RXLR effector diversity in Phytophthora infestans isolates determines recognition by potato resistance proteins; the case study AVR1 and R1

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Abstract: Late blight disease caused by the plant pathogenic oomycete pathogen Phytophthora infestans is one of the most limiting factors in potato production. P. infestans is able to overcome introgressed late blight resistance by adaptation of effector genes. AVR1 is an RXLR effector that triggers immune responses when recognized by the potato resistance protein R1. P. infestans isolates avirulent on R1 plants were found to have AVR1 variants that are recognized by R1. Virulent isolates though, lack AVR1 but do contain a close homologue of AVR1, named A-L, of which all variants escape recognition by R1. Co-expression of AVR1 and R1 in Nicotiana benthamiana results in a hypersensitive response (HR). In contrast, HR is not activated when A-L is co-expressed with R1. AVR1 and A-L are highly similar in structure. They share two W motifs and one Y motif in the C-terminal part but differ in the T-region, a 38 amino acid extension at the carboxyl-terminal tail of AVR1 lacking in A-L. To pinpoint what determines R1-mediated recognition of AVR1 we tested elicitor activity of AVR1 and A-L chimeric and deletion constructs by co-expression with R1. The T-region is important as it enables R1-mediated recognition of A-L, not only when fused to A-L but also via trans-complementation. Yet, AVR1 lacking the T-region is still active as an elicitor of HR, but this activity is lost when certain motifs are swapped with A-L. These data show that A-L circumvents R1 recognition not only because it lacks the T-region, but also because of differences in the conserved C-terminal effector motifs.

Key words: Effector-triggered immunity (ETI), Effector variation, Host defence, Late blight disease, Nucleotide-binding and leucine-rich repeat (NLR) protein, Potato resistance.

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INTRODUCTION

Plant pathogenic oomycetes cause devastating diseases on a wide range of crop plants resulting into significant losses in agriculture. The most notorious oomycete plant pathogens belong to the genus Phytophthora, which includes over 120 species (Kroon et al. 2012). One of the best studied and economically important species is Phytophthora infestans, the causal agent of late blight and a major yield limiting factor in potato production.

Multiple loci conferring late blight resistance were identified in wild Solanum species and have been successfully introgressed into different potato cultivars (Vleeshouwers et al. 2011). To date, the majority of cloned potato late blight resistance (R) genes encode NLR proteins, intracellular immune receptors containing conserved Nucleotide-binding and Leucine-rich Repeat domains (Vleeshouwers et al. 2011). These NLR proteins are activated upon recognition of effectors secreted by P. infestans, resulting in effector-triggered immunity (ETI), a process known as a hypersensitive response (HR) that is associated with a rapid local cell death and prevents pathogen colonization. According to the classical gene-for-gene interaction model each R gene in the host has its own matching avirulence (AVR) gene in the pathogen.

P. infestans AVR genes hitherto identified encode RXLR effectors, small proteins named after a conserved N-terminal host-translocation motif composed of the amino acids arginine-any amino acid–leucine–arginine (RXLR) and secreted during infection to modulate host defence (Bouwmeester et al. 2009, Stassen & van der Ackerveken 2011, Whisson et al. 2016). P. infestans has over 580 RXLR effector genes that are mainly located in gene-sparse and repeat-rich regions in the genome and that evolve more rapidly in comparison to genes in gene-dense regions (Haas et al. 2009). Hence, RXLR effectors are able to undergo rapid adaptation thereby avoiding recognition by R proteins. P. infestans overcome R-gene mediated resistance by adapting its matching effectors in various ways. For example, by mutation of just two amino acids in AVR3a (K86M103) to E80M103, the pathogen can overcome recognition mediated by the NLR protein R3a (Bos et al. 2006). In the case of AVR4, various frame-shift mutations in the coding region of AVR4 result into truncated peptides that are no longer recognized by R4 (van Poppel et al. 2008). Unlike above-mentioned examples, the piO gene family (AVR-bib1) is highly diverse and encodes effector variants that can be grouped into three different classes (Champouret et al. 2009). Most P. infestans isolates contain multiple PI-O variants. Isolates lacking class I variants can circumvent recognition and are virulent on Rpi-bib1-containing
Solanum plants (Champouret et al. 2009). Isolates that have (a) class I variant(s), usually in combination with variants from class II and/or III, are recognized by the late blight R protein Rpi-blb1.

Apart from the conserved host translocation motif in the N-terminus, RXLR effectors show extensive sequence diversity in the C-terminal region, the part that is required for effector function and recognition by R proteins (Whisson et al. 2007, Dou et al. 2008). Yet, in these highly diverse regions Hidden Markov Model (HMM) searches revealed certain motifs that were named W, Y and L after a conserved amino acid residue at a fixed position in the respective motifs. These motifs, found in over one-half of all RXLR effectors, are 21-to-30 amino acids in length and can occur in modules in the order W–Y–L. The number of modules and motifs varies in each RXLR effector (Jiang et al. 2008). Their role in inducing effector-triggered HR was analysed for a number of RXLR effectors, including AVR-bib1, AVR-bib2, AVR4 and AVR3a from P. infestans and AVR1b from Phytophthora sojae (Bos et al. 2006, Dou et al. 2008, van Poppel et al. 2008, Champouret et al. 2009, Oh et al. 2009), and these studies showed that these conserved motifs are essential to efficiently initiate ETI. For example, P. infestans AVR4 contains three W motifs, of which W2 in combination with either W1 or W3 is needed to elicit an HR on R4-containing potatoes (van Poppel et al. 2009).

Efforts to introgress late blight resistance in the cultivated potato Solanum tuberosum from the wild Mexican species Solanum demissum started at the beginning of the previous century and resulted in differential lines with R1 recognition specificities, named R1–R11 (Black et al. 1953, Malcolmson & Black 1966, Waste 1991). Only a few years after the first R1-containing potato cultivars were introduced, P. infestans populations became virulent on these cultivars. R1 was the first major late blight R gene that was cloned and identified as a NLR encoding gene (Ballvora et al. 2002). With the cloning and identification of the P. infestans avirulence gene AVR1 the molecular basis of the loss of recognition by R1 could be investigated. AVR1, isolated by map-based cloning; was shown to trigger HR when co-expressed with R1 in Nicotiana benthamiana (Guo 2008, Du et al. 2015a). Similar to other P. infestans AVR genes, AVR1 encodes an RXLR effector. The 208-amino acid protein consists of a signal peptide, an RXLR domain, and a C-terminal region with two W motifs and one Y motif. P. infestans isolates that are virulent on R1 potato plants lack the AVR1 locus but possess a homologous variant named A-L (AVR1-like) at another locus. At the protein level, A-L shows 82 % homology to AVR1. It also has the WWY motifs but is shorter. Due to a premature stop codon A-L lacks a 38 amino acid extension after the Y motif, named the T-region. Unlike AVR1, A-L is not able to trigger HR when co-expressed with R1 (Du et al. 2015a, b).

In this study we first analysed the occurrence and sequence variation of AVR1 and A-L in P. infestans isolates that vary in their virulence on R1 potato lines and this confirmed that the presence of AVR1 is correlated with avirulence on potato carrying R1. We then focussed on the role of the WWY motifs and the T-region in R1 recognition. By deletion and domain swapping various AVR1 and A-L chimeric and deletion constructs were generated and their elicitor activity was tested by co-expression in N. benthamiana with R1. The results give further insight into the determinants in AVR1 that are important for R1-mediated recognition of this RXLR effector.

**RESULTS**

**Multiple variants of AVR1, but none of A-L, trigger R1-mediated cell death**

To obtain additional effector variants of AVR1 and A-L we performed PCR amplification on genomic DNA of several P. infestans isolates using specific primers (Table S1). The fragments were cloned and sequence analysis resulted in the identification of five AVR1 and four A-L variants (Fig. 1A, Fig. S1). All variants have the signal peptide and the RXLR domain followed by the conserved WWY motifs. The region linking W1 and W2 is named In1 and the one linking Y and the T-region is named In2. Since A-L lacks the T-region the A-L protein ends with In2. Sequence alignments revealed few polymorphisms among the variants (Fig. S1). Among the AVR1 variants nine residues are polymorphic and among the A-L variants only six (Fig. 1A). However, when comparing the overall sequence of AVR1 with A-L there are 29 amino acids positions that differ in the A-L variants, 28 of which are entirely conserved in the AVR1 variants and one highly conserved. Seventeen of these are located in the C-terminal effector domain (Fig. S1). All obtained AVR1 and A-L variants have a W or Y residue at the characteristic position in the respective motifs with the exception of AVR1 variant #97 in which the W in the W2 motif is mutated in R (Fig. 1A, Fig. S1).

To verify that the phenotypes of the isolates are in line with what is predicted based on the AVR1 or A-L variants obtained from those isolates, we tested their virulence on potato lines/ cultivars with and without R1. Results show that the R1 potato breeding line Cebeeco45-154-5 is resistant to isolates carrying AVR1 variants, but susceptible to those lacking AVR1 but carrying A-L variants (Table 1, Fig. S2).

To determine the activity of the various AVR1 and A-L variants we performed agroinfiltrations in N. benthamiana leaves. We co-expressed each AVR1 variant and each A-L variant with R1 and monitored HR. Despite multiple sequence polymorphisms, all variants of AVR1 were found to trigger HR upon co-expression with R1 in N. benthamiana leaves but not in the absence of R1 (Fig. 1B, Table 1). In contrast, no such R1-mediated HR was observed upon agroinfiltration with any of the A-L variants. We verified these findings by agroinfiltrating the AVR1 and A-L variants into leaves of potato with and without R1. Also in this case, all AVR1 variants were able to trigger HR in the R1 breeding line Cebeeco44-31-5 and in Nicola, a cultivar containing R1, but not in those of cultivar Bintje that lacks known R genes or in Cebeoco44-31-5 that has R4 (Fig. 1C; Table 1). Collectively, these results indicate that all tested AVR1 variants are recognized by R1, and act as genuine avirulence factors in R1 potato cultivars.

**The T-region of AVR1 is important but not sufficient to trigger R1-mediated HR**

In order to investigate which C-terminal motifs of AVR1 are needed to trigger R1-mediated HR various deletion constructs were made, which were then co-expressed with R1 and without R1 in N. benthamiana. Since AVR1 has a 38 amino acid C-terminal extension that is lacking in A-L, we first tested whether this T-region is required for R1 recognition. A version of AVR1
that lacks the T-region (AVR1ΔT) was still able to induce R1-mediated HR in 100 % of the infiltrated sites, but the HR development was delayed for 1 day when compared to AVR1 (Fig. 2). In the absence of R1, AVR1ΔT did not trigger a response and this shows that the necrosis is not due to toxicity of the truncated AVR1. The T-region by itself was not able to trigger R1-mediated HR. However, when the T-region was fused to A-L, the chimeric protein (A-LST) showed HR inducing activity. Similar to AVR1ΔT, A-LST did not trigger a response in the absence of R1 and also in this case the HR was delayed by one day (Fig. 2). However, since A-L by itself is unable to trigger R1-mediated HR, these findings show that the T-region in one way or another boosts recognition by R1 (Fig. 2).

The T-region of AVR1 trans-complements A-L to trigger R1-mediated HR

The fact that the T-region of AVR1 by itself was unable to trigger R1-mediated HR could be due to instability of this relatively small protein in planta. Therefore we fused a N-terminal GFP-tag to the T-region, and showed by western blot analysis that this fusion protein, named GFP-T, is stable in planta (Figs 3B, C). Nevertheless, also GFP-T was not able to trigger R1-mediated HR upon co-expression with R1 in N. benthamiana (Fig. 2) demonstrating that the T-region as such has no effector activity. However, when we co-expressed the T-region or GFP-T with A-L and R1 in N. benthamiana we observed HR (Fig. 3A, middle and right panel). This result is in line with the finding that the chimeric protein A-LST is recognized by R1 (Fig. 2). In addition, it shows that the T-region can confer effector activity to A-L by trans-complementation. Trans-complementation might be due to a physical association between two proteins and therefore we investigated if the T-region can bind to A-L in planta. Co-immunoprecipitation assays showed that GFP-T is in a complex with AVR1, but not with A-L (Fig. 3B). Apparently trans-complementation does not require binding of the T-region to A-L.

In our assays we repeatedly observed a higher protein accumulation of AVR1 than of A-L and this led to the hypothesis that R1-mediated recognition is dependent on the amount of effector protein present in the cell. It could be that the T-region can stabilize A-L in such a way that A-L accumulates and reaches higher levels thereby triggering R1 recognition. To test this, we co-expressed GFP-T with AVR1-myc, A-L-myc or IPI-O1-myc, isolated proteins from the infiltrated mixture in such a way that

![Image](image-url)
the relative amounts of the T-region were higher but this did not result in an enhancement of the HR (Fig. S3).

Conserved motifs in the AVR1 effector domain are important for recognition by R1

To test whether other regions and motifs in the C-terminus of AVR1 are responsible for R1-mediated HR, additional AVR1 and A-L chimeric and deletion constructs were made. Transient co-expression of R1 with these various constructs in N. benthamiana showed that deletion of W1 does not abolish the recognition of AVR1 by R1. However, by deleting W2, Y or ln2 the recognition is completely lost (Fig. 4). Since these deletions may change the three-dimensional structure of the protein, we swapped W2, Y or ln2 from A-L to AVR1. These reconstituted chimeric constructs were again able to trigger HR when co-

![Diagram](image)

**Fig. 2.** The T-region of AVR1 is important but not sufficient for R1 recognition. Agrobacterium stains containing the depicted constructs were co-agroinfiltrated with or without R1 in Nicotiana benthamiana leaves. The final OD of the Agrobacterium suspension was adjusted to 0.5. Pictures were taken at 3 dpi. Experiments were repeated three times with a total of 19 infiltrated sites per construct. HR was observed in either all infiltrated sites (100 %; +) or none of the infiltrated sites (0 %; −). +: HR at 2 dpi; ±: HR at 3 dpi; −: no HR.
expressed with R1 but not in the absence of R1, and this suggests that the overall structure of AVR1 is important for its activity as effector of R1-mediated recognition.

The finding that the T-region of AVR1 can be deleted without losing activity should allow us to use chimeric constructs to pinpoint which of the conserved motifs in the C-terminal effector domain are required to trigger R1-mediated recognition. We made several domain swaps between AVR1 and A-L, but none of these chimeric constructs — that all lack the T-region — were able to induce R1-mediated HR in N. benthamiana (Fig. 5).

Although these data suggest that actually the complete C-terminal domain of AVR1 including W1, W2 and Y is required for recognition by R1, the lack of recognition could in theory also be due to instability of the chimeric proteins. To test this we took advantage of the trans-complementation of A-L by the T-region. We repeated the assays but now included the T-region in the co-infiltration mixtures. With the exception of A-LSRXLR-W1 and A-LSRXLR-W1-W2, all the chimeric constructs induced HR (Fig. 5). This shows that the majority of the chimeric proteins is produced and stable in planta. Based on the lack of recognition of the chimeric proteins in the absence of the T-region we can conclude that W1, W2 and Y in AVR1 are equally important for recognition by R1.

**DISCUSSION**

*Phytophthora infestans* is notorious for its rapid adaptation and can often easily escape recognition by R genes from wild *Solanum* species that have been introduced in potato cultivars by breeding. With the molecular identification of key players in the gene-for-gene interaction we can now unravel the mechanisms that cause this rapid adaptation. This case study focussed on the RXLR effector AVR1 and its recognition by the NLR protein R1. *P. infestans* isolates that escape recognition have a deletion at the AVR1 locus but do have a close homologue, named A-L, elsewhere in the genome. By comparing the domain structure of AVR1 and A-L and by making use of deletion constructs and domain swapping we determined that the 38 amino extension in AVR1 and A-L includes W1, W2 and Y, and largely overlaps with the second W and Y motifs in VR-1. Fusin the T-region to A-L results in gain of recognition by R1, likely because the T-region modifies A-L in such a way that it resembles AVR1 and thereby activates R1. Intriguingly, this gain of recognition is also accomplished when the T-region is not fused to A-L, but is present as a 38 amino acid peptide. This trans-complementation point to a role for the T-region, for example by bridging the interaction between the R protein and the RXLR effector.

Previously we have shown that activation of HR mediated by R1 requires nuclear localisation of R1 and AVR1 (Du et al. 2015a). When expressed in planta both AVR1 and A-L showed a nucleocytoplasmic distribution albeit that the signal for A-L in the nucleus was less intense than that for AVR1. Prediction of the secondary structure revealed four α-helixes in the C-terminal part of AVR1, one of which is embedded in the T-region (Ye et al. 2015). We can speculate that this extra α-helix in AVR1 plays a role in balancing the subcellular distribution and this might explain the one day delay in HR when the T-region of AVR1 is removed. It may also explain the trans-complementation of A-L by the T-region whereby the T-region helps in shuttling A-L to the nucleus. The finding that the T-region co-immunoprecipitates with AVR1, but not with A-L, suggests that the interaction with AVR1 occurs at the T-region and this is in line with preliminary observations that AVR1 can dimerize. As yet we have no indications for dimerization of A-L, but this needs to be confirmed by analysing the interaction between either AVR1 or the T-region with the fusion protein A-LST and by including AVR1ST as extra control.

Intriguingly the T-region by itself also promotes virulence of *P. infestans* and it slightly boosts the virulence function of A-L (Du et al. 2015b). When the T-region is produced in plants lacking R1 lesion growth is significantly increased compared to the controls. AVR1 suppresses host defence by inhibiting its host target Sec5, a subunit of the exocyst complex, and — as shown in yeast-2-hybrid assays — the T-region is indispensable for AVR1-Sec5 interaction. There is, however, no interaction between Sec5 and A-L. This points to a direct interaction between the T-region of AVR1 and Sec5 and since Sec5 is a cytoplasmic protein this interaction presumably takes place in the cytoplasm (Du et al. 2015b). This differs from AVR1/R1 where there is no evidence for a direct interaction. The same holds for any other interaction between a *Phytophthora* RXLR effector and its matching R protein. In fact there are only a few R-AVR pairs known that show a direct protein–protein interaction.

All these findings together emphasize the importance of the T-region for the activity of AVR1, either in triggering ETI or in suppressing defense. But what about the other C-terminal domains, the conserved W and Y motifs that are characteristic for over half of the *Phytophthora* RXLR effectors? Apart from the missing T-region we found changes in the primary sequence in the C-terminal A-L effector domain at 17 positions where the amino acid residues in the AVR1 variants are conserved. Both AVR1 and A-L have two W motifs and one Y motif and also the spacing between these motifs is the same. The predicted secondary structure of AVR1 and A-L is quite similar (Ye et al. 2015) and with respect to the predicted three dimensional structure both have the conserved adaptable α-helical fold that is defined by Boutemy et al. (2011) as the WY-domain. In AVR1 and A-L this domain is 49 aa in length and largely overlaps with the second W domain and the Y domain (position 104-153). Based on the crystal structure of two RXLR effectors Boutemy et al. (2011) showed that in the WY domain the highly conserved tryptophan and tyrosine residues contact each other to form the hydrophobic core of the fold. They hypothesize that the WY domain forms a flexible scaffold that supports rapid changes in the primary sequence and structural architecture of the RXLR effectors. The core α-helical fold provides stability but also enables functional adaptation driven by co-evolution between plant and pathogen, for example via insertions and deletions in the loop regions, N- or C-terminal extensions and amino acid replacements in surface residues.

With the deletion and chimeric constructs used in this study we have not been able to pinpoint specific epitopes for recognition by R1. However, the fact that deletion of W2, Y or ln2 in AVR1 abolishes recognition and that swapping with W2, Y or ln2 of A-L restores recognition, stresses the importance of the three dimensional structure and the α-helical fold in AVR1. The T-region as C-terminal extension is the hallmark for AVR1 and the determinant for recognition by R1. Likely this extra α-helix also harbours the sites for dimerization of AVR1 and functions in binding to the host target Sec5 (Du et al. 2015b). Since *P. infestans* exploits RXLR effectors to...
A

T + R1  |  T + A-L + R1  |  GFP-T+ A-L + R1

B

| Protein      | 28 | 31 | 36 | 32 |
|--------------|----|----|----|----|
| GFP-HA       | +  | -  | +  | -  |
| GFP-T        | -  | -  | +  | +  |
| AVR1-myc     | +  | -  | +  | -  |
| A-L-myc      | -  | +  | -  | +  |

C

| Protein      | 28 | 31 | 36 | 32 | 30 |
|--------------|----|----|----|----|----|
| GFP-HA       | +  | -  | +  | -  | -  |
| GFP-T        | -  | -  | -  | +  | +  |
| AVR1-myc     | -  | -  | -  | +  | -  |
| A-L-myc      | -  | +  | -  | -  | +  |
| IPI-O1-myc   | -  | -  | -  | +  | -  |
suppress defence by targeting host proteins, binding to Sec5 is probably the original and primary role of the T-region. However, through co-evolution the T-region has gained an additional but unwanted role and that is activation of R1-mediated HR.

**EXPERIMENTAL PROCEDURES**

**Cloning of *P. infestans* AVR1 and A-L variants**

Variants of *AVR1* (PITG_16663.2) and A-L (AVR1-like; PITG_06432.1) were amplified from genomic DNA of five *P. infestans* isolates (Table S1) using *Pfu* DNA polymerase and primers Fw-AVR1/A-L and Rv-AVR1/A-L (Table S2). Obtained amplicons were cloned after A-tailing into vector pGEM-T Easy. Sequencing was performed using universal M13 primers, and DNA sequences were analysed using Vector NTI software. Fragments containing *AVR1* and A-L excluding their signal peptide-coding sequences were PCR amplified from the pGEM-T Easy clones using primers containing *EcoRI* and *NotI* sites, and ligated via restriction digestion into the binary vector pGRAB. Amino acid sequences of the various AVR1 and A-L variants are listed in Table S1.

**Deletion and hybrid constructs**

PCR fragments encoding the T-region of AVR1 were amplified with appropriate primers (Table S2) using *AVR1#170* as template. Amplicons were cloned into the Gateway vector pENTR/D-TOPO followed by recombination using Gateway LR Clonase II (Invitrogen) into the plant binary vector pSOL2094 (Liebrand et al. 2012) resulting into plasmid GFP-T. Motif deletion and chimeric constructs were constructed by overlap extension PCR using *AVR1#170* and A-L#117 as template with appropriate primers (Table S2; Du et al. 2015b), and subsequently cloned into pGRAB using *Ascl* and *NotI*, or *EcoRI* and *NotI* restriction sites. Construction of vectors AVR1-myc and A-L-myc was described previously by Du et al. (2015b). Binary plasmids were transformed via electroporation into Agrobacterium tumefaciens strain AGL1.

**Plant growth conditions and agroinfiltration**

*N. benthamiana* and potato plants were grown in a greenhouse under standardized conditions. Leaves from 4-to-5 week old plants were used for agroinfiltration. Transgenic *A. tumefaciens* strains were grown in YEB medium (per L: 5 g beef extract, 1 g yeast extract, 5 g peptone, 0.5 g MgCl₂) at 28 °C with
appropriate antibiotics for 18–24 h. Agrobacteria were resuspended in infiltration medium (per L: 10 mM MES pH 5.6, 5 g MS salts (w/o vitamins), 20 g sucrose, and 150 μM acetosyringone) and adjusted to a final OD600 of 1 and 0.2 prior to agroinfiltration of N. benthamiana and potato leaves, respectively. For HR assays, Agrobacterium cultures were mixed in a 1:1 or 1:1:1 ratio.

Pathogen inoculation

P. infestans isolates (Table S1) were grown on rye sucrose agar medium at 18 °C for 10 days before zoospore isolation. Detached leaf assays were performed as described (Champouret et al. 2009). The inoculum concentration was set to 1x10^5 zoospores/mL. Lesion development on potato leaves was observed and pictures were taken at 6 days after inoculation (dai).

Protein isolation and co-immunoprecipitation

Proteins were isolated from N. benthamiana leaves 2 days after agroinfiltration. Leaf material was ground in liquid nitrogen, of which one gram of grinded sample was incubated with 2 mL of extraction buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1 % NP-40, 5 mM DTT, and 1 proteinase inhibitor tablet (Roche) per 50 mL) for 30 min on ice. Cell debris was pelleted and 100 μl supernatant was kept as input sample. Remaining volumes were incubated with 15 μl GFP-trap_A (ChromoTek) beads at 4 °C for 2 h. Subsequently, the beads were collected by centrifugation at 720 g for 2 min and washed 8 times with extraction buffer. After washing, the beads were collected and pipetted into a new 1.5 mL tube containing 100 μl extraction buffer. Protein samples were boiled in 4x loading buffer (200 mM Tris-HCl pH 6.8, 8 % SDS, 400 mM DTT, 40 % glycerol and 0.2 % bromophenol blue) for 5 min before gel electrophoresis.

Immunoblotting and protein detection

Proteins separated by SDS-PAGE were transferred to Immuno-Blot PVDF membrane (Bio-Rad), and subsequently incubated with a blocking buffer containing 5 % skimmed milk powder at room temperature for two hours. Membranes were incubated with α-GFP-HRP (Milenyi Biotec, 130-091-833) or α-myc (Sigma–Aldrich, 9E10) antibodies for approximately two hours. Myc-tagged proteins were hereafter probed with HRP-conjugated anti-mouse IgG secondary antibody (Amersham). Protein bands were visualized with SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific). Membranes were stained with Coomassie blue to visualize RuBisCO proteins.

Fig. 5. Conserved motifs in AVR1 are important for recognition by R1. Agrobacterium stains containing the depicted constructs were co-agroinfiltrated with R1 in Nicotiana benthamiana leaves and in the absence (+R1) or presence of the T-region (T + R1). The chimeric constructs are coded with the prefix S followed by the domains swapped with AVR1 (shown in light gray). Trans-complementation by the T-region is a marker for production and stability of the chimeric proteins and adjusted to a 50 mL for 30 min on ice. Cell debris was pelleted and 100 μl supernatant was kept as input sample. Remaining volumes were incubated with 15 μl GFP-trap_A (ChromoTek) beads at 4 °C for 2 h. Subsequently, the beads were collected by centrifugation at 720 g for 2 min and washed 8 times with extraction buffer. After washing, the beads were collected and pipetted into a new 1.5 mL tube containing 100 μl extraction buffer. Protein samples were boiled in 4x loading buffer (200 mM Tris-HCl pH 6.8, 8 % SDS, 400 mM DTT, 40 % glycerol and 0.2 % bromophenol blue) for 5 min before gel electrophoresis.
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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.simyco.2018.01.003.

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