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ORIGINAL ARTICLE

Evolution of *Hyaloperonospora* effectors: ATR1 effector homologs from sister species of the downy mildew pathogen *H. arabidopsidis* are not recognised by RPP1^{WsB}

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Abstract Like other plant-pathogenic oomycetes, downy mildew species of the genus *Hyaloperonospora* manipulate their hosts by secreting effector proteins. Despite intense research efforts devoted to deciphering the virulence and avirulence activities of effectors in the *H. arabidopsidis*/*Arabidopsis thaliana* pathosystem, there is only a single study in this pathosystem on the variation of effectors and resistance genes in natural populations, and the evolution of these effectors in the context of pathogen evolution is studied even less. In this work, the identification of *Arabidopsis thaliana* recognised (ATR)1-homologs is reported in two sister species of *H. arabidopsidis*, *H. thlaspeos-perfoliati*, and *H. crispula*, which are specialized on the host plants *Microthlaspi perfoliatum* and *Reseda lutea*, respectively. ATR1-diversity within these sister species of *H. arabidopsidis* was evaluated, and the ATR1-homologs from different isolates of *H. thlaspeos-perfoliati* and *H. crispula* were tested to see if they would be recognised by the previously characterised RPP1-WsB protein from *A. thaliana*. None of the effectors from the sister species was recognised, suggesting that due to the adaptation to altered or new targets after a host jump, features of variable effectors might vary to a degree that recognition of orthologous Avr-causing effectors is no longer effective and probably does not contribute to non-host immunity.

Keywords Oomycetes · ATR-effectors · Resistance proteins

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Introduction

Oomycetes are a group of eukaryotic organisms with a wide distribution and include a number of notorious plant pathogens of economically important crop plants. Among these oomycetes are *Pythium* species, which cause root rot on numerous glasshouse crop plants, as well as *Phytophthora* species, e.g. *Phytophthora infestans*, the causal agents of potato late blight, and *Phytophthora sojae*, causing soybean root and stem rot. In addition to these necrotrophic to hemibiotrophic pathogens, the order Peronosporales also includes the obligate biotrophic downy mildew pathogens, such as *Pseudoperonospora cubensis*, *Plasmopara viticola*, *Peronospora tabacina*, *Hyaloperonospora brassicae*, and *Bremia lactucae*, which cause disease on cucumbers, grapes, tobacco, cabbage, and lettuce, respectively (Thines et al. 2009; Thines and Kamoun 2010).

Like other plant pathogens, oomycetes manipulate their hosts by secreting an arsenal of effector proteins. Within the context of host–pathogen interactions, effectors are molecules that are secreted by pathogens to target plant molecules and

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alter plant processes (Hogenhout et al. 2009). Effector proteins modulate the plant's innate immunity and enable parasitic colonisation and reproduction (for review, see Hogenhout et al. 2009; Schornack et al. 2010; Thines and Kamoun 2010; Bozkurt et al., 2012). Although effectors function primarily in virulence, they can also elicit defence responses leading to resistance in plants. Effector-triggered immunity is mediated by a large group of structurally related immune receptors encoded by resistance *(R)* genes (for review, see Glowacki et al. 2011). R-proteins recognize pathogen effectors either directly or indirectly and trigger a hypersensitive response (HR), resulting in the cell death of infected cells, successfully halting the infection. In such cases, effectors have an avirulence (Avr) activity.

Great effort was devoted to studying the *Hyaloperonospora arabidopsidis*/*Arabidopsis thaliana* pathosystem, which became established as a laboratory model in the 1990s (Slusarenko and Schlaich 2003).

Four effectors with avirulence activity, ATR1 (Rehmany et al. 2005), ATR13 (Rentel et al. 2008), ATR5 (Bailey et al. 2011), and ATR39 (Goritschnig et al. 2012) have been cloned from *H. arabidopsidis* until recently (ATR stands for *A-rabidopsis thaliana* recognised).

Effectors with avirulence activity trigger resistance of *A. thaliana* mediated by RPP-proteins (RPP stands for recognition of *Peronospora parasitica*, a name previously used for *Hyaloperonospora arabidopsidis*). The recognition of the ATR-effectors is highly specific. Only a subset of ATR1 alleles has shown to be recognised by either RPP1 from the accession Wassilewskija (Ws) or RPP1 from the accession Niederzenz (Nd). Only one clade from a phylogenetic tree of known variants of RPP13 contains alleles that recognize ATR13 from some *H. arabidopsidis* isolates (Allen et al. 2004; Hall et al. 2009).

A common feature of ATR-effectors is the presence of an N-terminal secretion signal (signal peptide), followed by an RxLR-EER motif that is considered to be involved in translocation of proteins from the pathogen to the host cell cytoplasm (Rehmany et al. 2005; Allen et al. 2004; Whisson et al. 2007). The *ATR5* effector gene recently cloned from *H. arabidopsidis* isolate Emoy2 encodes a putative effector without a canonical RxLR sequence, but containing the EER motif in the translocation domain, which is also present in most RxLR proteins (Bailey et al. 2011).

The ATR-effectors reveal extreme levels of allelic diversity in different strains of the pathogen. The recognition specificity of ATR-effectors resides in the C-terminal region of the proteins (Rehmany et al. 2005). Site-directed mutagenesis performed for ATR13 identified three amino acid positions (Glu147, Thr152, and Arg181) that are important for recognition specificity (Allen et al. 2008). The ATR5 effector from *H. arabidopsidis*-Emoy2 is recognised by RPP5 from the accession *Landsberg erecta* (Ler-0) (Bailey et al. 2011). The recently identified ATR39 effector was found to trigger a resistance response in the *Arabidopsis* ecotype Weiningen (Wei-0) and two amino acid residues were shown to be critical for the resistance (Goritschnig et al. 2012).

Despite extensive sequence diversity in the C-terminal regions of RxLR effectors structural conserved sequence motifs termed WY domains [named after the W(Trp) and Y(Tyr) residues] that often form tandem repeats have been described for *Phytophthora* RxLR effectors (Boutemy et al. 2011). The WY domains were also identified in the RxLR effector ATR1 from *H. arabidopsidis* (Win et al. 2012). The C-terminal part of ATR1 from HpaEmoy2 contains a five-helix WY-domain, as revealed by analysis of the crystal structure of this protein (Chou et al. 2011).

Deciphering the virulence and avirulence activities of effectors is crucial for understanding how pathogens interact and coevolve with their host plants. Thus, this has become an emerging topic in the field of oomycete and fungal pathology. So far, little is known about the variation of effectors and resistance genes in natural populations, and even less about the evolution of these effectors in the context of pathogen evolution.

Before the onset of molecular phylogenetics in downy mildews, it was often assumed that downy mildew species have wide host ranges (e.g. Yerkes and Shaw 1959), despite earlier taxonomic works suggesting a narrow species concept (e.g. Gäumann 1918, 1923). However, molecular phylogenetic investigations have revealed that the narrow species concept for downy mildews is generally more appropriate (Riethmüller et al. 2002; Göker et al. 2003; Voglmayr 2003; Voglmayr et al. 2004; Voglmayr and Constantinescu 2008; Choi et al. 2007, 2009, 2011, 2015; Thines et al. 2009; Sökücü and Thines 2014), with very few exceptions, e.g. in *Pseudoperonospora* (Runge et al. 2011).

As a consequence, the view that only one species, *Hyaloperonospora parasitica,* is parasitic to a broad range of crucifers, including the downy mildew pathogens of *Brassica* species and *Arabidopsis thaliana*, had to be given up. Six diverse clades could be recognised within the genus *Hyaloperonospora* (Göker et al. 2003, 2009). Clade 3, which includes *H. arabidopsidis*, comprises several closely related pathogens of a rather diverse assemblage of hosts within the *Brassicaceae* (e.g. *Microthlaspi perfoliatum*, *Arabidopsis thaliana*, *Erophila verna*), but also from the *Resedaceae* (e.g. *Reseda lutea*) (Thines et al. 2009). The species closely related to *H. arabidopsidis* have evolved through host jumping to adapt to the new hosts and show a high degree of host specificity.

Despite the research efforts devoted to the *A. thaliana*/*H. arabidopsidis* pathosystem, there are currently no studies related to the immediate sister species of *H. arabidopsidis*. The molecular identification of homologous effectors in sister species may provide information on the functional and evolutionary processes of diversification of pathogens. One important question regarding plant pathogen interactions concerns

the arms race between pathogenicity effectors and resistance proteins. It could be assumed that R-proteins would be generally able to recognize a specific effector protein, but that the selection pressure exerted on the pathogen would lead to the evolution of new variants that are no longer recognised. This would mean that Avr-causing effectors from closely related sister species might be generally recognised and thus in these cases serve as non-host R-genes triggering resistance.

Given the close relationship of the species in clade 3 of *Hyaloperonospora*, it seemed feasible to clone effector homologs known from *H. arabidopsidis* from these to obtain a first glimpse into this topic. The aim of this study was to identify homologs of one of the most intensively studied downy mildew effectors, ATR1, to infer its diversity within sister species of *H. arabidopsidis* and to test if these orthologs would be recognised by a corresponding resistance protein from *A. thaliana*.

Materials and methods

Hyaloperonospora isolates

Collection and storage of *Hyaloperonospora* isolates was done according previous publications (Dangl et al. 1992; Holub et al. 1994; Rehmany et al. 2000). *Hyaloperonospora* isolates of *H. arabidopsidis* sister species used in this study were collected from naturally infected *M. perfoliatum* and *R. lutea* populations; the locations of various populations are summarized in Table 1.

The isolates were named *Htp*, *Hc*, or *Hpa* for *H. thlaspeos-perfoliati*, *H.crispula*, or *H. arabidopsidis*, respectively, plus a number (for *Htp*) or the location abbreviated as two letters (for *Hc* and *Hpa*), indicating where the strains were collected. Different subclones of the ATR1-gene produced from the same single host plant were given a distinguishing number. For example, *Htp-2-1* and *Htp-2-2* both were produced from the same *Htp2* strain, but were derived from different subclones of the ATR1-genes. These subclones may represent the same allele or different alleles or paralogs of the gene in the respective strain.

Cloning of *ATR1 genes*

All *ATR1* variants were amplified by PCR from genomic DNA templates extracted from single, systemically infected plants or single-spore laboratory strains. DNA extraction was performed using the Innu PREP Plant DNA Kit from Analytik Jena (Jena, Germany) according to the instructions of the manufacturer. PCR protocol: 95 °C for 10 min; 30 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s; 72 °C for 5 min with the primers atr1mtslF: 5′-GTSTGAACCACAACAGGC AG-3′ and atr1mtslR: 5′-TAGTTGCACCATGCTACTC-3′. PCR products were cloned in to a pSC-B vector using the StrataClone PCR Cloning Kit (Agilent Technologies, USA).

Sequencing of subclones, sequences assembly, and phylogenetic analysis

Colonies with the correct insert size were sent as a culture for sequencing to a commercial sequencing provider (GATC Biotech AG, Konstanz, Germany).

Table 1 The locations of the populations of the *Hyaloperonospora* isolates used in this study

Population	Pathogen	Host	Location (Germany)	Collected by:
Htp-02	*H. thlaspeos-perfoliati*	*M.perfoliatum*	CastleTeck, BW	Ploch/Schmuker
Htp-03	*H. thlaspeos-perfoliati*	*M.perfoliatum*	Weilheim a.d.Teck, BW	Ploch/Schmuker
Htp-04	*H. thlaspeos-perfoliati*	*M.perfoliatum*	Weilheim a.d.Teck, BW	Ploch/Schmuker
Htp-05	*H. thlaspeos-perfoliati*	*M.perfoliatum*	Reichenbach i.T, BW	Ploch/Schmuker
Htp-06	*H. thlaspeos-perfoliati*	*M.perfoliatum*	Reichenbach i.T, BW	Ploch/Schmuker
Htp-08	*H. thlaspeos-perfoliati*	*M.perfoliatum*	Ohnastetten, BW	Ploch/Schmuker
Htp-16	*H. thlaspeos-perfoliati*	*M.perfoliatum*	Hütten, BW	Schmuker
Htp-22	*H. thlaspeos-perfoliati*	*M.perfoliatum*	Villingendorf, BW	Schmuker
Htp-25	*H. thlaspeos-perfoliati*	*M.perfoliatum*	Döggingen, BW	Schmuker
Htp-30	*H. thlaspeos-perfoliati*	*M.perfoliatum*	Widdern, BW	Ploch/Schmuker
Htp-32	*H. thlaspeos-perfoliati*	*M.perfoliatum*	Ballenberg, BW	Ploch/Schmuker
Htp-40	*H. thlaspeos-perfoliati*	*M.perfoliatum*	Castle Teck, BW	Schmuker
Htp-LB	*H. thlaspeos-perfoliati*	*M.perfoliatum*	Limburg, BW	Laboratory strain derived from a single conidiospore
Htp-RT	*H. thlaspeos-perfoliati*	*M.perfoliatum*	Reichenbach i.T, BW	Laboratory strain derived from a single conidiospore
Hc-939	*H. crispula*	*Reseda lutea*	Mahlstetten, BW	Thines
Hc-Ab1	*H. crispula*	*Reseda lutea*	Abtsdorf, ST	Thines

The SeqMan module of the Lasergene computer software, version 5 (DNAStar, USA) was used to edit the obtained sequence data and to assemble them with the published sequence data of several *H. arabidopsidis atr1*-sequences from the GenBank (http://www.ncbi.nml.nhi.gov/Genbank).

Alignment of the *ATR1* dataset was done using the ClustalW as implemented in MEGA version 5.1 (Tamura et al. 2011).

Minimum Evolution phylogenetic reconstruction was performed using MEGA version 5.1 (Tamura et al. 2011) with 1000 bootstrap replicates (Felsenstein 1985). In addition, maximum likelihood phylogenetic reconstruction was performed using RAxML-v.7.2.6 program (Stamatakis et al. 2008), again with 1000 bootstrap replicates. All other parameters were set to default values.

Strains and growth conditions

Escherichia coli DH5α (Hanahan 1983) and *Agrobacterium tumefaciens* GV3101 (Holsters et al. 1980) were grown in Luria–Bertani medium supplemented with the appropriate antibiotics at 37 °C and 28 °C, respectively. Bacterial DNA transformation was conducted using chemically competent cells of *E. coli* (Invitrogen, USA) and through electroporation of competent *A. tumefaciens* cells according to Weigel and Glazebrook (2006).

Tobacco (*Nicotiana tabacum* cv. Petite Gerard) plants were grown in a controlled growth chamber at 21 °C with a 16-h-light/8-h-dark cycle.

Vector construction

The sequences for all primers used in this study are shown in Supplemental Table 1 in 5′ to 3′ orientation, restriction sites are indicated in boldface, the sequence encoding the FLAG-tag is underlined.

For co-expression we used ATR1Δ50 constructs, lacking both the signal peptide sequence and the RXLR motif. The ATR1 constructs were fused with a FLAG epitope tag on the N-terminal end of the protein, allowing protein detection in Western blots.

All *atr1Δ50* variants were first cloned into a pJL48 binary vector (Lindbo 2007). The forward primer included an in-frame FLAG epitope tag in order to create an N-terminal fusion protein with ATR1.

For the infiltration experiments, the constructs were introduced into the pEG202 binary vector (35S promoter, N-terminal Flag-tag fusion, Earley et al. 2006). The *atr1Δ50* variants were amplified by PCR from the corresponding pJL48-clones with the primers indicated in Supplemental Table 1. The PCR products were directly subcloned in pENTR/D-TOPO vector (Invitrogen, USA) and were introduced in the pEG202 binary destination vector via LR recombination. $RPP1^{WsB}$ used for the co-infiltration experiments was expressed from the pEarleyGate vector pEG201 (35S promoter, N-terminal HA-tag fusion, Krasileva et al. 2010).

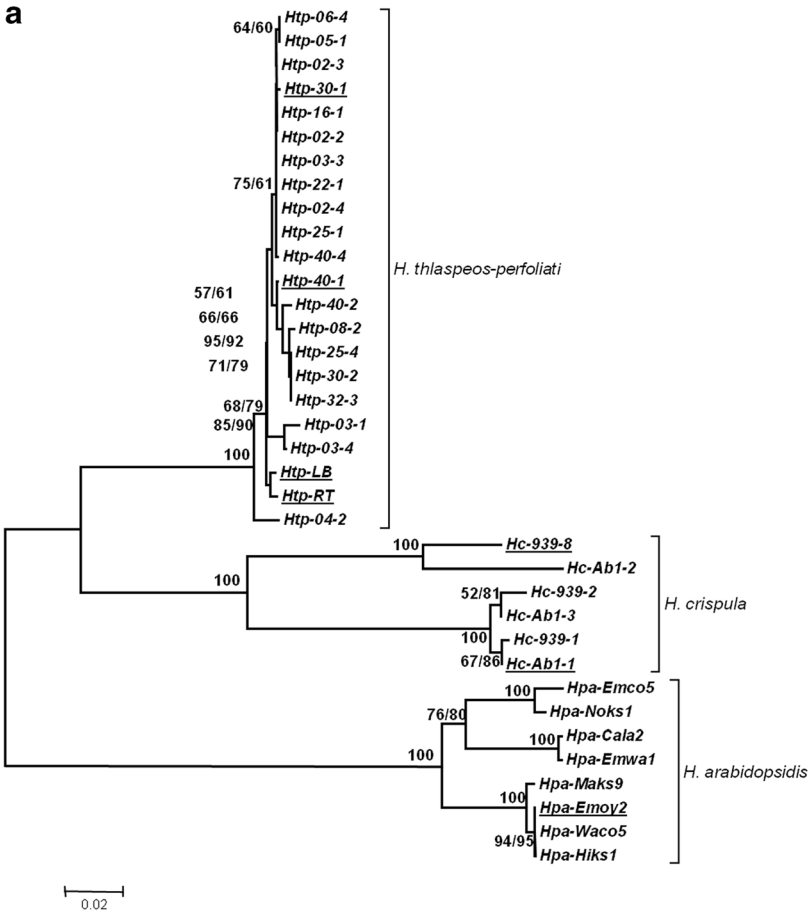
Agrobacterium tumefaciens mediated transient expression

Agrobacterium tumefaciens was grown in Luria–Bertani broth cultures (supplemented with 50 μg/ml gentamycin, 20 μg/ml rifampicin, and 50 μg/ml kanamycin) overnight at 28 °C with constant shaking. The cultures were pelleted using a benchtop centrifuge at 2000*g* for 5 min. The resulting pellet was resuspended in an induction medium (10 mM MgCl, 10 mM MES, and 150 mM acetosyringone, adjusted to pH 5.6 with KOH). Bacterial concentrations were measured and adjusted with the induction medium to OD600=0.8. For co-infiltrations, cultures carrying individual constructs were mixed in a 1:1 ratio and pre-induced 2–3 h at 28 °C before infiltration. Young tobacco leaves (5–6 weeks) were inoculated with *Agrobacterium* cultures using a blunt syringe.

To detect transient protein expression in tobacco, two leaf discs (1.5 cm diameter) were collected 48–72 h post infiltration from each leaf. The samples were frozen in liquid nitrogen and ground with a prechilled plastic pestle. Protein was extracted with 100 μL of Laemmli buffer [0.24 M Tris-Cl, pH 6.8, 6 % SDS, 30 % glycerol, 0.006 % bromophenol blue, and 1 mM DTT (Laemmli 1970)]. Samples were boiled for 5 min and centrifuged at 13,000*g* for 10 min in a bench top centrifuge at room temperature; supernatants were transferred to fresh tubes.

The samples, 15 μL per lane, were separated on 12 % SDS-PAGE gels, transferred to a nitrocellulose membrane (Fisher Scientific GmbH, Schwerte, Germany), and analyzed by immunoblotting using mouse α-FLAG antibodies (Sigma-Aldrich Chemie GmbH, Germany) and goat anti-mouse horseradish peroxidase antibodies (BioRad, Germany). The Dual Electrophoresis System DCX-700 from C.B.S. Scientific (USA) was used for performing SDS-PAGE and electro-blotting.

Fig. 1 Evolutionary relationships of ATR1-effectors. **a** Minimum evolution tree with bootstrap values from ME/ML analyses on branches. The *bar* indicates genetic distances based on the number of base substitutions per site. **b** Alignment of amino acid sequences of ATR1-effectors from *Htp*, *Hc*, and *Hpa*. *Dots* indicate amino acids identical to $HtpATR1^{30-1}$ sequences, *dashes* indicate gaps in the alignment. The predicted RXLR and EER motifs are boxed in the $HtpATR1^{30-1}$ sequence. Numbers at the site correspond to amino acid positions



b

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Htp-30-1    M R V C C F A P L H A A A L A V M A T E S S E T S S S I V H L A S L R D I A H H H S D A L I N R A L R A Q A V L D D G E E R M P F [65]
Htp-40-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [65]
Htp-LB-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [65]
Htp-LB-2    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [65]
Htp-RT      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [65]
Ho-939-8    . . . . . . . . . . . . . . . . . . . . . . . . H . . . . . V P . . . V . D . R D Y V . T . . . . K . . . . . . . D . . . W . S [65]
Ho-Ab1-1    . . . . . L . . . . . . . . . . I . . . . . . . H . . F . V V P . . . V . N N R D Y V . . . . . . . . . T G I . . D . . . W . S [65]
Hpa-Cala2   . . . . Y . V L . P S V . . . . I . . . . . . . . R T . . . V F P . . . V . D . R N . . . . . . . . . . . . A . . . D . . . W . . [65]
Hpa-Emoy2   . . . . Y . V L V P S V . . . . I . . . . . . . . G T . . . V F P . . . V . D . R N . . . . . . . . . . . . T A . . . D . . . W . . [65]

Htp-30-1    G L L T T K A L A N S I E R H - - T V Y G D Q A K M E K A V Q T W K K L T E G D G M V H K M G S H Y F D A P G P P F L S G D E A L [130]
Htp-40-1    . . . . . . . . . . . . . . - - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [130]
Htp-LB-1    . . . . . . . . . . E . . . . . - - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [130]
Htp-LB-2    . . S A . . . . . K . . . . . - - I . . . . . . . . . . . . . . E . . . . . . . . . . . . . . . . . . . . . . . . . [130]
Htp-RT      . . S A . . . . . Q . . . . . - - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [130]
Ho-939-8    R F S A . . . . . E I . D . . . - S L T D V . . . . . E . . . . . I . . K K . . . S . L T K . . L K R . . . V . D F P . . . . [130]
Ho-Ab1-1    K T S A . E . . . E . . H . . . . K L S K R . . R . D . . I . . . . . . . K . . . . N E . V . . . . R T A R - - - - A T . . T . [130]
Hpa-Cala2   . P S A V . . . I R T . D S . . . A G L N . E . . . K E V . . . . N . . I . R . N L I D E I . K . . S K . . . . V Y D . Y . . . . [130]
Hpa-Emoy2   . P S A V E . . I E T . D . . G R V S L N . E . . . K . V . R . . . . . I . R . D L I G E I . K . . . E . . . . L H D T Y . . . . [130]

Htp-30-1    A Y K L L T T Y S D R G V A R A I L H T R A S D P L S P K A E Q A S R L Q D I V V A L W Q Q R G H A V D E V L L K F D T G K D V D [195]
Htp-40-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [195]
Htp-LB-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [195]
Htp-LB-2    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [195]
Htp-RT      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [195]
Ho-939-8    . H . . V . . . . N . . A . . . . H I . . . . . H . L E . M . V G E . . . M . L K . . A E . . . T T . D . . S Y . . . . . [195]
Ho-Ab1-1    . H R . Q L S . W . . . I . W . . R . . . . . . . . . V . . R . . . . M . I T . . K . E . R . P N D . . S Y . . . . . [195]
Hpa-Cala2   . T M . V . . . . Y . . . . . . . . . P . P . . . I . N . . G R . H . . E E A . A S . . K E . . Y T S H N . V S N I A . D D . . . [195]
Hpa-Emoy2   . T R . V . . . . . . . . . . . . . . . P . . . . . K . . G . . H . . E E A . A S . . K G . . Y T S . N . V S S I A . . H . . . [195]

Htp-30-1    F F A S T A F T F L E K Y V G S E E N A N A A I F A Y F G S N S S R Y L S A V L N A M E K T G A D I R A L D R S K I W M F R Y C A [260]
Htp-40-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [260]
Htp-LB-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [260]
Htp-LB-2    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [260]
Htp-RT      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . [260]
Ho-939-8    . . . P . . . . . . K . A . . . . . . . . E . D . Y C . F . . . K . K E S . P . . . V . G . T . . . . R . . [260]
Ho-Ab1-1    . . . P . . . . . . K . A . . . . . . . . E . D . Y C . F . . . K . K E S . P . . . V . G . T . . . . R . . [260]
Hpa-Cala2   . . . . . . . . . . V . C . E . D D . N . . E . . . . . P . . . F . . . . H . . . . P . . . . . K . E N . . K . . . L Y . [260]
Hpa-Emoy2   . . . P . . . . . . V . C . E . D D . N . . E . . . . . P . . . F . . . . H . . . . P D . . S . V . E S . . K . . . Q C Y . [260]

Htp-30-1    . . . N E A S A I A F K N S L A I L Q R G E Y A P S D A S N H Y K M L S P S Q I K K L G K E F A E Y F S V [313]
Htp-40-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . E . . . . I . . . [313]
Htp-LB-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . N . . . [313]
Htp-LB-2    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . N . . . [313]
Htp-RT      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . N . . . [313]
Ho-939-8    . . . S V D . . S T . E S . . T N . . Q . . N . . N E . . . . F . E . . S E . . Q A . V . . . . . N . . . [313]
Ho-Ab1-1    . . . S V D . . S T . E S . . T N . . Q . . N . . N E . . . . F . E . . S E . . Q A . V . . . . . N . . . [313]
Hpa-Cala2   Q A P E P F . P P D . E W A . . R F . D E D H . F V G . Q . D . . K . . . . . E . . V E . Y S G I Y . . [313]
Hpa-Emoy2   . . . K Q F P T P V . E R T . . A Y . S E D . . I R G . R . . . E K . . L . . . E E . V E . Y S R I Y . . [313]
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Results

Phylogenetic reconstruction of ATR1-effectors from *Hyaloperonospora* isolates

ATR1 homologues were identified in two sister species of *H. arabidopsidis*, *H. thlaspeos-perfoliati* and *H. crispula*, and phylogenetic inference of the effectors by minimum evolution (ME) and maximum likelihood (ML) analysis revealed three highly distinct groups corresponding to the three species from which the effectors were cloned. The ME and the ML analysis showed similar topologies without significantly supported differences and the ME tree with bootstrap values from both analyses is shown in Fig. 1a.

All *ATR1* homologs from *H. thlaspeos-perfoliati* share the same sequence length (939 bp) and form a highly distinct clade with little sequence variation within the clade (up to 1.4 %).

Three paralogous *ATR1* homologs were detected in the two isolates of *H. crispula* on *Reseda lutea* (Hc-Ab1 and Hc-939). Hc-Ab1-2 and Hc-939-8 show a large genetic distance from the other two paralogs and have a slightly different sequence length (936 bp and 939 bp, respectively). The two other paralogs are closely related to each other (sequence divergence of 1.0 %) and have the same sequence length (927 bp).

ATR alleles of *H. arabidopsidis* showed a high sequence divergence of up to 7.7 % and were 933 bp ($ATR1^{Emoy2}$, $ATR1^{Waco5}$, $ATR1^{Hiks1}$, $ATR1^{Maks9}$), 936 bp ($ATR1^{Noks1}$, $ATR1^{Cala2}$), or 939 bp ($ATR1^{Emco5}$, $ATR1^{Emwa1}$) long.

The recognition response of $RPP1^{WsB}$ to ATR1 from sister species of *H. arabidopsidis*

To determine if *Arabidopsis thaliana* RPP1 recognises the ATR1-homologs from *H. thlaspeos-perfoliati* and *H. crispula*, an *A. tumefaciens*-mediated transient protein expression system in tobacco was used.

To avoid autoactivation of the overexpressed resistance protein, we choose $RPP1^{WsB}$ for the co-expression, as it had been shown that the expression of $RPP1^{WsB}$ in tobacco driven by the strong constitutive cauliflower mosaic virus 35S promoter does not exhibit autoactivation and cell death in the absence of the effector (Krasileva et al. 2010).

Six ATR1 homologs from sister species of *H. arabidopsidis* were selected for testing from different parts of the phylogenetic tree (underlined in Fig. 1a). The polymorphism between ATR1 homologs from the different species increased towards the C-terminus (Fig. 1b).

The six ATR1 homologs from *Htp* and *Hc* and two variants of ATR1 from *H. arabidopsidis* for positive and negative controls (one recognised and one not recognised) were co-expressed with $RPP1^{WsB}$. Fig. 2a illustrates the structure of the expressed proteins.

The testing of the response of $RPP1^{WsB}$ to ATR1 homologs revealed no recognition response for any of the effectors from sister species of *H. arabidopsidis* (Fig. 2b). As expected, RPP1-Ws induced an HR, which was observed 48 h post infiltration only when co-expressed with $ATR1^{Emoy2}$ as a positive control (Krasileva et al. 2010).

To ascertain that all ATR1 protein variants were expressed to equally high levels and the lack of recognition of ATR1-alleles was not due to a lack of protein expression, Western blot analysis was performed with FLAG-specific antibodies (Fig. 2c bottom panel). The Western blot analysis showed that, with the exception of the recognised allele of $ATR1^{Emoy2}$, for which the level of expression decreased upon the induction of HR, all tested ATR1 proteins were expressed to equal levels (Fig. 2).

Discussion

The study of the molecular interactions between species of *Hyaloperonospora* and their hosts is mostly limited to a few strains of *H. arabidopsidis* and inbred lines of the model plant *Arabidopsis thaliana*. The species closely related to *H. arabidopsidis*, which evolved to specialise on distinct hosts, offer the opportunity for comparative analyses with the *Arabidopsis* downy mildew pathosystem.

In the current study, the ATR1 effectors were identified in *H. thlaspeos-perfoliati* and *H. crispula,* sister species of *H. arabidopsidis*, which evolved as the result of host jumps to the unrelated hosts *M. perfoliatum* and *R. lutea*, respectively. It is generally assumed that there is a co-evolutionary arms race between avirulence effectors and the resistance proteins that recognize them (Jones and Dangl 2006). There are a high degree of high amino acid polymorphism and different sequence lengths for the alleles of ATR1 in *H. arabidopsidis* caused by segment deletions or duplications (Rehmany et al. 2005). Such sequence diversity is most likely due to co-adapting the interaction of the Avr protein ATR1 with the corresponding R protein RPP1. In an evolutionary arms race, such an interaction can produce diverse and rapidly evolving avirulence and resistance gene alleles. The high diversity of ATR1 in *H. arabidopsidis*, and the fact that it seems to be maintained in pathogens from various locations, suggests that this effector is important for the virulence of the pathogen and is thus maintained, despite recognition by resistance proteins from *A. thaliana* (Rehmany et al. 2005; Krasileva et al. 2011).

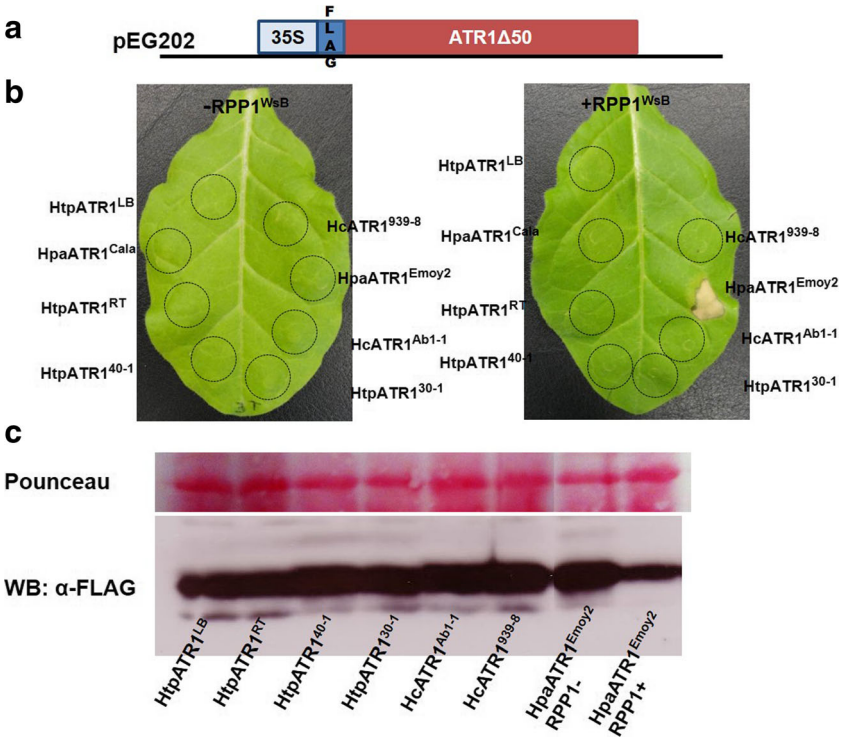

Fig. 2 RPP1^{Ws} causes no HR in co-infiltration with ATR1-homologs from *H. thlaspeos-perfoliati* and *H. crispula*. **a** Composition of the expressed *atr1*-genes. **b** *Agrobecterium*-mediated transient expression assay in *Nicotiana tabacum (Petite Gerard)*. The ATR1-constructs were infiltrated without the resistance protein (*left leaf*) or together with RPP1^{WsB} (*right leaf*) Inoculated areas are indicated by dashed circles. The picture was taken 72 h after infiltration. **c** Protein immunoblot of FLAG-ATR1 demonstrates similar protein expression levels in the leaves of tobacco. Pounceau staining is shown as a protein-loading control

The low genetic diversity within *ATR1* of *H. thlaspeos-perfoliati* (at most 1.4 %) provides evidence that ATR1 in the *H. thlaspeos-perfoliati*/*M. perfoliatum* pathosystem is stable, possibly because of the absence of recognition of this effector by a resistance protein of *M. perfoliatum*. An alternative explanation would be a low genetic variation resulting in homogeneity in virulence. But unpublished observations show that virulence among strains of *H. thlaspeos-perfoliati* is highly diverse, comparable to the situation observed in the *A. thaliana*/*H. arabidopsidis* pathosystem. At the same time, there is strong diversification between the two species, which is suggestive of adaptive evolution resulting from the adaptation to new or divergent target proteins after host jumps.

So far, little is known about the evolution of effector recognition apart from proteins engaged in an evolutionary arms race, and knowledge of the fate of effectors in sister species is at best limited (Sharma et al. 2014). It is also unknown, if R-proteins would initially recognize all variants of an effector and the pathogen than reacts to avoid this recognition, or if the evolutionary arms race would start from a resistance protein recognizing only a single variant. In the first case, it could be assumed that homologs of effectors inciting resistance from closely related species potentially also elicit defence, and that the corresponding R-genes might also act as non-host resistance factors, similar to the situation observed for the non-host R-genes already known. (Sumit et al. 2012; Mukhtar et al. 2011). However, our results of the recognition test, in which none of the ATR1 homologs from sister species of *H. arabidopsidis* was recognised by RPP1^{WsB} of *A. thaliana* seem to favour the model that the onset of an evolutionary arms race starts out from the recognition of only few or a single variant of an effector and not from broad scale recognition of effector variants. This is also in line with the findings of Allen et al. (2008) and Goritschnig et al. (2012), who found that for ATR13 and ATR39, only few amino acids are critical for recognition by the respective R-proteins of *A. thaliana*. In light of these studies, the conclusion could be deduced that homologs of effectors recognised in one pathosystem would usually not be recognised by divergent hosts of sister species. A first piece of evidence for this hypothesis was found in the present study.

However, for essential core pathogenicity effectors, the situation might be different as in this case, recognition in non-hosts would be a source of relatively durable resistance possibly outweighing the cost of maintaining multiple paralogs of resistance genes that might recognize effectors of related pathogens in the same habitat. Further comparative studies in other R-protein/Avr-effector pairs, for which the sister species in the *H. arabidopsidis* might be a valuable resource, could help to shed light onto this vastly unexplored field of pathogen evolution.

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