MATERIALS AND METHODS

Hyaloperonospora parasitica isolates and Arabidopsis thaliana accessions
All H. parasitica isolates used in this study were gathered from naturally infected Arabidopsis populations within the United Kingdom. The location of these populations is described in (1) and (2). The Col5::RPP13-Nd transgenic line was generated as described in (3). The F₀ Col5 x Nd inbred line HR3879 is described by (1) and deposited in the Nottingham Arabidopsis Stock Centre (UK).

Genomic DNA preparation from H. parasitica
Arabidopsis seedlings were inoculated with H. parasitica as described previously (2). DNA was prepared from 50 mg of infected Arabidopsis leaf material using the plant DNAeasy kit and following the protocol for plant DNA isolation (Qiagen, Crawley, UK). DNA was eluted into 50 µl of buffer and 0.5 µl was used in subsequent PCR reactions.

BAC hybridisations
A BAC library from H. parasitica isolate Emoy2 (4) was probed with 32P-labelled SSH clones Ppat5 and Ppat17 (6). Hybridisation was performed at 55°C in a solution of 10 x Denhardt’s solution; 4 x SSC; 1% SDS for 18 hours. Hybridised filters were washed in 5 x SSC; 0.1% SDS at 55°C and exposed to Kodak X-Omat AR film for 1-3 days at –70°C. PCR and primers designed to the ends of the SSH clones were used to verify that hybridising BAC clones contained either Ppat5 or Ppat17. The Ppat5 probe hybridised to BAC clones 4F16, 5H16, 7M23, 16D7, 18E17 and 21H20; the Ppat17 probe hybridised to BAC clones 8H5, 12N5, 14P17 and 18H5.

Mapping Ppat17
Sequence markers, obtained from one end of BAC clone 14P17 and within the SSH clone Ppat17, were used to map Ppat17 relative to known avirulence genes in an F₂ mapping population derived from a cross between H. parasitica isolates Emoy2 and Maks9 (5). This mapping population carries at least 5 segregating avirulence genes. Primers were designed to Ppat17: ATR13-1F (5’-ACG ATC GTC ATG TCT TCG) lay in the repeat region and ATR13-1R (5’-CGA TGC TAC ATC TAA GCC) lay 3’ to the predicted polyadenylation site. BAC end primers 14P17-1F (5’-TTA GGA TCA TCA CAG GCG) and 14P17-1R (5’-ATT CTG GAG TCG CAT TGG) were designed to the T7 end of 14P17. These two sets of primers were used to amplify DNA from the F₂ mapping population. The products were cycle-sequenced (Applied Biosystems, Foster City, CA, USA) and the resulting DNA polymorphisms scored. We found that the BAC end marker was linked to ATR13 with 2 recombinants among 206 F₂s and that the Ppat17 marker cosegregated over the same number of F₂s. These data indicated that Ppat17 was tightly linked to the avirulence gene ATR13.

Sequencing ATR13 alleles and ATR13-Maks9 cDNA
ATR13-Emoy2 was sequenced directly from the BAC clone 14P17 by a primer walking strategy using initial primers designed from the SSH (suppression subtractive hybridisation) clone fragment of Ppat17. ATR13-Maks9 was amplified from genomic DNA as a 564 bp product and sequenced directly using a primer pair designed from ATR13-Emoy2 (ATR13-2F 5’-CAA TGC GCC TTG TTC ACG CGG TAC; ATR13-2R 5’-CAA CTA CTG TCT GTC TGT GTC AAG GCC AGC
C). *ATR13* alleles from Aswa1, Emco5, Goco1 and Hind4 were amplified and sequenced directly using the following primer pair: (ATR13-3F) 5’-CGC TGG ACG AAC AAC GCA CCG TG and ATR13-3R 5’-CTA GAT AAA GGT AAA ACG AAA AGG. cDNA generated from Col5 seedlings infected with Maks9 (6) was used as template for 5’ and 3’ RACE analysis and to confirm the structure of the predicted *ATR13* open reading frame (ORF). 3’ RACE was carried out using gene specific primer ATR13-RACE (5’-CGC CAG CTC CGA GCA GCC GCC AGC G) located 111 bp downstream of the predicted ATG start codon and a universal primer (SMART cDNA synthesis kit, BD Biosciences Clontech, Palo Alto, CA, USA). Primary PCR products were reamplified using the universal primer and a nested primer ATR13-RACEN (5’-TTG GAC TCT CAC GAG CTA GTT TCG) located 142 bp downstream of the predicted ATG start codon. Sequence analysis of the 3’ RACE product confirmed the predicted structure of the ORF for this region of the gene. 5’RACE analysis failed to define the start of the transcript. However, a 5’ cDNA fragment was obtained using primer ATR13-2F, which straddles the predicted ATG start codon, and primer ATR13-4R (5’-CTC CTA CTT CGT CGT GAC G) located 45 bp upstream of the stop codon. Sequence analysis of this cDNA fragment confirmed that *ATR13-Maks9* carries no introns and that it encodes a 187 amino acid protein. An in-frame stop codon lies between the ATG start codon and a predicted transcriptional start point consensus sequence at −51 bp (5’-TGT CAT TTC CGC ATT CGC C). This sequence lies within the range seen for transcription start points that have been mapped in other oomycete genes (26-91bp) and shows 74% identity to a 19 nucleotide consensus sequence (5’-GCYCATTYYNCAWTTTNYY) (7, 8).

**Sequencing Ppat5 alleles**
Ppat5-Emoy2 was sequenced directly from the BAC clone 5H16 by a primer walking strategy using initial primers designed from the SSH clone fragment of Ppat5. Ppat5 alleles were amplified from Maks9, Aswa1, Emco5, Goco1 and Hind4 genomic DNA as two overlapping fragments using the following primer pairs: 2G3-1F (5’-TTT TGC CAC CAC AGA GAG AC and 2G3-1R  (5’-ATC AGC TGC TGC ACC TTG G); 2G3-2F (5’-TAC CGA GTC GTT GAG TCG TG) and 2G3-2R  (5’-AAG CAT GTG GTT CGC ACT TAC). PCR products were purified by Qiaquick PCR purification kit (Qiagen) and sequenced directly by cycle-sequencing.

**Cloning ATR13 alleles and transient expression in detached Arabidopsis leaves**
*ATR13-Maks9* and *ATR13-Emoy2* were cloned into the plant expression vector pKEX4tr (9) as BamHI/SacI digested PCR products. The following first primer pairs, incorporating either BamHI or SacI restriction sites (sites underlined), were used: ATR13-5F (5’-GTC CCG GAT CCG AAC AAT GCG CCT TG) or ATR13-6F (5’-TCT TCG GAT CCA ACA TGA ATC TGC TCG TG) with ATR13-5R (5’-GTG GTA GGA GCT CAA CTA CTG ACT GGC) for cloning *ATR13-Emoy2* with or without its signal peptide, respectively; ATR13-5F or ATR13-6F with ATR13-6R (5’-GTG GTA GGA GCT CAA CTA CTG TCT GTC) for cloning *ATR13-Maks9* with or without its signal peptide, respectively. *ATR13* from Aswa1, Emco5, Goco1 and Hind4 were cloned into the plant expression vector pK2GW7 (Plant Systems Biology, University of Ghent, Belgium) using Gateway recombination (Invitrogen, Carlsbad, CA, USA). Entry clones containing the full-length ORF were initially created in pDONR221 (Invitrogen) using primer pairs ATR13-7F (5’-AAA AAG CAG GCT CAA TGC GCC TTG TTC ACG CGG TAC) and ATR13-7R (5’-AGA AAG CTA GGT AGG TAC ACA ACT ACT GTC TGT C) (Aswa1, Emco5 and Goco1) or ATR13-7F and ATR13-8R (5’-AGA AAG CTG GGT AGG TAC ACA ACT ACT GAC TGG C) (Hind4) and full-length attB1 and attB2 primers (Invitrogen). All
pKEX4tr and pK2GW7 expression clones were sequence verified before being used in the biolistic assay. The \textit{uidA} gene from pBI121 (BD Biosciences Clontech, Palo Alto, CA, USA) was cloned on a BamHI/SacI fragment into the vector pKEX4tr to produce pKEX4tr::GUS for \textit{GUS} expression.

Arabidopsis leaves from accession Col5 and Col5::RPP13-Nd (3) were used for bombardments. Plants were grown in 10 hour light at 18°C until 7-10 weeks old. Detached leaves were placed abaxially on 1% agar plates on a piece of Whatman 3mm paper. DNA (2.5µg) from the pKEX4tr (\textit{ATR13-Emoy2} and \textit{ATR13-Maks9}) or pK2GW7 (\textit{ATR13-Aswa1}, \textit{ATR13-Emco5}, \textit{ATR13-Goco1} and \textit{ATR13-Hind4}) expression clones was mixed with pKEX4tr::GUS (2.5µg) and loaded onto M17 tungsten particles (Biorad, Hercules, CA, USA) and used to make 5 replicate bombardments using the manufacturer’s recommended protocol. Bombardments were performed using a Biorad PDS-1000(He) apparatus with 1100psi rupture discs. After bombardment, leaves were incubated at 25°C for 20 hours. Histochemical \textit{GUS} staining was performed using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) (Duchefa Biochemie, Haarlem, Netherlands) at 37°C for 4 hours (10) and the tissue was cleared with methanol. The control plasmids used were pBluescript II SK (Statagene, La Jolla, CA, USA), pK2GW7 and pKEX4tr.

**Generation of glucocorticoid-inducible \textit{ATR13-Maks9} and \textit{ATR13-Emoy2} transgenic Arabidopsis**

The binary plant transformation vector pBDex was a kind gift from Hiro Kaminaka and Jeff Dangl (University of North Carolina, Chapel Hill, USA). pBDex is a derivative of the glucocorticoid-inducible plant promoter vector pTA7001 (11) containing a phosphinothricin acetyl transferase gene for selecting transgenic plants. \textit{ATR13} was PCR-amplified from the appropriate pKEX4tr clone using \textit{Pfu} polymerase (Promega, Madison, WI, USA) and the following primer pairs: \textit{ATR13-9F} (5'-TAA TGT CTC TTG TTC ACG CGG TAC TGC TA) and \textit{ATR13-9R} (5'-CGA CGG CGC TAT TCA GAT CCT CT) (full-length ORF); \textit{ATR13-10F} (5'-TAT TGT CTT CGT ATC CAT GGG GAA TC T) and \textit{ATR13-10R} (5'-CTT CTG AGA TGA GTT TTT GTT CTA CG) (ORF minus signal peptide sequence). PCR products were purified using a Qiaquick PCR purification kit (Qiagen) and ligated into EcoRV-digested pBluescript II SK (Stratagene) thereby obtaining \textit{ATR13} flanked by \textit{XhoI} and \textit{SpeI} restriction sites. Cycle-sequencing using T3 and T7 primers was used to verify the integrity and orientation of selected clones. pBluescript \textit{ATR13} clones were digested with \textit{XhoI} and \textit{SpeI} (Emoy2) or \textit{SalI} and \textit{SpeI} (Maks9) and gel-purified inserts ligated into \textit{XhoI} and \textit{SpeI} digested pBDex to obtain pBDex/\textit{ATR13-Emoy2} (with and without signal peptide) and pBDex/\textit{ATR13-Maks9} (with and without signal peptide).

The floral dip transformation procedure (12) was used to generate all transgenic Arabidopsis lines. \textit{T1} transformants and subsequent homozygous lines were selected by spraying 1-2 week-old seedlings with Challenge herbicide (Hoescht). Transgene expression was induced by dexamethasone (Sigma, St Louis, MO, USA) application as described in (11). When required, plant crosses were performed as described in (1). For Northern analysis, total RNA was isolated from transgenic plants using the RNeasy plant mini kit (Qiagen), 10 µg samples were separated on 1.2% agarose-formaldehyde gels and transferred to Hybond N nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were hybridised to random primed \textsuperscript{32}P-labelled DNA probes for 18 hours at 55°C in 250 mM Na\textsubscript{2}HPO\textsubscript{4} (PH 7.2), 7% SDS. Membranes were washed at 55°C for 15 minutes in 62 mM Na\textsubscript{2}HPO\textsubscript{4} (PH 7.2), 1% SDS and exposed to
Kodak X-Omat AR film for 1-5 days. DNA for the GVG hybridisation probe was obtained by PCR using pBDex as template and the following primers: GVG-F (5'-TTT CTA CTG ATT TTT CCT AGG TCG); GVG-R (5'-TGG TAA AAC CGT TGC ACAG TT). Primers used to generate an ACTIN probe standard have been described previously (6) and ATR13 probes were obtained through PCR using the ATR13 primers described above and the appropriate pBluescript clone.

**GenBank accession numbers**
All DNA sequences described can be accessed under the following Genebank accession numbers: AY785301, AY785302, AY785303, AY785304, AY785305, AY785306, AY785307, AY785308, AY785309, AY785310, AY785311, AY785312.

**References**


