium (Kamoun *et al.*, 1993), grown for another 2 wk at 18°C and then harvested for genomic DNA isolation using Omniprep kit (G-Biosciences, Maryland Heights, MO, USA; catalogue number 786-136) with minor modifications. For sequencing, the flow cells were prepared according to the manufacturer's instructions using the Illumina pair read cluster generation kit, PE-203-4001. Sequencing reactions were performed mostly on 2G GAs (Illumina Inc., Chesterford Research Park, Essex, UK). The reference genome sequence of the *P. infestans* strain T30-4, annotation and gene/exon locations was downloaded from http://www.broad.mit.edu (GenBank project accession number AATU01000000). The generated raw reads with abnormal lengths and reads containing Ns were removed from the datasets. Filtered reads were use to align to the reference genome strain T30-4. Alignments were obtained with BWA software v0.5.7 (Li & Durbin, 2010) using as parameters a seed length (*l*) of 38 and a maximum of mismatches (*M*) of 3.

Table 1 Details of isolates used in this study and details of PCR product amplification with primers sets (shown in Supporting Information, Fig. S1) specific to each of *PiAVR2* or *PiAVR2-like*

				Amplification with primers below				Amplification with primers below		
Genotype[a]	Isolate name	Origin	*R2*[b] virulence	AVR2 F2 and R2[c]	AVR2 F1 and R1	AVR2 F4 and R4	SNP[d] within *PiAVR2*	avr2 F7 and R7	avr2 F6 and R6	SNP[e] within *PiAVR2-like*
3_A2	2006_4012F	UK	+	–	–	–	n/a	+	+	MI/TV
3_A2	2006_4244E	UK	+	–	–	–	n/a	+	+	—
13_A2	2006_3884B	UK	+	–	–	–	n/a	+	+	—
13_A2	2006_3928A	UK	+	+/–	–	–	n/a	+	+	MI/TV
13_A2	2006_3964A	UK	+	–	–	–	n/a	+	+	—
13_A2	2006_4132B	UK	+	–	–	–	n/a	+	+	—
5_A1	01/29	UK	+	+/–	–	–	n/a	+	+	MI
5_A1	1996_9_5_1	UK	+	–	–	–	n/a	+	+	—
Misc	MP618	Poland	+	+/–	–	–	n/a	+	+	MI/TV
7_A1	2006_4168B	UK	+	+	+	+	K	+	+	MI
7_A1	2006_4168C	UK	+	+	+	+	K	+	+	—
17_A2	2006_4388D	UK	+	+	+	+	K/N	+	+	MI
1_A1	2006_3984C	UK	–	+	+	+	K/N	+	+	TV
2_A1	2006_3888A	UK	–	+	+	+	K	+	+	—
2_A1	2006_4068B	UK	–	+	+	+	K	+	+	MI
8_A1	2006_4256B	UK	–	+	+	+	K	+	+	MI
8_A1	SC_95 17_3_2	UK	–	+	+	+	K	+	+	MI
22_A2	2003_25_1_3	UK	–	+	+	+	K	+	+	MI/TV
22_A2	2003_25_3_1	UK	–	+	+	+	K	+	+	MI/TV
Misc	88069	The Netherlands	–	+	+	+	K	+	+	MI
EC1	EC1	Ecuador	–	+	+	+	K	+	+	—
4_A1	2006_4352E	UK	–	+	+	+	K	–	–	n/a
6_A1	2006_4100A	UK	–	+	+	+	K/N	–	–	n/a
6_A1	2006_3920A	UK	–	+	+	+	K/N	–	–	n/a
10_A2	2006_4440C	UK	–	+	+	+	K	–	–	n/a
10_A2	2006_3936C2	UK	–	+	+	+	K	–	–	n/a
15_A2	2004_7804B	UK	–	+	+	+	K/N	–	–	n/a
Misc	Ca65	USA	–	+	+	+	K/N	–	–	n/a
Misc	T30-4	n/a	–	+	+	+	K/N	–	–	n/a

SNP, single nucleotide polymorphism; n/a, not applicable.
[a]*Phytophthora infestans* genotypes are based on defined simple-sequence repeat marker profiles that will be reported in detail elsewhere.
[b]'+' indicates ability to infect *R2* plants and '–' indicates the isolate triggers HR on *R2* plants.
[c]'+/–' indicates PCR amplification only when the annealing temperature was decreased.
[d]SNP results in amino acid polymorphism N31K in PiAVR2. K/N is heterozygous.
[e]Two SNPs result in amino acid polymorphisms M10T and I92V in PiAVR2-like. MI/TV is heterozygous
Protein alignments from the isolates above, indicating the N31K, M10T and I92V polymorphisms, are shown in Fig. S4.

Western analyses of protein stability

PiAVR2, *PiAVR2-like* and PITG_08949 sequences were cloned into pDNR221 using almost identical primers as described earlier except with one nucleotide of TAA stop codon changed to make a sense codon AAA. LR clonase® (Invitrogen) was used to recombine correct sequences into pB7FWG2.0 (Karimi *et al.*, 2002) C-terminal GFP-tagged plant expression vector, transformed into *E. coli* by electroporation, sequenced, and plasmids with confirmed inserts were transformed into *A. tumefaciens* strain AGL1, pSoup, pVirG cells by electroporation. Positive transformants were grown for 2 d at 27°C in LB supplemented with rifampicin, chloroamphenicol, and spectinomycin. For transient expression in *N. benthamiana*, the conditions described earlier were used. A final OD_{600} = 0.25 of pB7FWG2.0 containing cultures was achieved by mixing with an OD_{600} = 0.2 pJL3-p19 containing culture in a 2 : 1 ratio. Whole leaves were infiltrated with culture to allow 1 cm^2 leaf discs to be cut out at 3 dpi for the protein extraction protocol. Leaf discs of *c.* 100 mg were ground in liquid N_2, 200 μl extraction buffer was added (20 mM HEPES, 13% sucrose, 1 mM EDTA, 1 mM DTT, proteinase inhibitor cocktail tablet, 0.1% Triton – the DTT (dithiothreitol), Triton and proteinase inhibitor cocktail tablet were added fresh each time) and left to thaw on ice. Twenty microlitres of the whole lysate was mixed with 20 μl sodium dodecyl sulphate loading buffer. Samples were boiled for 5 min at 95°C, loaded on to a 12% Bis-Tris NuPAGE® Novex® Mini gel (Invitrogen) and run at 200 V, 120 mA and 25 W for 1 h, then membrane-blotted for 1 h at 130 V. Four per cent% milk powder was used to block the membrane. The primary antibody was monoclonal mouse GFP antibody (Sigma-Aldrich) at 1 : 10 000 dilution. The membrane was washed with PBS-T 0.1% before addition of secondary goat antimouse immunoglobulin (Ig) horseradish peroxidise antibody (Sigma-Aldrich) at 1 : 2000 dilution. ECL-Plus Western Blotting Detection Reagents (Amersham) were used for detection, according to the manufacturer's instructions.

P. infestans transformations

For expression of $PiAVR2^{K31}$ in *P. infestans* isolate 06_3928A, the gene was cloned from genomic DNA of isolate 88069 using primers shown in Fig. S1. The *Cla*I and *Sac*I restriction sites generated were used to clone $PiAVR2^{K31}$ into *P. infestans* expression pTOR, and transformation of isolate 06_3928A was carried out as previously described (Bos *et al.*, 2010). To generate a *P. infestans* strain expressing PiAVR2::Tdtomato, *PiAVR2* was amplified from isolate 88069 using an alternative reverse primer (Fig. S1) altering the stop codon to a sense codon, and cloned into pTOR::Tdtomato (based on pTOR::mRFP described in Whisson *et al.*, 2007; but with the fluorescent protein gene *Tdtomato* in place of *mRFP*) using *Cla*I and *Sac*I restriction sites. This was transformed into isolate 88069 as described in Bos *et al.* (2010). Isolate 06_3928A and this genotype expressing $PiAVR2^{K31}$ and isolate 88069 were inoculated on to potato cv Craigs Royal and R2 differential 1512 c(16) as described previously (Bos *et al.*, 2010) and lesions were observed 1 wk later. Inoculation of the 88069 strain expressing $PiAVR2^{K31}$*::Tdtomato* on to *N. benthamiana*, and confocal microscopy were conducted as described in Whisson *et al.* (2007), but to image Tdtomato, it was excited with the 561 nm laser line, and the emissions were collected between 570 and 600 nm.

Results

Map-based cloning of *PiAVR2* gene candidates

Previously, F_1 progeny of a cross between *P. infestans* parental isolates 80029 (race 2.4.7; A1 mating type) and 88133 (race1.3.7.10.11; A2 mating type) were found to segregate for six dominant *Avr* genes, allowing these to be positioned within a genetic linkage map (Van der Lee *et al.*, 1997, 2001). To facilitate positional cloning of *AVR* genes, a BAC library was constructed from T30-4, an F_1 individual from this cross that contains all six segregating avirulence genes (*AVR1*, *AVR2*, *AVR3*, *AVR4*, *AVR10* and *AVR11*) (Whisson *et al.*, 2001). The BAC library was screened with AFLP markers E + GA/M + ATs513 and E + TG/M + CTs338 (Fig. 1a), defining the *AVR2* map location. Four positive BAC clones that contained either one or other of the two markers were assembled into two contigs that did not completely span the *AVR2*-containing region (Fig. 1a). BAC end-sequencing allowed the contigs to be anchored to supercontig 1.16 of the *P. infestans* isolate T30-4 genome sequence (http://www.broad.mit.edu/annotation/genome/phytophthora_infestans/Home.html; Haas *et al.*, 2009). Sequences at the outer ends of the BAC contigs indicated that the cloned fragments spanned 742.6 kb, within which was a 36.6 kb region containing three predicted RXLR-dEER effector-encoding genes (PITG_08943, PITG_08949 and PITG_22870) (Fig. 1a). Two of these (PITG_08943 and PITG_22870) are identical, apparently duplicated copies 2 kb apart. We confirmed that this is a genuine duplication in the T30-4 genome using PCR primers AVR2F10 and AVR2R10 (Fig. S1), which amplified between the duplicated gene copies to generate a PCR product of the expected 2.2 kb size (results not shown). As they are identical in T30-4, we refer only to PITG_22870 in the following. The third gene (PITG_08949) encodes a predicted protein that is similar across the first 79 amino acids, but which diverges considerably at the C-terminus (Fig. 1b), probably as a result of a DNA recombination event (Fig. S2).

PITG_22870 triggers R2-dependent HR

Transcripts of RXLR effector genes accumulate predominantly during the early, biotrophic stage of *P. infestans* infection (Whisson *et al.*, 2007; Haas *et al.*, 2009). Real-time qRT-PCR was used to investigate transcript accumulation of PITG_22870 and PITG_08949 during potato infection by isolate 88069, which is avirulent on *R2* plants and possesses gene sequences that are identical to those in T30-4. Transcripts of both genes accumulated significantly in preinfection stages and during the biotrophic phase (1–3 d postinoculation), showing the characteristic expression (Whisson *et al.*, 2007) of RXLR effector genes (Fig. 2a).

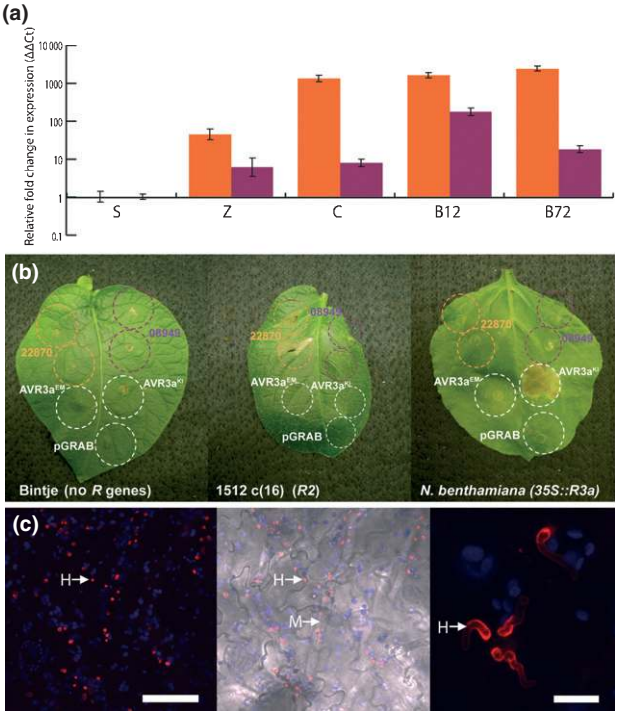

Fig. 2 PITG_22870 is *PiAVR2*. (a) qRT-PCR showed that, relative to the control gene *ActA*, the expression of both PITG_22870 (orange) and PITG_08949 (purple) was up-regulated in *Phytophthora infestans* isolate 88069 in zoospores (Z), germinating cysts (C), and 12 and 72 h postinoculation of susceptible cv Bintje (B12 and B72), relative to expression in sporangia (S), which was given a value of 1. Error bars indicate ± SD for three biological replicates. (b) Whereas expression of PITG_22870 (orange), PITG_08943 (purple) and, as controls, $AVR3a^{EM}$, $AVR3a^{KI}$ and empty pGRAB vector (all white) yielded no response in cv Bintje (left), $AVR3a^{KI}$ alone caused a hypersensitive response (HR) in transgenic *Nicotiana benthamiana* expressing *R3a* (right), and only PITG_22870 triggered an HR in the *R2* potato differential 1512 c(16) (middle). (c) A *P. infestans* transformant expressing *PiAVR2* (PITG_22870)::*Tdtomato* fusion. The left panel shows the accumulation of the fusion protein in haustoria (H), which can be seen in relation to mycelium (M) in the middle panel. The right panel shows a higher magnification of haustorial accumulation of PiAVR2::Tdtomato fluorescence (H). White bars, 50 μm (left panel); 10 μm (right).

To test whether PITG_22870 and PITG_08949 are potentially *PiAVR2*, each was expressed, minus SP-encoding sequences, in potato cv Bintje, which lacks known late blight *R* genes; in the potato *R2* differential 1512 c(16); and, as a further control, in transgenic *N. benthamiana* expressing *R3a*. PITG_08949 failed to elicit an HR in any of these plants, whereas $AVR3a^{KI}$ elicited an HR, as expected (Armstrong *et al.*, 2005), only in *N. benthamiana* expressing *R3a*. PITG_22870 elicited an HR exclusively in the *R2* potato differential (Fig. 2b). Both PITG_22870 and PITG_08949, fused at the C-terminus to GFP, are stable when expressed in *N. benthamiana* (see Fig. 4).

To verify R2-dependent recognition of PITG_22870, the full-length construct (minus SP) was expressed, via co-bombardment with *GUS* as a marker of cell vitality, in cv Bintje and the *R2* differential clone 1512 c(16). Whereas no difference in GUS activity was seen following co-bombardment with PITG_22870 in Bintje, a considerable reduction in GUS activity, consistent with triggering the HR, was seen following co-bombardment with PITG_22870 exclusively on the *R2* differential (Fig. S3). Independently, both PITG_22870 and PITG_08949 were coexpressed in *N. benthamiana* with the *R2* gene, or with the orthologous genes *Blb3*, *ABPT* and *R2-like*, each of which provides a similar spectrum of resistance to *P. infestans* isolates. HR was observed with each *R2* orthologue only when coexpressed with PITG_22870 (Lokossou *et al.*, 2009). Taking these results together, PITG_22870 was tentatively renamed *PiAVR2*.

Previously, effector AVR3a was shown to accumulate at the site of haustorium formation during the biotrophic phase of late blight infection (Whisson *et al.*, 2007). Similar localization patterns have been observed for AVR4 and AVR-blb1 (Van Poppel, 2009). To investigate whether this was also the case for PiAVR2, isolate 88069 was transformed to express PiAVR2, translationally fused at its C-terminus to the fluorescent protein Tdtomato. Confocal microscopy revealed that PiAVR2 exclusively accumulated at haustoria during infection (Fig. 2c), a property consistent with a potential virulence role during biotrophy.

Presence/absence and sequence polymorphisms in *PiAVR2*

To investigate polymorphisms that may explain *P. infestans* virulence on *R2* potato plants, three PCR primer pairs, one located in 3′ and 5′-flanking regions (primers AVR2F1 and R1), one spanning flanking and coding regions (AVR2F2 and R2; Fig. S1) and one set located within the coding region (AVR2F4 and R4; Fig. S1), were used to amplify *PiAVR2* from a diverse collection of 29 *P. infestans* isolates, 12 of which are virulent on *R2* plants and 17 of which are avirulent. Strikingly, no PCR amplification was observed with any of the three primer sets from nine of the 12 viru-

lent isolates, suggesting that *PiAVR2*, and its flanking regions, are either absent or highly divergent in these genomes. In the case of primer set AVR2F2/R2, lowering the annealing temperature resulted in weak PCR amplification products of the expected size from three of the nine virulent isolates that showed no amplification with AVR2F1/R1 and AVR2F4/R4 primers (Table 1). By contrast, all three primer sets amplified PCR products of the expected size from the remaining three virulent isolates and from all 17 avirulent isolates (Table 1).

The strong PCR amplification products generated with the AVR2F2/R2 PCR primer set from 20 of the tested isolates were sequenced. Only a single amino acid polymorphism, N31K, was observed in the predicted mature protein sequences derived from these *PiAVR2* genes. Seven isolates were heterozygous for $PiAVR2^{N31}$ and $PiAVR2^{K31}$, and 13 isolates were homozygous for $PiAVR2^{K31}$ (Table 1; Fig. S4). Both $PiAVR2^{N31}$ and $PiAVR2^{K31}$ sequences were represented within the three virulent and 17 avirulent isolates, indicating that this polymorphism is unlikely to specify differential recognition by R2. The *PiAVR2* sequence was PCR-amplified using the AVR2F2/R2 primer set and sequenced from a further 30 European and North American isolates of undetermined virulence on *R2* plants. Again, only the single N31K polymorphism was observed; 20 of these isolates were heterozygous for $PiAVR2^{N31}$ and $PiAVR2^{K31}$, and 10 isolates were homozygous for *PiAVR2*^{K31} (Table S2). Intriguingly, out of the 50 tested isolates containing this gene, no $PiAVR2^{N31}$ homozygotes were observed.

The C-terminal effector domain of PiAVR2 is recognized by R2

To test whether the N31K polymorphism in PiAVR2 proteins determined recognition by R2, each form, minus SP-encoding sequences, was coexpressed with *R2* in *N. benthamiana*. *R2* was also coexpressed with the N-terminal half (from the cleavage site of the SP to the end of the EER motif) or C-terminal half (from the first amino acid after the EER motif to the stop codon) coding regions of $PiAVR2^{N31}$ (the sequence within the assembled genome of avirulent clone T30-4; Haas *et al.*, 2009) (Fig. 3a). We found that R2-dependent HR occurred, to a similar extent, with both $PiAVR2^{N31}$ and $PiAVR2^{K31}$ full-length (minus SP-encoding) sequences (Fig. 3b,c). Given the presence of the $PiAVR2^{N31}$ and/or $PiAVR2^{K31}$ forms in three isolates that can infect *R2* plants, additional factors are needed to explain the virulence of these isolates. In agreement with this, no recognition of the N-terminus-encoding half of $PiAVR2^{N31}$ was observed, whereas a stronger and faster HR was observed with the C-terminus-encoding half alone (Fig. 3b,c). Western analyses with each *PiAVR2*-derived construct, fused at the C-terminus to *GFP*, revealed that

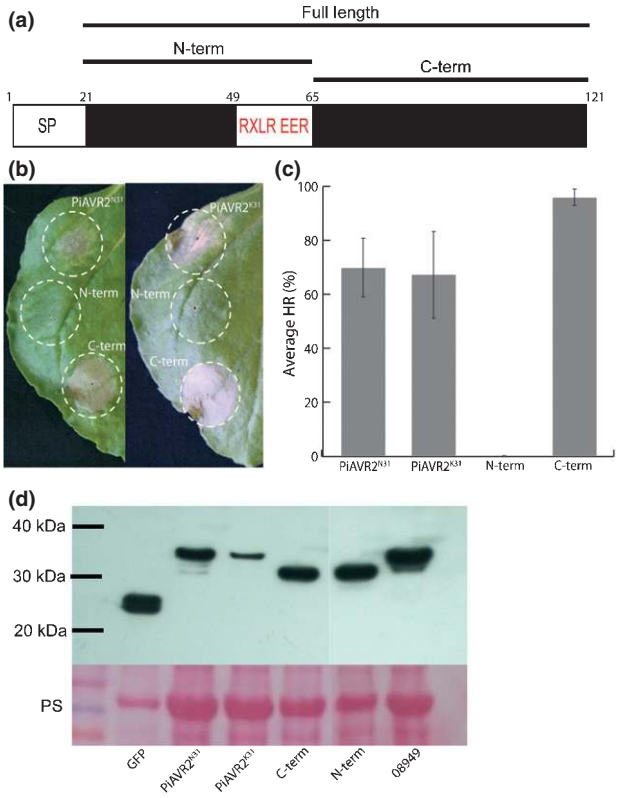

Fig. 3 Resistance protein R2 recognizes the C-terminus of PiAVR2. (a) Schematic of portions of *PiAVR2* that were cloned and coexpressed with *R2*. (b) Coexpression of *R2* with full length of $PiAVR2^{N31}$ or $PiAVR2^{K31}$ (both minus SP), or the N-terminal or C-terminal coding portions of $PiAVR2^{N31}$, as indicated, in *Nicotiana benthamiana*. (c) Percentage of inoculation sites developing hypersensitive response (HR) following coexpression of *R2* with full length (minus SP) of $PiAVR2^{N31}$ or $PiAVR2^{K31}$, or the N-terminal or C-terminal coding portions of $PiAVR2^{N31}$, as indicated, in *N. benthamiana*. These results are the averages of three independent experiments each involving 24 inoculation sites per construct combination. Error bars indicate ± SE. (d) Western hybridization of green fluorescent protein (GFP) antibody to protein extracted 3 d postinoculation of plants expressing free *GFP*, full length (minus SP) of $PiAVR2^{N31}$ or $PiAVR2^{K31}$*::GFP*, the N-terminal or C-terminal coding portions of $PiAVR2^{N31}$, or PITG_08949, all translationally fused at the C-terminus to GFP, in *N. benthamiana*. Ponceau stain (PS) and size markers (kDa) are indicated.

each fusion protein was detectable following agroinfiltration and expression in *N. benthamiana*, albeit $PiAVR2^{K31}$ was apparently less stable than $PiAVR2^{N31}$ (Fig. 3d; see also Fig. 4g). These results indicate that, similar to $AVR3a^{KI}$ (Bos *et al.*, 2006), recognition of PiAVR2 is not dependent on the N-terminal half of the protein.

An alternative form, PiAVR2-like, evades recognition by R2

No PCR product was obtained using primer sets AVR2F1/R1 or AVR2F4/R4 from nine of the *P. infestans* isolates that are virulent on *R2* potato, suggesting that

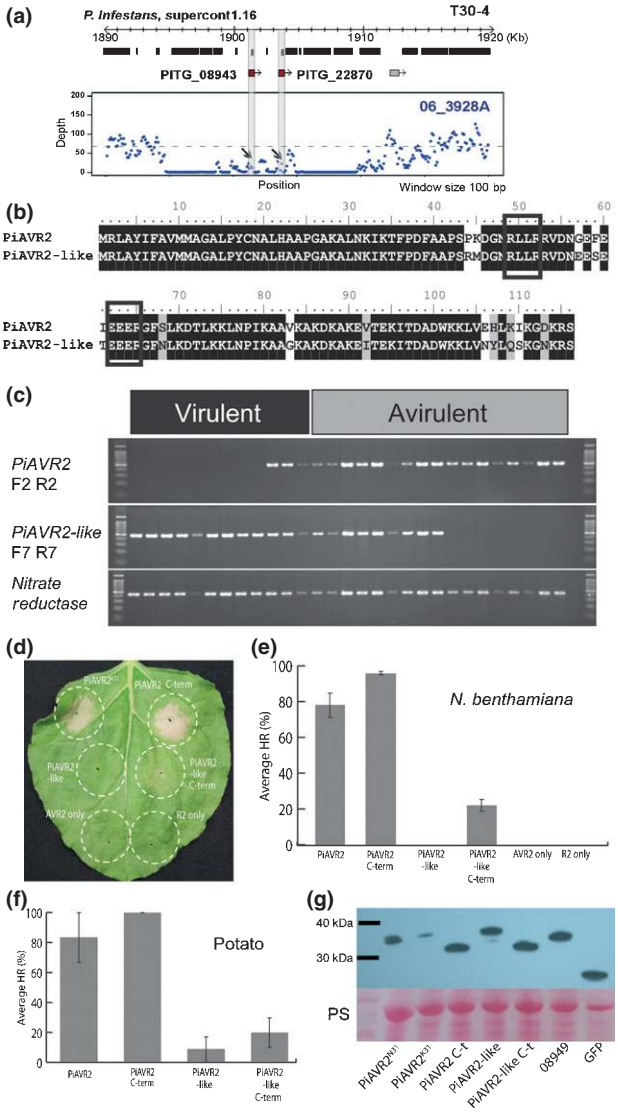

Fig. 4 PiAVR2-like evades detection by R2. (a) Plot of sequencing depth of coverage of Illumina reads from isolate 06_3928A aligned to the region of supercontig 1.16 from isolate T30-4 containing the two *PiAVR2* paralogues (in red): PITG_22870 and PITG_08943. Arrows indicate regions where sequence reads from 06_3928A are aligned to *PiAVR2* genes highlighted within grey vertical bars. The horizontal dashed line indicates the average coverage of the 06_3928A genome. Note the *c.* 14.8 kb subregion (from 1894.9 to 1909.7 kb) that shows reduced coverage in reads from isolate 06_3928A, indicating high sequence divergence in this isolate (dark square, repeat; red square, RXLR effector; grey square, gene). (b) Protein alignment of PiAVR2 and PiAVR2-like, revealing 13 amino acid polymorphisms between the two mature proteins. The RLLR and EER motifs (dark boxes) is indicated. (c) Presence/absence polymorphisms between *PiAVR2* (PCR amplified with AVR2F2/R2 primers, annealing temperature 60°C) and *PiAVR2-like* (PCR amplified with AVR2F7/R7 primers, annealing temperature 58°C) across 12 virulent and 17 avirulent *Phytophthora infestans* isolates (arranged in the order shown, from top to bottom, in Table 1). PCR amplification of the control gene *nitrate reductase* is indicated. (d) Whereas the full-length (minus SP-coding) and C-terminus coding regions of *PiAVR2* trigger HR when coexpressed with *R2* in *Nicotiana benthamiana*, the equivalent regions of *PiAVR2-like* do not. Expression of *PiAVR2* and *R2* alone are indicated as controls. (e) Average percentage HR for 24 inoculation sites replicated in three experiments following expression of *PiAVR2* or *R2* alone, or coexpression of each construct combination in *N. benthamiana* (as in d) as indicated. Error bars indicate ± SE. (f) Average percentage HR across 24 inoculation sites for full-length (minus SP-coding) and C-terminus coding regions of *PiAVR2*, or equivalent regions of *PiAVR2-like* following expression in the *R2* differential 1512 c(16). (g) Western hybridization of green fluorescent protein (GFP) antibody to protein extracted 3 d postinoculation of plants expressing free *GFP*, full length (minus SP) of *PiAVR2*^{N31}*::GFP* or *PiAVR2*^{K31}*::GFP*, the C-terminal coding portion of *PiAVR2* (PiAVR2 C-t) fused to *GFP*, full-length (minus SP) *PiAVR2-like::GFP*, the C-terminal portion of *PiAVR2-like* (Piavr2 C-t) fused to *GFP*, or PITG_08949::*GFP*, in *N. benthamiana*. Ponceau stain (PS) and size markers (kDa) are indicated.

PiAVR2, if present, is divergent at the DNA sequence level. Nevertheless, three of these isolates yielded a weak PCR product with the AVR2F2/R2 primer set when the annealing temperature was lowered (Table 1). One of these isolates, 06_3928A, is a representative of the 13_A2 genotype that is currently prevalent in western Europe (Cooke *et al.*, 2009; Fry *et al.*, 2009). The genome of this isolate has recently been sequenced using Illumina technology and will be reported elsewhere. Alignment of 06_3928A reads against the genomic region of T30-4, including the two *PiAVR2* paralogues, PITG_22870 and PITG_08943, revealed that a subregion of 14.8 kb is highly divergent in this strain (Fig. 4a). Within the 14.8 kb subregion, very few reads could be aligned to the *PiAVR2* coding sequence, suggesting the gene is divergent in the 06_3928A strain. The weak AVR2F2/R2 PCR products from the three *P. infestans* isolates were thus sequenced. They revealed a gene sequence that was conserved between the three isolates and which showed striking similarity to *PiAVR2*; 25 SNPs were observed between the coding regions (Fig. S1), resulting in 13 amino acid polymorphisms between the mature proteins (Fig. 4b). The combined assembly of the aligned and unaligned sequence reads from 06_3928A corresponding to the *PiAVR2* gene confirmed the presence of the variant form (termed *PiAVR2-like*) within the 06_3928A genome. *PiAVR2-like* was absent from both assembled and unassembled reads of the T30-4 genome.

PCR primers (avr2F6/R6 and avr2F7/R7; Fig. S1) designed specifically to amplify this sequence variant yielded amplification products from all 12 isolates that are virulent on *R2*, and from nine of the 17 avirulent isolates (Fig. 4c; Table 1). PCR products were sequenced from 13 isolates that represent distinct *P. infestans* genotypes, as defined by simple sequence repeat markers (Lees *et al.*, 2006). Only two polymorphisms in the predicted proteins were observed, M10T and I92V (Table 1; Fig. S4). The first is within the predicted signal peptide, which is cleaved from the mature protein during secretion, and is thus unlikely to

affect recognition by R2. In the case of the I92V polymorphism, the Val residue is shared with the recognized PiAVR2 protein. If *PiAVR2-like* evades recognition by R2, the presence of the allele encoding a protein with the Val^{92} residue in virulent isolates indicates that this polymorphism is unlikely to specify recognition by R2 (Fig. S4; Table 1). Although a number of isolates contain both *PiAVR2* and *PiAVR2-like*, all avirulent isolates contain the recognized *PiAVR2* sequence, and all virulent isolates contain *PiAVR2-like*. This prompted us to test whether *PiAVR2-like* is recognized by R2.

Regions encoding the full length (minus SP) and C-terminal halves (from the first amino acid after the EER motif to the stop codon) of *PiAVR2* and *PiAVR2-like* were independently coexpressed with *R2* in *N. benthamiana*. Whereas both *PiAVR2* constructs triggered *R2*-dependent HR, full-length *PiAVR2-like* did not, and the C-terminal portion very rarely triggered an HR (Fig. 4d,e). Moreover, expression of each construct in the potato *R2* differential, 1512 c(16), again revealed an HR with *PiAVR2*, but an HR was infrequently observed with only the C-terminal half of *PiAVR2-like* (Fig. 4f). As before for PiAVR2, western analysis of all forms translationally fused at the C-terminus to GFP indicated that PiAVR2-like was stable on expression in *N. benthamiana* (Fig. 4g).

R2 orthologues from wild *Solanum* species show a similar spectrum of recognition

Previously, three *R2* orthologues from wild *Solanum* species, *BLB3*, *ABPT* and *R2-like*, were all shown to possess the same spectrum of resistance and to respond with a strong HR when coexpressed with *PiAVR2* (Lokossou *et al.*, 2009). We coexpressed the full-length (minus SP) forms of $PiAVR2^{N31}$, $PiAVR2^{K31}$ or *PiAVR2-like* with each of these *R2* orthologues in *N. benthamiana*. As expected, whereas both $PiAVR2^{N31}$ and $PiAVR2^{K31}$ triggered a clear HR with each, there was seldom a response to *PiAVR2-like* (Fig. 5). Thus, none of these *R* genes is expected to extend resistance to additional *P. infestans* isolates, such as 06_3928A, a representative of the prevalent genotype 13_A2.

Virulence on *R2* plants is associated with presence or expression of only *PiAVR2-like*

Whereas *PiAVR2* is present in all avirulent isolates, *PiAVR2-like* is present in all virulent isolates. Nevertheless, nine of the avirulent isolates, and three of the tested virulent isolates, possess both forms (Table 1). A possible explanation for the phenotypic differences in this class of isolate is differential expression of *PiAVR2* and *PiAVR2-like*. To investigate this, PCR primers specific to the coding regions of *PiAVR2* (F4/R4; Fig. S1) and *PiAVR2-like* (Fig. S1) were used to amplify the corresponding fragments from

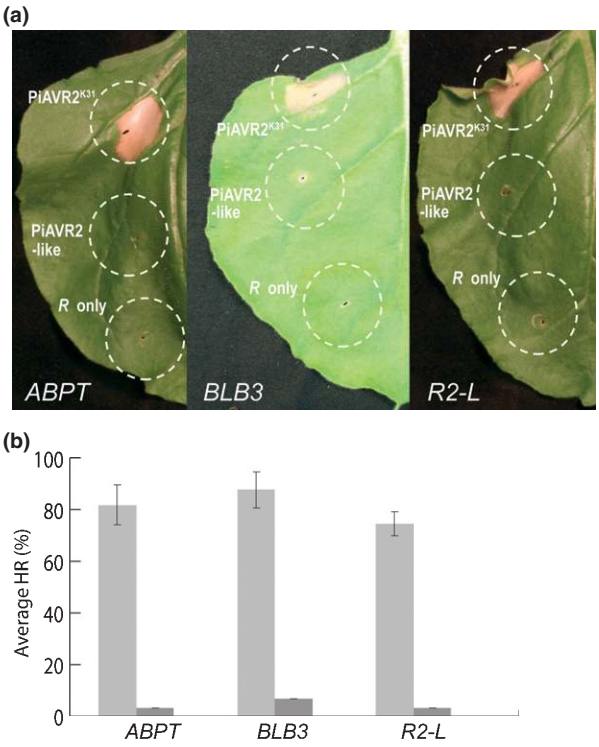

Fig. 5 Resistance genes *ABPT*, *BLB3* and *R2-like* show similar specificity to *R2*. (a) Panels showing coexpression of full-length (minus SP-coding) regions of $PiAVR2^{K31}$ or *PiAVR2-like* with *ABPT* (left), *BLB3* (middle) or *R2-like* (right) in *Nicotiana benthamiana*. Expression of the relevant *R* gene alone is shown in each panel. (b) Average percentage hypersensitive response (HR) for 24 inoculation sites replicated in three experiments following coexpression of $PiAVR2^{N31}$ or $PiAVR2^{K31}$ (combined in the graph as PiAVR2, as both forms were recognized to a similar degree; light grey bars), or *PiAVR2-like* (mid-grey bars) with *ABPT* (left), *BLB3* (middle) or *R2-like* (right) in *N. benthamiana*. (*R* gene only, black bars) Error bars indicate ± SE.

cDNA prepared 2 d after leaf inoculation. The following isolates were tested: T30-4 (which possesses only *PiAVR2*); 06_3928A (which possesses only *PiAVR2-like*); 06_4168B and 06_4168C (which are virulent but possess both forms); and 88069 and 06_4256B (which are avirulent but possess both forms) (Table 1). As expected, expression of only *PiAVR2* was detected in T30-4, and of only *PiAVR2-like* in 06_3928A. In addition, expression of both *PiAVR2* and *PiAVR2-like* was detected in the avirulent isolates 88069 and 06_4256B. However, in the virulent isolates 06_4168B and 06_4168C, expression of only *PiAVR2-like* was detected (Fig. 6a). Thus, both presence/absence and expression polymorphisms of *PiAVR2* and *PiAVR2-like* explain virulence on *R2* potato. qRT-PCR primers which amplify both forms (Fig. S1) were used to show that the corresponding *PiAVR2* and *PiAVR2-like* genes show the expected biotrophic pattern of transcript accumulation early in infection in T30-4 and 06_3928A, respectively (Fig. 6b).

PiAVR2 is a dominant gene triggering *R2*-dependent resistance

To investigate whether R2-dependent recognition of *PiAVR2* confers disease resistance, isolate 06_3928A, which is virulent on *R2* potato plants and contains only the *PiAVR2-like* variant, was transformed to express the $PiAVR2^{K31}$ allele cloned from the avirulent isolate 88069. Isolate 88069 and both transformed and untransformed 06_3928A strains infected cv Craigs Royal, which lacks *R2*. By contrast, the 06_3928A strain transformed to express *PiAVR2* elicited a clear HR on the *R2* differential 1512 c(16), whereas untransformed 06_3928A was able to infect this plant (Fig. 6c). *PiAVR2* is thus a dominant gene that triggers *R2*-dependent HR and disease resistance.

Discussion

A map-based cloning strategy was used to identify an RXLR-EER effector gene, *PiAVR2,* from the *P. infestans* T30-4 genome sequence (Fig. 1). Coexpression of *PiAVR2* with the potato resistance gene *R2* results in a clear HR (Figs 3, 4). Moreover, transformation of a virulent isolate of the pathogen to express *PiAVR2* resulted in R2-mediated HR (Fig. 6), indicating that this is a dominant gene responsible for triggering R2-dependent disease resistance. Expression in plants of either full-length PiAVR2 protein minus the signal peptide, or of its C-terminal effector domain (from the amino acid following the EER motif to the stop codon) resulted in R2-dependent HR (Figs 3, 4), indicating that, as shown for other RXLR effectors (Birch *et al.*, 2008; Schornack *et al.*, 2009), recognition occurs within the host cell. Again consistent with other RXLR effector genes, such as *AVR3a* from *P. infestans* (Armstrong *et al.*, 2005; Whisson *et al.*, 2007), *PiAVR2* transcripts accumulate during biotrophy and encode a protein that accumulates at haustoria (Fig. 2), which form an intimate association with the host cell during this stage of infection.

PCR amplification and sequencing of *PiAVR2* from a diverse set of *P. infestans* isolates revealed that this gene was present in all avirulent isolates and was highly conserved (Fig. 4). Only a single amino acid polymorphism, N31K, was observed between the signal peptide and the RLLR-EER motifs in the predicted proteins. Of 50 isolates containing this gene, 27 were heterozygous for the $PiAVR2^{N31}$ and $PiAVR2^{K31}$ alleles, and 23 were homozygous for the $PiAVR2^{K31}$ allele (Table 1; Table S2). The strong bias towards the $PiAVR2^{K31}$ allele is reminiscent of the bias towards $AVR3a^{EM}$, rather than the $AVR3a^{KI}$ allele, across a similar number of diverse *P. infestans* isolates (Armstrong

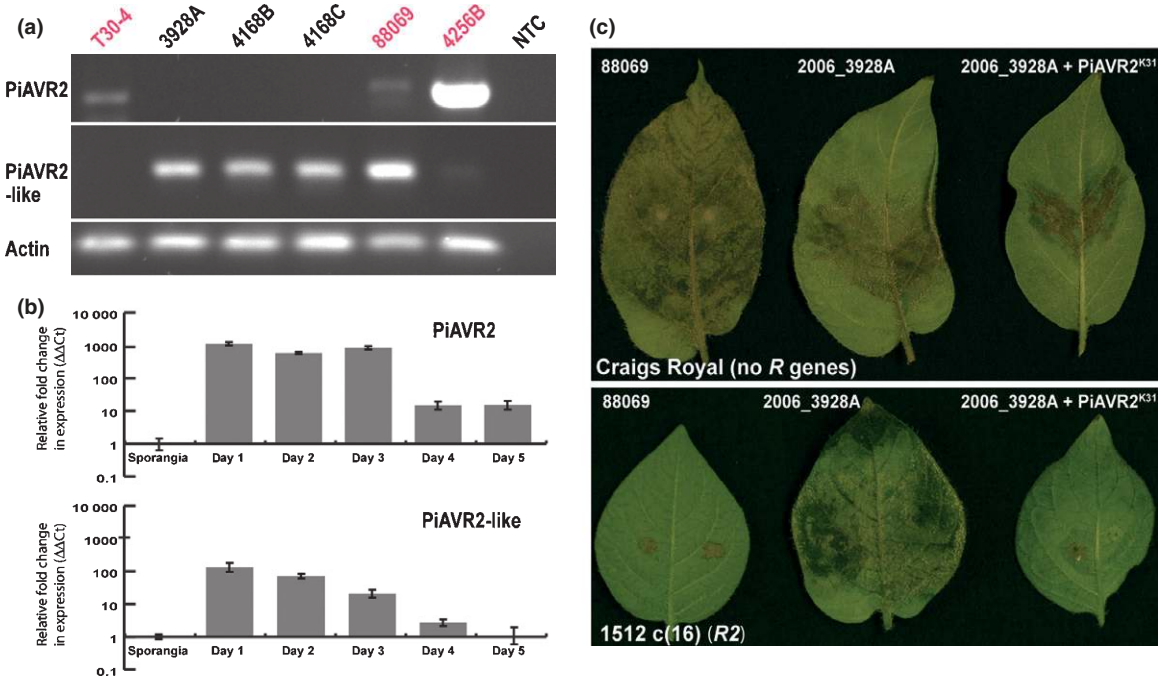

Fig. 6 *PiAVR2* is a dominant gene specifying *R2*-dependent disease resistance. (a) RT-PCR showing expression of *PiAVR2* (upper panel), *PiAVR2-like* (middle panel) and control gene *ActA* (lower panel) in isolates T30-4, 06_3928A, 06_4168B, 06_4168C, 88069 and 06_4256B, 24 h postinoculation of potato cv Bintje. Isolates in red are avirulent and those in black are virulent on *R2* plants. (b) qRT-PCR showing up-regulation, relative to the control gene *ActA*, of *PiAVR2* in T30-4 (upper graph) and *PiAVR2-like* in 06_3928A (lower graph) during the first 5 d of potato cv Bintje infection, relative to that within sporangia (given a value of 1). (c) Infection of leaves from cv Craigs Royal (upper panel; CR), which lacks *R2*, and the *R2* potato differential 1512 c(16) (lower panel; R2), 6 d after inoculation with isolates 88069 (avirulent on *R2*), 06_3928A (virulent on *R2*) and 06_3928A transformed to express $PiAVR2^{K31}$.

et al., 2005). However, whereas *AVR3a*^{EM} evades recognition by the potato resistance gene *R3a*, both the *PiAVR2*^{N31} and *PiAVR2*^{K31} alleles are recognized equally by *R2*. Indeed, the strong recognition of the C-terminal effector domain, which is conserved between the proteins encoded by these alleles, indicates that any genotypic bias in pathogen populations is not associated with evasion of *R2*-mediated recognition.

Gene duplication and recombination to create a new RXLR-EER effector

PiAVR2 was apparent as two identical, adjacent copies (PITG_08943 and PITG_22870) within the genome of *P. infestans* isolate T30-4, the consequence of a recent duplication event. Approx. 30 kb away is a related gene, PITG_08949, which is up-regulated before and during biotrophy, a hallmark of RXLR effector genes. The predicted protein sequences of PiAVR2 and PITG_08949 are highly similar across the first 79 amino acids, strongly suggestive of a common ancestry. After this they diverge significantly, providing distinct C-termini of 37 and 20 amino acids, respectively. These differences are not the result of frameshift mutations, but rather are likely the result of a DNA recombination event (Fig. S2).

The N-terminal signal peptide and RXLR-EER domains of RXLR effectors can be regarded as a functional unit required for secretion and delivery to their site of action within the host cell. It is therefore reasonable to expect greater sequence diversity in response to selection pressure on the C-terminal 'effector' domains which, in addition to their proposed roles in manipulating host defences, must evade detection by R proteins. Indeed, positive selection has been detected mainly in the C-terminal portions of RXLR genes (Win *et al.*, 2007). Regarding the N-terminal and C-terminal regions of these effectors as functionally distinct modules, a likely mechanism by which the latter could evolve distinct virulence specificities is through recombination, and this appears to be the case to generate either *PiAVR2* or PITG_08949, the latter of which evades detection by R2 (Fig. 2). Further work is needed to determine the potential host targets of PiAVR2 and PITG_08949 and to investigate whether they play distinct, or related, roles in promoting *P. infestans* disease development.

Virulence on *R2* plants

Copy number variation has been a feature of avirulence loci in *P. infestans* and *P. sojae* and has contributed to phenotypic variation (Jiang *et al.*, 2006; Qutob *et al.*, 2009; Dong *et al.*, 2009). This is well documented particularly for *PsAvr1a* and *PsAvr3a*, with some avirulent *P. sojae* isolates containing multiple copies and some virulent isolates revealing complete deletion of recognized forms (Qutob *et al.*, 2009). Both PCR and genome sequencing suggest that *PiAVR2* is deleted from the genomes of many virulent isolates. Alignment of sequence reads from the virulent isolate 06_3928A against the genome sequence of the avirulent clone T30-4 revealed that a 14.8 kb subregion, including *PiAVR2* paralogues, is highly divergent (Fig. 4a). In addition, for both *PsAvr1a* and *PsAvr3a*, transcriptional differences have contributed to virulence on plants containing the associated *R* genes (Qutob *et al.*, 2009). It is apparent that not only sequence but also transcriptional variation has occurred in *PiAVR2*, as no expression was detected in two virulent isolates that contain this gene (Fig. 6a).

In addition to *PiAVR2,* and by using the genomic and PCR sequencing data, we discovered a divergent form, *PiAVR2-like,* which is present in the genomes of all tested virulent isolates. The predicted protein of this sequence differs from PiAVR2 in 13 amino acids, eight of which reside in the C-terminal effector domain. As this domain alone, from PiAVR2, is detected by R2, one or more of these polymorphisms must specify the evasion of R2-mediated HR by PiAVR2-like.

We found that nine of the 12 virulent isolates tested lacked *PiAVR2* but possessed *Piavr2*. By contrast, eight of the 17 avirulent isolates tested possessed *PiAVR2* but lacked *PiAVR2-like*. The remaining three virulent and nine avirulent isolates contain both forms. Testing two of these three virulent isolates revealed that *PiAVR2-like* was expressed whereas *PiAVR2* was not. Therefore, not only does a combination of presence/absence polymorphisms and transcriptional silencing explain virulence on *R2* plants, but the data may also suggest functional complementarity between *PiAVR2* and *PiAVR2-like*, as absence or silencing of one form coincides with presence or expression of the other. It will thus be interesting to determine whether these sequences share a common function in pathogenicity. We noted that, of the two avirulent isolates tested that possess *PiAVR2* and *PiAVR2-like*, both forms were expressed during infection, albeit to different levels (Fig. 6a), indicating that expression of one does not preclude expression of the other. However, critically, every isolate investigated in this study expresses one form or the other, or both, and it will thus be important to determine whether, like *AVR3a* (Bos *et al.*, 2010), these sequences provide an essential role during infection.

Proposed strategy for durable disease resistance

Although both *PiAVR2* and *PiAVR2-like* differ from each other by 25 nucleotides (13 amino acids), within each sequence we observed little variation. A single nonsynonymous change, N31K, was observed in the PiAVR2 protein sequences from 50 isolates, and only two polymorphisms, M10T and I92V, were observed in the PiAVR2 protein

from 13 isolates, one of which is in the signal peptide and therefore cleaved from the mature protein (Table 1; Fig. S4). Whereas *R2*, and related sequences from wild *Solanum* species, *BLB3*, *ABPT* and *R2-like*, all recognize *PiAVR2*, none of these resistances extends specificity to *PiAVR2-like*. Given the apparent conservation of each effector sequence, and the ubiquitous presence of either or both forms, we propose that a search for *R* genes that detect *PiAVR2-like* is a high priority. Deployed in combination, *R2* and an *R* gene that recognizes *PiAVR2-like* may provide effective and durable late blight resistances for the European potato industry, given that the pathogen genotypes studied here are representative of the wider European population. This study highlights the importance of understanding the molecular basis not only of recognition, which leads to disease resistance, but also of how the pathogen, at the molecular level, is equipped to evade such recognition. Armed with such knowledge, we propose that the targeted search for specific resistances, and their judicious deployment, can be carefully directed to control this versatile and economically devastating pathogen.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Locations of PCR primers on the *PiAVR2* and *PiAVR2-like* gene sequences.

Fig. S2 DNA sequence alignment of PITG_22870 and PITG_08949, showing the location of a likely recombination event.

Fig. S3 Co-bombardment reveals *R2*-mediated recognition of *PiAVR2*.

Fig. S4 Protein sequence alignments of PiAVR2 and PiAVR2-like from isolates virulence tested on *R2* plants.

Table S1 PCR primers for cloning *PiAVR2* and *PiAVR2-like* regions for transient expression

Table S2 *PiAVR2* genotypes of 30 *Phytophthora infestans* isolates of unknown virulence on *R2* plants

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