PNAS

Supporting Information for

Structural polymorphisms within a common powdery mildew effector scaffold as a driver of co-evolution with cereal immune receptors

Yu Cao[a, b, 1], Florian Kümmel[a, 1], Elke Logemann[a], Jan M. Gebauer[b], Aaron W. Lawson[a], Dongli Yu[a, b], Matthias Uthoff[b], Beat Keller[c], Jan Jirschitzka[a, b], Ulrich Baumann[b], Kenichi Tsuda[a, d], Jijie Chai[a, b, e, f, 2], Paul Schulze-Lefert[a, g, 2]

[a]Department of Plant Microbe Interactions, Max Planck Institute for Plant Breeding Research, 50829 Cologne, Germany
[b]Department of chemistry, Institute of Biochemistry, University of Cologne, 50674 Cologne, Germany
[c]Department of Plant and Microbial Biology, University of Zurich, 8008 Zurich, Switzerland
[d]State Key Laboratory of Agricultural Microbiology, Hubei Hongshan Laboratory, Hubei Key Lab of Plant Pathology, College of Plant Science and Technology, Huazhong Agricultural University, 430070, Wuhan, China
[e]Westlake Laboratory of Life sciences and Biomedicine, School of Life Sciences, Westlake University, 18 Shilongshan Road, Hangzhou 310024, China
[f]Beijing Frontier Research Center for Biological Structure, Center for Plant Biology, School of Life Sciences, Tsinghua University, 100084 Beijing, China
[g]Cluster of Excellence on Plant Sciences, Max Planck Institute for Plant Breeding Research, 50829 Cologne, Germany

[1]These authors contributed equally to this work.
[2]Correspondence to: Jijie Chai - chai@mpipz.mpg.de and Paul Schulze-Lefert - schlef@mpipz.mpg.de .

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Supporting References

Other supporting materials for this manuscript include the following:

Datasets S1–S2

SI Materials and Methods

Transient gene expression in *N. benthamiana* and protein detection by immunoblotting

For *N. benthamiana* transient gene expression, AVR_{A6}, *CSEP0333,* and effector chimeras and mutants were cloned into the *pDONR221* vector (Invitrogen). The obtained plasmids were recombined by an LR clonase II (Thermo Fisher Scientific) into the *pXCSG-mYFP* vector with a C-terminally fused mYFP epitope tag. Constructs were verified by Sanger sequencing. The *Mla6* and *Mla7* expression clones were previously described in refs. 1 and 2. Expression constructs were transformed into *Agrobacterium tumefaciens* GV3101 (pMP90RK) by electroporation. Transformants were grown on LB media selection plates containing rifampicin (15 mg ml^{-1}), gentamycin (25 mg ml^{-1}), kanamycin (50 mg ml^{-1}), and spectinomycin (50 mg ml^{-1}) for transformants harboring *pGWB517-Mla6-4×Myc* or carbenicillin (50 mg ml^{-1}) (3) for *pXCSG-mYFP* effector constructs (4).

Individual *Agrobacterium* transformants were cultured in LB medium containing respective antibiotics at 28 °C for 16 h. Bacterial cells were harvested by 2500 *g* for 15 min and resuspended with infiltration buffer containing 10 mM MES pH 5.6, 10 mM $MgCl_2$, and 150 µM acetosyringone. Construct expression was conducted in leaves of four-week-old *N. benthamiana* plants via *Agrobacterium*-mediated transient expression assays in the presence of the P19 and CMV2b suppressors of RNAi silencing (5). The final OD_{600} of receptor, effector, and RNAi silencing suppressor strains was adjusted to 0.5 each. Phenotypic data were recorded at day 6 after infiltration. For protein detection, the leaf material from four individual plants was harvested 48 h after infiltration, flash-frozen in liquid nitrogen and ground to powder using a Retsch bead beater. Plant powder was mixed with 4 x Laemmli buffer in a 1:2 ratio. After centrifugation at 16,000 *g* for 15 min, 5 µl of supernatant were loaded onto a 10% SDS-PAGE. Separated proteins were transferred to a PVDF membrane and probed with monoclonal mouse anti-Myc (1:3,000; R950-25, Thermofisher), polyclonal rabbit anti-GFP (1:3,000; pabg1, Chromotek) followed by polyclonal goat anti-mouse IgG-HRP (1:7,500; ab6728, Abcam) or polyclonal swine anti-rabbit IgG-HRP (1:5,000; PO399, Agilent DAKO) antibodies. Protein was detected using SuperSignal West Femto: SuperSignal substrates (ThermoFisher Scientific) in a 1:1 ratio.

Microscale Thermophoresis (MST)

For microscale thermophoresis experiments, total RNA was isolated from 7-day-old barley cv. Golden promise plants by phenol/chloroform extraction. Briefly, 5 g of leaf material were ground to a fine powder in liquid nitrogen. In a 50-mL propylene tube, the powder was resuspended in 10 mL lysis buffer (100 mM TRIS pH 8.0, 100 mM NaCl, 20 mM EGTA, 2% SDS) and 100 µL 2-mercaptoethanol, followed by the addition of 1 volume of phenol. Tubes were incubated for 20 min

while mixing in a revolving rotator, followed by the addition of 0.5 volume mL chloroform and another 15 min of mixing. Samples were centrifuged for 10 min at maximum speed, and the upper aqueous phase was transferred to a fresh tube. Phenol/chloroform extraction was repeated a total of three times, followed by a fourth time with chloroform only. Then, nucleic acids were precipitated by the addition of 0.1 volumes of DEPC-treated 3 M sodium acetate pH 5.2 and 2.5 volumes of ethanol, following by incubation at -70 °C for >30 min. After centrifugation at 30 min at max. speed, pellets were resuspended in 5 mL of DEPC-treated water, followed by addition of 5 mL DEPC-treated LiCl and incubation on ice at 4 °C for >3 hrs. Finally, after centrifugation at 30 min at max. speed, pellets were resuspended in 1.8 mL DEPC-treated water and precipitated one more time using sodium acetate and ethanol, followed by three washing steps with 70% ethanol. RNA pellets were resuspended in 500 µL DEPC-treated water. To obtain RNA concentrations >5 µg μL^{-1}, the RNA pellets from 24 extractions were pooled.

The fluorescent dye NT-647 (MO-L001, NanoTemper Technologies) was used to label effector proteins GST or BSA. The labeled proteins were eluted with the reaction buffer (20 mM phosphate-buffered saline, 150 mM NaCl, and 0.05% (v/v) Tween 20, pH 7.4), and mixed with different concentrations of barley total RNA (phenol/chloroform) before loading onto Monolith NT.115 (NanoTemper Technologies). Data were treated by the KD Fit function of the Nano Temper Analysis Software (version 1.5.3).

Supplementary figures

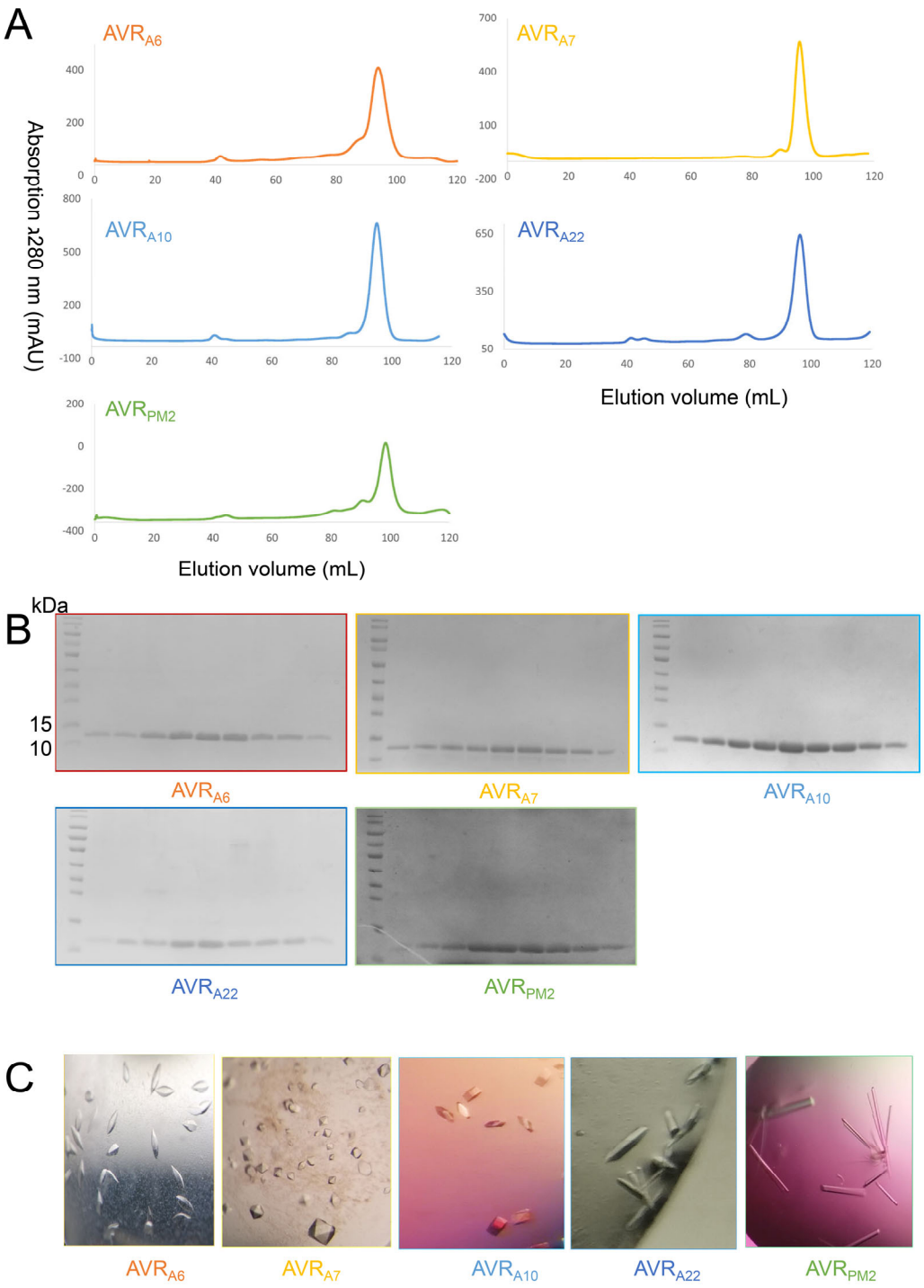


Figure S1. Purification and crystallization of *Blumeria graminis* AVR effector proteins. (A) Absorption spectra at 280 nm (mAU) for the effectors that were purified using size-exclusion

chromatography (SEC). The AVR_{A6}, AVR_{A10}, AVR_{A22}, and AVR_{PM2} proteins were obtained from *E. coli* SHuffle cells (NEB). Although AVR_{A7} could be purified from *E. coli*, the successfully crystallized protein was purified from insect cells. (B) Coomassie staining of selected peak fractions. (C) Representative pictures of crystals obtained for five AVR effectors.

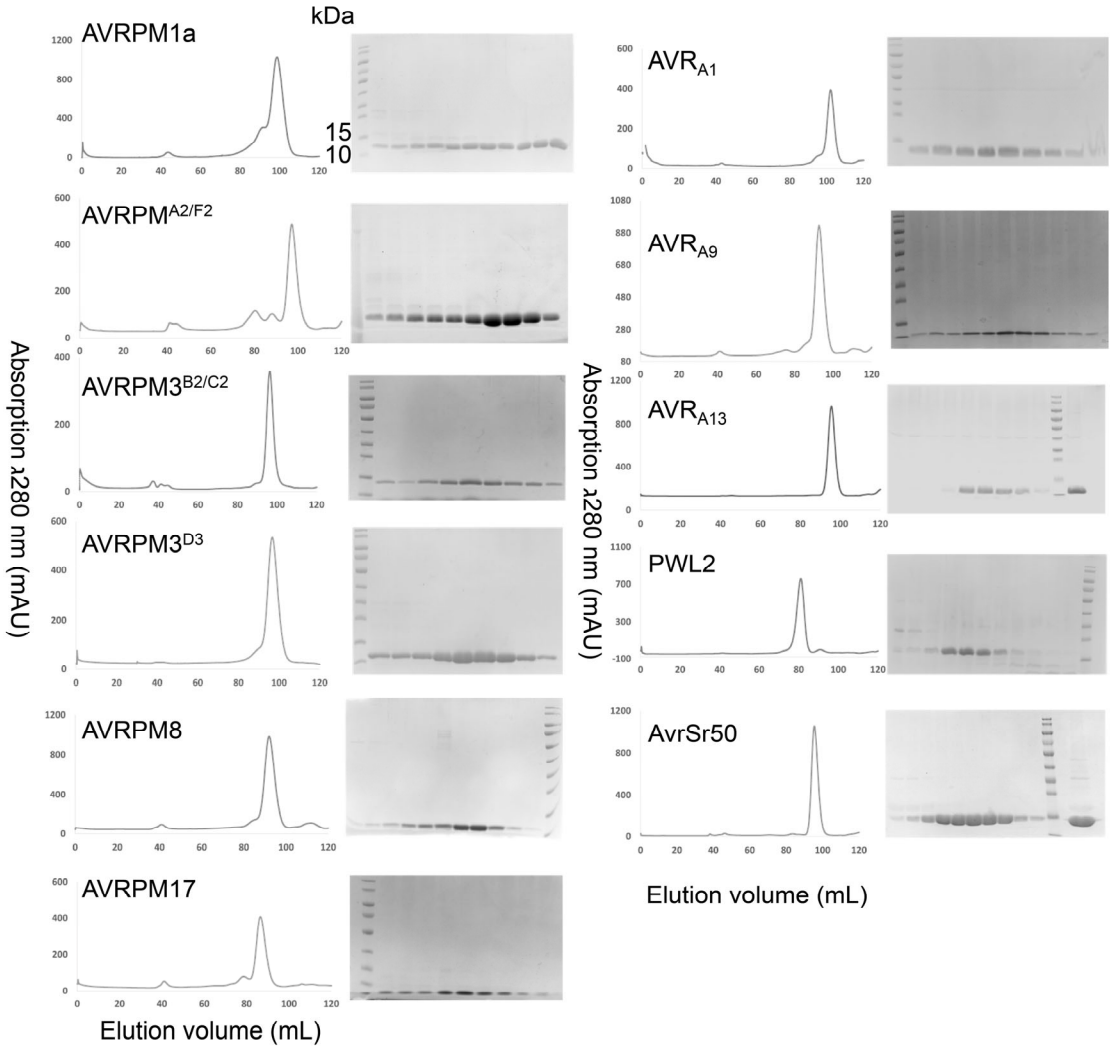
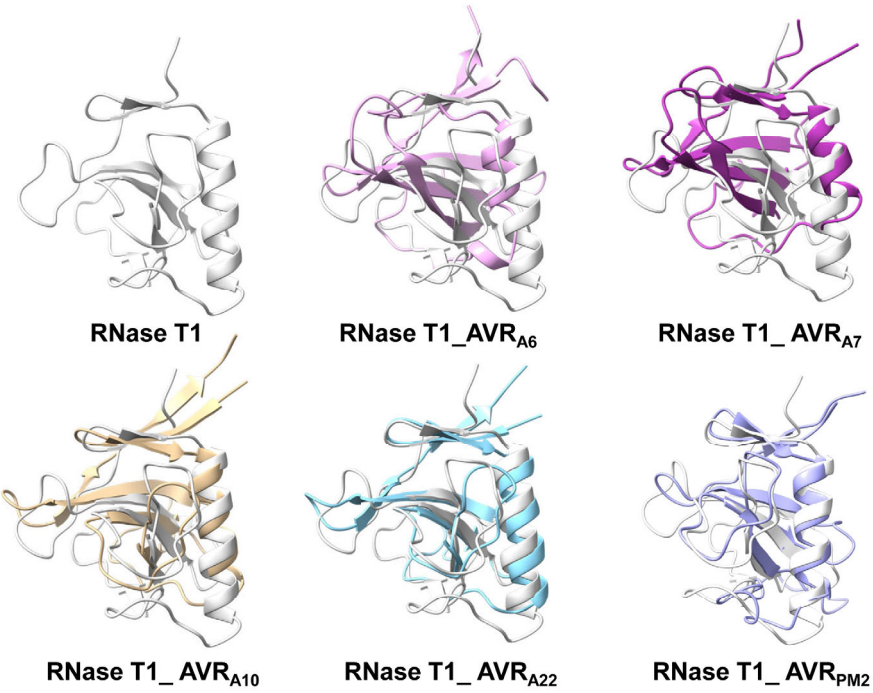


Figure S2. Purification of additional ascomycete effectors. The AVR effectors proteins were obtained from *E. coli* SHuffle cells (NEB), except for AVR$_{A13}$ and AvrSr50, which were expressed in insect cells. These 11 additional effectors were purified to homogeneity using SEC but failed to subsequently yield well-diffracting crystals.

A



B

Chain	Z	RMSD	LALI	# residues	% identity
AVR$_{PM2}$	10.7	2.5	87	99	16
AVR$_{A10}$	10.4	2.4	85	94	13
AVR$_{A22}$	10.3	2.4	86	94	13
AVR$_{A6}$	5.8	3.5	77	92	6
AVR$_{A7}$	3.7	3.2	63	92	10

C

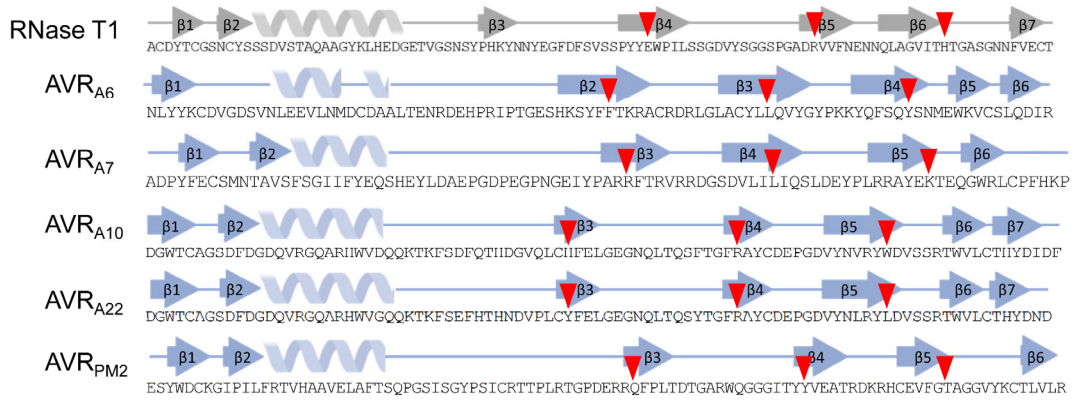


Figure S3. Structural comparison of *Blumeria graminis* AVR effectors with RNase T1 from *Aspergillus oryzae*. (A) Superimposition of the AVR structures with RNase T1 (PDB: 9RNT) in

cartoon representation. (B) Pairwise structural comparisons using the DALI server. (C) 2D-representation of the RNase T1 structure and AVR effectors with residues corresponding to the catalytic triad in RNase T1 highlighted with a red triangle.

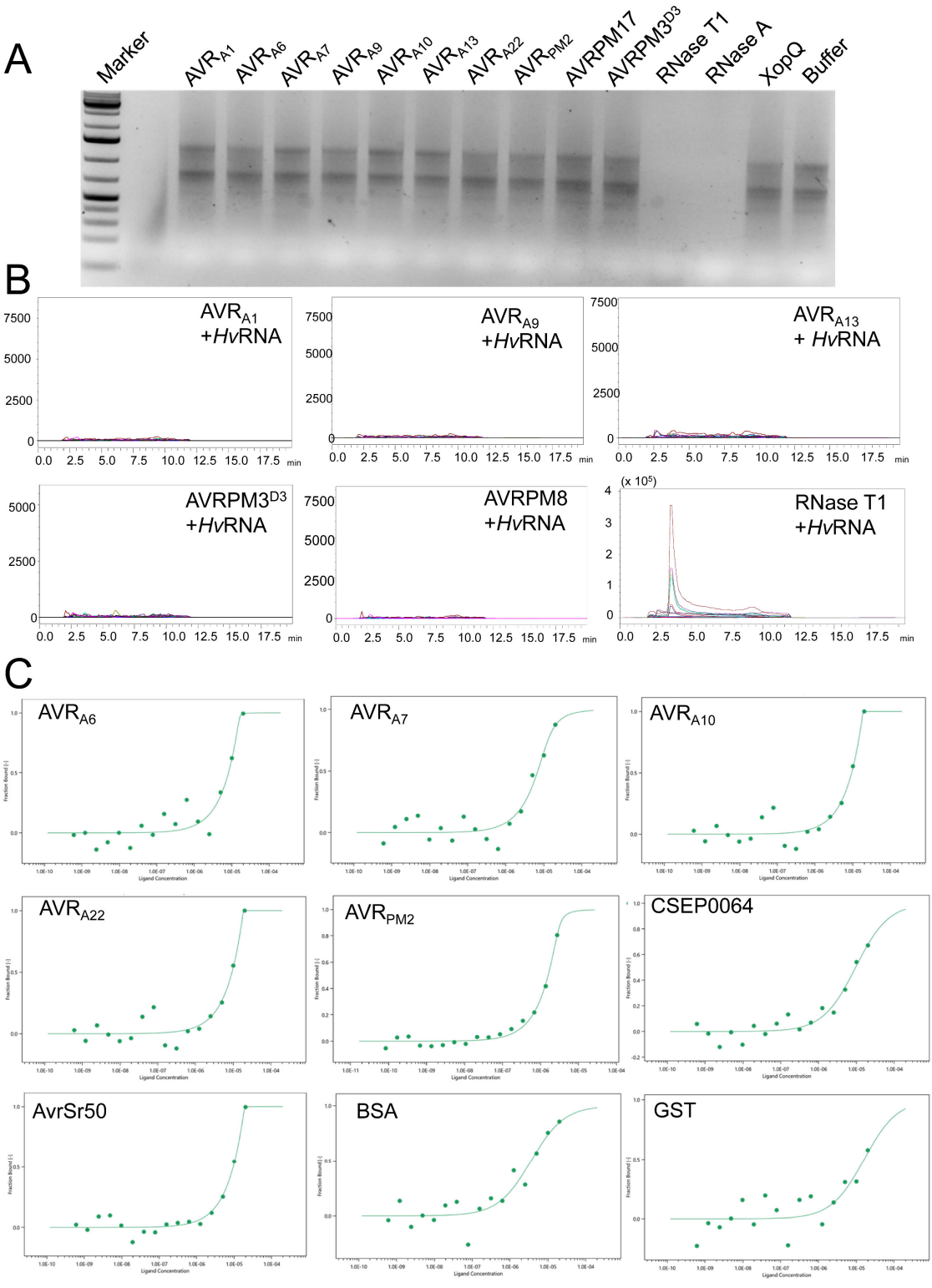


Figure S4. ***Blumeria graminis*** **AVR effectors are pseudo RNases.** (A) Co-incubation of RNA with AVR effectors for 16 hours and subsequent gel electrophoresis (1% agarose gel, 100 V, 45

min) Marker: GeneRuler 1kb Plus DNA ladder (Invitrogen). (B) Detection of 2′, 3′-cNMPs synthetase activity using LC-MS for additional AVR effector proteins. (C) MST traces of AVR effectors as well as non-RNase-like fold proteins BSA, GST and AvrSr50 with *Hv*RNA. All proteins were recombinantly expressed, purified, and subsequently labelled using the MO-L001 labelling kit (NanoTemper). The highest RNA concentration was set to 3750 ng μl^{-1}. Traces are representative of three experimental replicates with similar results.

Figure S5

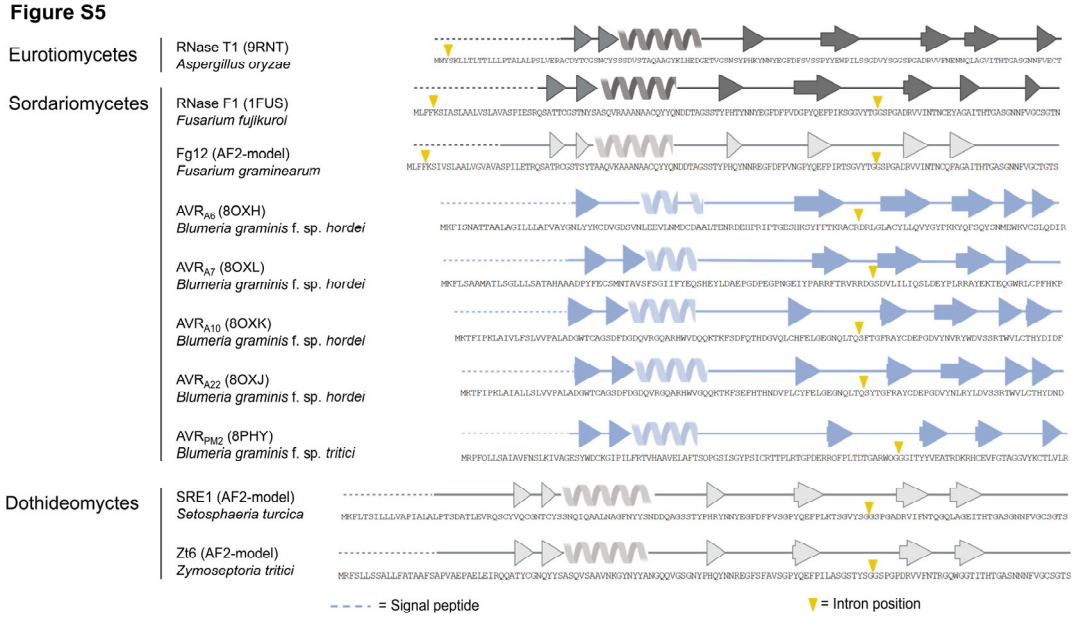


Figure S5. *Blumeria graminis* AVR effectors share a single intron with RNase F1 family members. 2D-representation of structures or AlphaFold2-models of characterized secreted ribonucleases and RALPH effectors with intron positions in the translated sequences highlighted with a yellow triangle. Signal peptide sequences are pictured with a dashed line. NCBI gene identifiers used: RNase T1: AP007171.1; RNase F1: AB355898.1; Fg12: FG11190.1; Zt6: NC_018216.1; SRE1: NW_007360025.1 (SETTUDRAFT_163271); AVR_{A6}: UNSH01000074 (BLGHR1_15960); AVR_{A7}: UNSH01000097 (BLGHR1_17217) AVR_{A10} and AVR_{A22}: CAUH01000387.1 (BGHDHI4_bgh03730) AVR_{PM2}: KX765276.1.

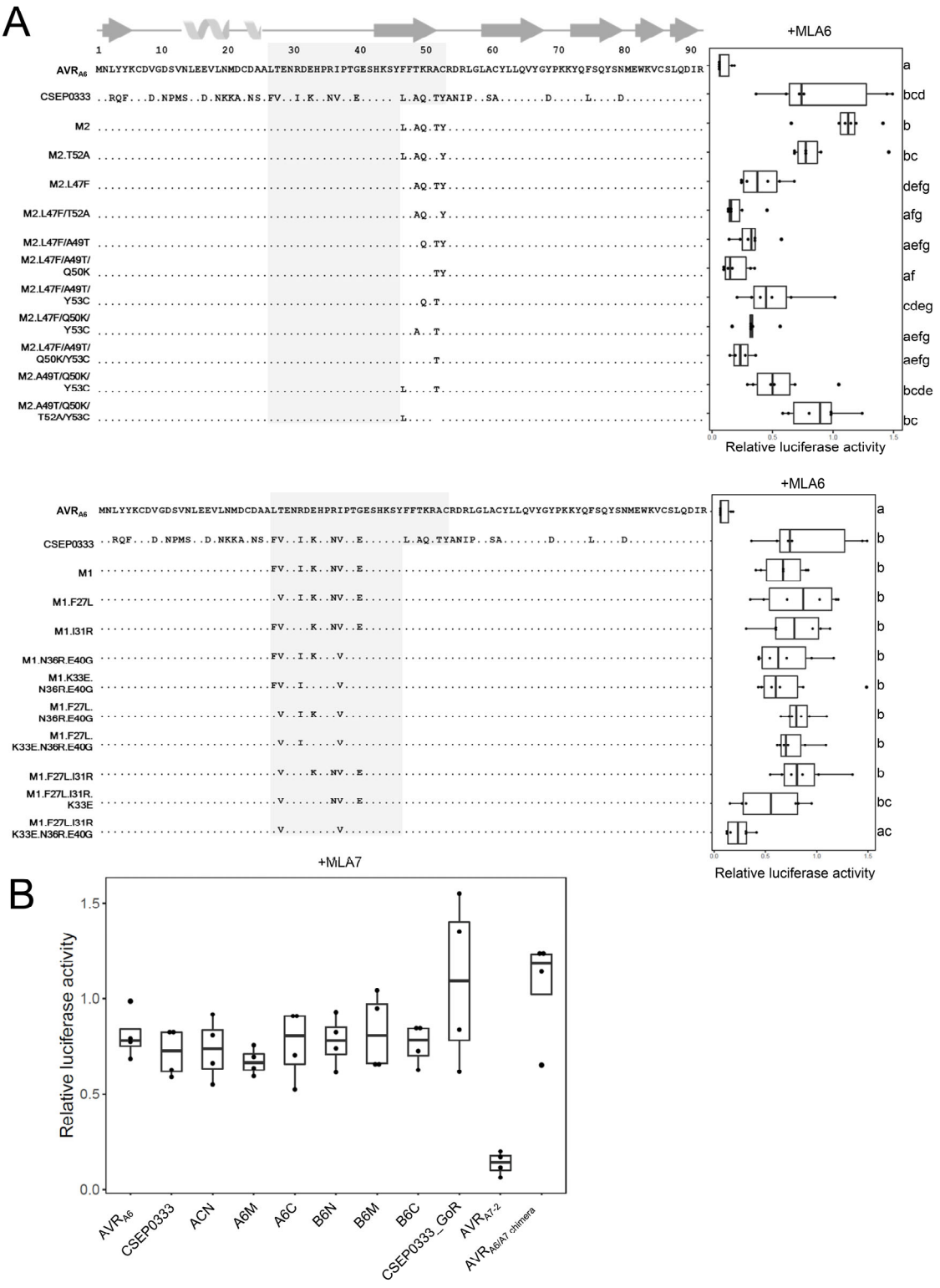


Figure S6. **Six amino acids in the central segment of AVR$_{A6}$ are essential for the detection**

by MLA6 in barley protoplasts. (A) Hybrid effectors with targeted substitutions were co-transfected with MLA6 into barley protoplasts and cell death was quantified by measuring luciferase reporter activity. Letters indicate results of statistical variance analysis using the Kruskal–Wallis test followed by Dunn's post hoc tests ($P < 0.05$). Raw relative luciferase measurements and P-values for all protoplast plots are provided in Supplementary Dataset S1. (B) Selected effector constructs were co-transfected with MLA7 into barley protoplasts as a control for receptor specificity.

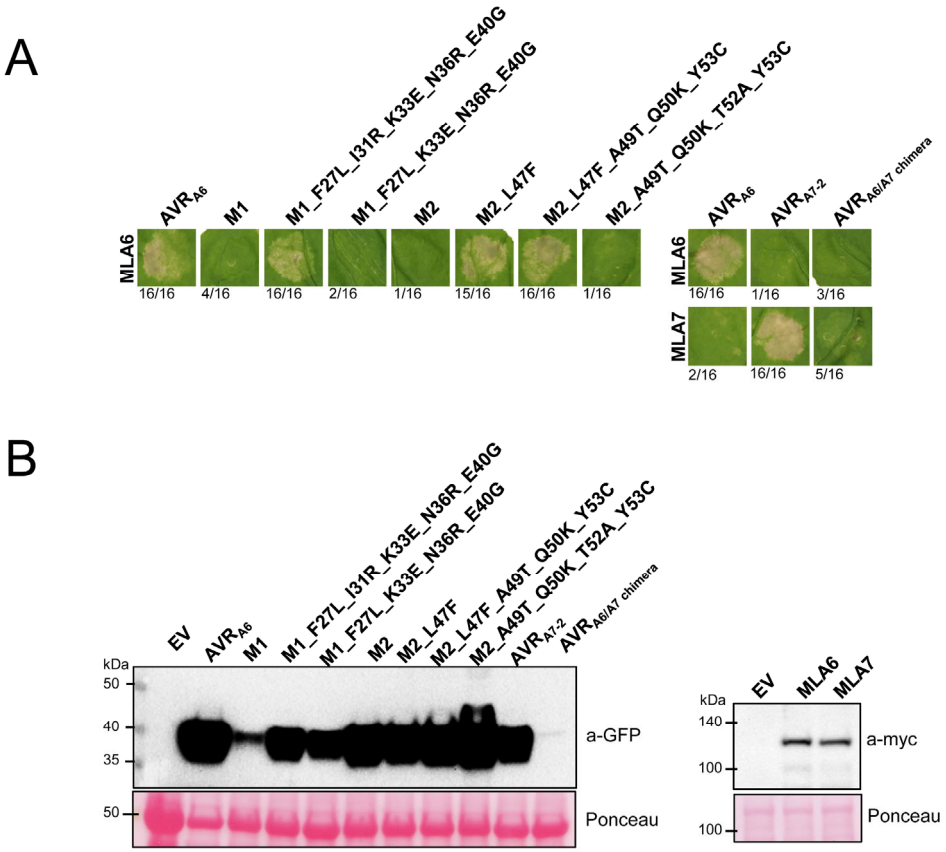


Figure S7. Six amino acids in the central segment of AVR$_{A6}$ are essential for the detection by MLA6 in *N. benthamiana*. (A) Selected effector hybrids with targeted amino acid substitutions were co-expressed in *N. benthamiana* using *Agrobacterium*-mediated infiltration. The cell death score is indicated below the representative pictures from 16 replicates. (B) Immunoblot to detect accumulation of effector and receptor proteins.

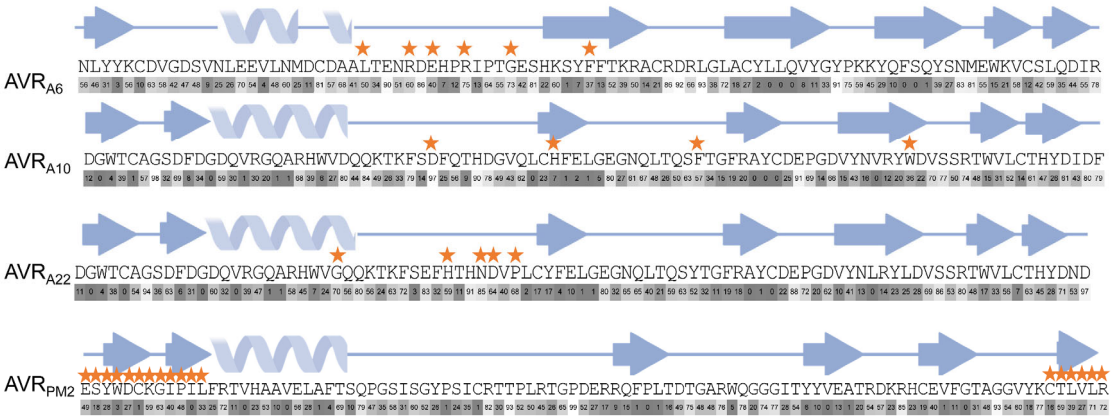
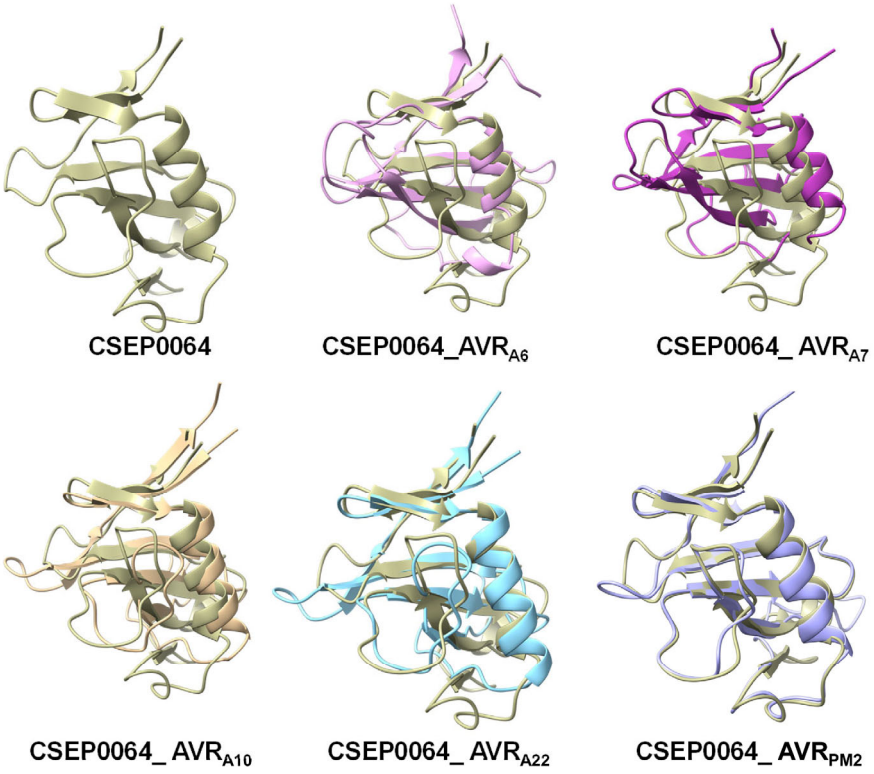


Figure S8. Relative solvent accessibility of *Blumeria graminis* AVR effector sequences. Relative solvent accessibility was computed using PyMOL. Orange asterisks indicate the residues important for recognition by the cognate NLR receptor. A darker color means less solvent accessibility, a lighter color means more accessibility, and the number indicates the percentage of solvent accessibility.

A



B

	scoring								target structure	
	Q	P	Z	RMSD	N_align	N_gaps	%seq	Query %sse	%sse	Nres
AVR$_{A6}$_CSEP0064	0.267	2.971	4.939	2.249	61	9	11	56	83	92
AVR$_{A7}$_CSEP0064	0.208	2.033	3.817	3.389	65	6	9	67	86	92
AVR$_{A10}$_CSEP0064	0.442	5.912	6.961	2.142	78	7	18	67	75	94
AVR$_{A22}$_CSEP0064	0.4397	5.707	6.836	2.158	78	7	15	67	75	94
AVR$_{PM2}$_CSEP0064	0.7065	14.14	11.32	1.228	89	3	44	78	100	99

C

Figure S9. Structural comparison of *Blumeria graminis* AVR effectors with CSEP0064. (A) Superimposition of the AVR structures with CSEP0064 (BEC1054) (PDB: 6FMB) in cartoon

representation. (B) Pairwise structural comparisons using the DALI server. (C) Sequence alignment of AVR structures with CSEP0064.

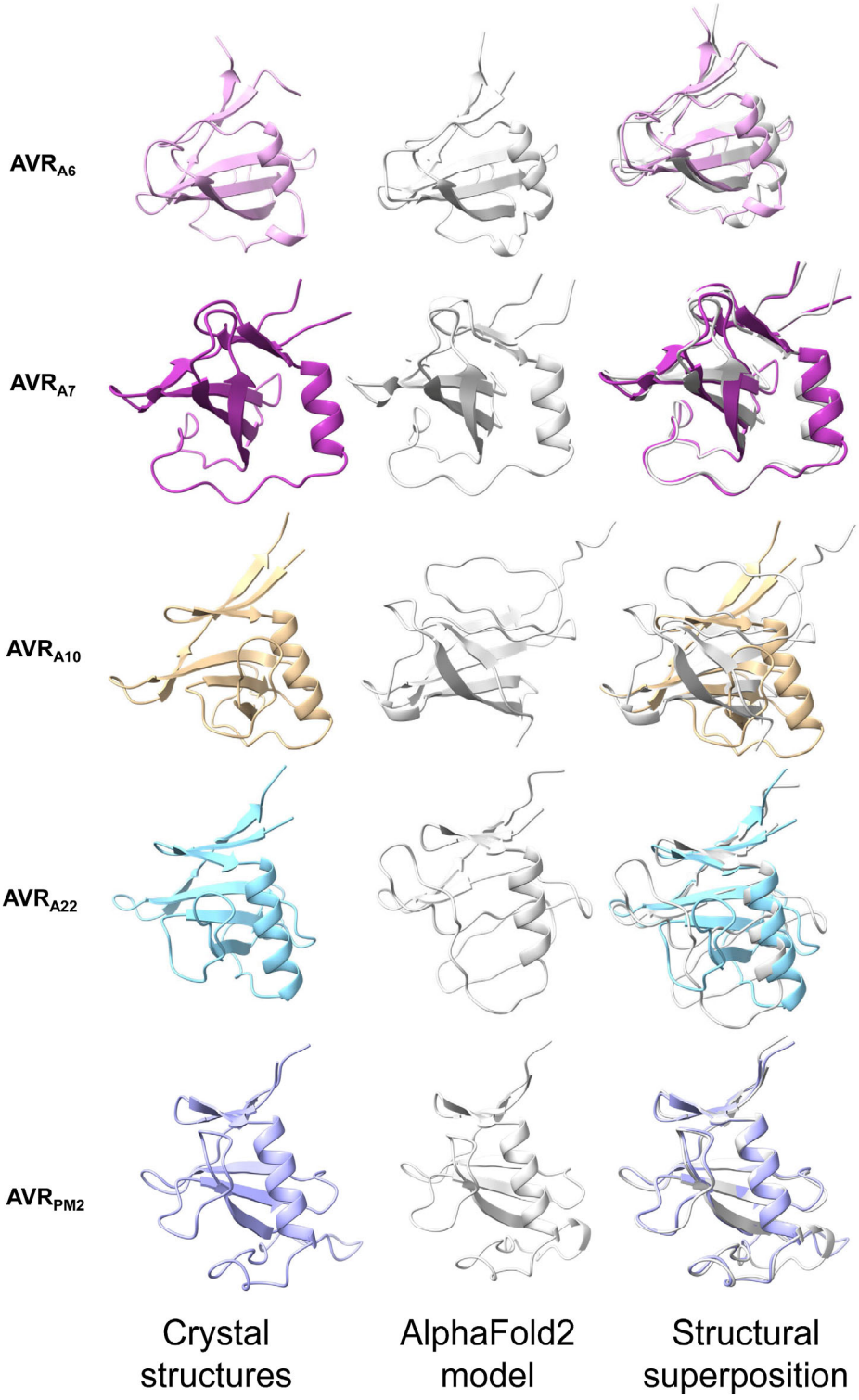


Figure S10. Structural comparison of the AlphaFold2-predicted and experimentally determined structure of AVR effectors. (A) For structural modelling, Colabfold v1.3

(colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb) was used to predict the structures of the AVR effectors without their signal peptides. The top-ranking model was used for superimposition with the experimental structure.

Table S1. Crystallographic data collection and refinement.

Crystallographic data collection and refinement			
	AVR_{A6}	**AVR_{A7}**	**AVR_{A10}**
Data collection			
Beamline	P13 / EMBL@DESY	ID30B / ESRF	P14 / EMBL@DESY
Wavelength (Å)	0.9762	0.9763	0.9763
Space group	P 65 2 2 (No. 179)	P 41 21 2 (No. 92)	C 1 2 1 (No. 5)
Cell dimensions			
a, b, c (Å)	62.47, 62.47, 202.37	57.55, 57.55, 121.64	125.94, 28.96, 29.18
α, β, γ (^{O})	90, 90, 120	90, 90, 90	90, 96.67, 90
Resolution (Å)[§]	42.20-2.50 (2.65-2.50)	33.82 - 1.56 (1.65-1.55)	31.27-1.38 (1.47-1.38)
Rmeas (%) §	7.7 (380.2)	8.2 (245.3)	8.2 (250)
CC 1/2 §	100(40.2)	99.8 (18.2)	99.9 (51.6)
Mean I/sigI §	24.36 (0.63)	8.18 (0.46)	12.25 (0.68)
Completeness (%)§	99.9 (99.9)	97.7 (93.2)	79.3 (33.4)
Multiplicity§	18.3 (19.0)	3.3 (3.1)	6.9 (6.4)
Refinement			
No. reflections #	8696 (816)	29943 (2878)	21484 (2109)
Rwork/Rfree (%)	27.8 / 31.3	19.3 / 21.6	16.8 / 18.6
No. of atoms	1477	3290	1536
a.a. residues	183	184	94
protein chains per asu	2	2	1
Bond lengths (Å)	0.003	0.017	0.0158
Bond angles (^{O})	0.674	0.92	1.34
Ramachandran plot			
Favored (%)	95.48	99.44	98.91
Allowed (%)	2.82	0.56	1.09
Outlier (%)	1.69	0	0

	AVR_{A22}	**AVR_{PM2} #1**	**AVR_{PM2} #2**
Data collection			
Beamline	X06SA /SLS	X06SA /SLS	X06SA /SLS
Wavelength (Å)	0.9999	0.9999	0.9999
Space group	P 21 21 21 (No. 19)	C 2 2 21 (No. 20)	P 21 (No. 4)
Cell dimensions			
a, b, c (Å)	48.10, 60.76, 66.35	50.41, 53.86, 61.81	36.67, 61.85, 37.16
α, β, γ (^{O})	90, 90, 90	90, 90, 90	90, 94.14, 90.00
Resolution (Å)[§]	44.81 - 1.65 (1.74-1.65)	36.80-1.51 (1.55 - 1.51)	36.57 - 1.06 (1.10 - 1.06)
Rmeas (%) §	10.4 (160.1)	10.6 (828.1)	10.5 (268.5)
CC 1/2 §	99.7 (40.7)	99.7 (8.8)	99.9 (19.8)
Mean I/sigI §	7.51 (0.93)	14.78 (2.85)	10.64 (0.56)
Completeness (%)§	99.1 (99.1)	94.4 (93.5)	93.02 (44.81)
Multiplicity§	3.1 (3.1)	6.9 (6.9)	10.6 (2.5)
Refinement			
No. reflections #	24155 (2348)	12843 (642)	68774 (3431)
Rwork/Rfree (%)	21.9 / 25.0	19.4 /20.7	17.2 / 19.23
No. of atoms	2893	822	1806
a.a. residues	184	95	207
protein chains per asu	2	1	2
Bond lengths (Å)	0.0083	0.01	0.01
Bond angles (^{O})	1.4	1.23	1.2
Ramachandran plot			
Favored (%)	98.89	97.8	97.04
Allowed (%)	1.11	2.2	2.46
Outlier (%)	0	0	0.5

§ Values in parentheses indicate outer shell # Values in parentheses indicate reflections in test set

Table S2. Primers used in this study.

Name	Sequence 5'-3'
35S promoter	CTATCCTTCGCAAGACCCTTC
attB1_AVR$_{A6}$_noSP	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAACCTATATTACAAATGTGATGTTGGCG
attB1_AVR$_{A7}$_noSP	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCGGATCCATACTTTGAATGC
attB1_CSEP0333	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAACCGACAATTCAAATGTGATGATGG
attB1_MLA6	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGATATTGTC
attB1_MLA7	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGATATTGTCACCGGTGCC
attB1 AVR$_{A6}$_ML27_K44	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAACCTATATTACAAATGT
attB1AVR$_{A22}$_a6central	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATGGCTGGACATGTGCC
attB1CSEP0333	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAACCGACAATTCAAATGT
attB2_AVR$_{A7}$_Nostop	GGGGACCACTTTGTACAAGAAAGCTGGGTTGGGTTTATGGAAGGGACATAGTCG
attB2_AVR$_{A7}$_stop	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGGGTTTATGGAAGGGACATAGTCG
attb2_CSEP0333+stop	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAACGAATATCTTGCAGAGAACATACTTTCCA
attb2_CSEP0333nostop	GGGGACCACTTTGTACAAGAAAGCTGGGTTACGAATATCTTGCAGAGAACATACTTTCCA
attB2_MLA6_nostop	GGGGACCACTTTGTACAAGAAAGCTGGGTTGTTCTCCTCCTC
attB2_MLA6_stop	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAGTTCTCCTC
attB2_MLA7_nostop	GGGGACCACTTTGTACAAGAAAGCTGGGTTGAAATCAGTTCTCCTCCTCTCCTCAC
attB2_MLA7_stop	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAGAAATCAGTTCTCCTCCTCTCCT
attB2A6ML27_K44noStop	GGGGACCACTTTGTACAAGAAAGCTGGGTCACGAATATCTTGCAGAGAACA
attB2A6ML27_K44Stop	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAACGAATATCTTGCAGAGA
attB2AVR$_{A22}$_a6centralStop	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAACGGTAATCGCTTTCAAA
attB2AVR$_{A22}$_a6centralnoStop	GGGGACCACTTTGTACAAGAAAGCTGGGTCACGGTAATCGCTTTCAAAATC
attB2CSEP0333ML27_K44noStop	GGGGACCACTTTGTACAAGAAAGCTGGGTCACGAATATCTTGCAGAGAACA
AttB2CSEP0333ML27_K44Stop	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAACGAATATCTTGCAGAGA
AVR$_{A1}$_XhoIRNostop	CCGCTCGAGGGTGCATTCTTCAATGAATT
AVR$_{A1}$_XhoIRstop	CCGCTCGAGCTAGGTGCATTCTTCAATGA
AVR$_{A10}$_XhoIRNostop	CCGCTCGAGACGGTAATCGCTTTCAAAA
AVR$_{A10}$_XhoIRstop	CCGCTCGAGCTAACGGTAATCGCTTTCAAAA
AVR$_{A10}$NBamHI	CGCGGATCCATGGATGGCTGGACATGTGCC
AVR$_{A13}$_Nde1NSTR	CCGCTCGAGTTCAGGGCTTGAAACCAT
AVR$_{A13}$_Nde1STR	CCGCTCGAGCTATTCAGGGCTTGAAACCAT
AVR$_{A13}$-1NBamHI	CGCGGATCCATGGCTGGCGATGGTTATA

Table S2. Primers used in this study – continued.

AVR_{A22}_XhoIRNostop	CCGCTCGAGACGGTAATCGCTTTCAAAAT
AVR_{A22}_XhoIRstop	CCGCTCGAGTTAACGGTAATCGCTTTCAAAAT
AVR_{A6} MF27LF	ATGCTGCTTTGGTAGAAAACATAG
AVR_{A6} MI31RR	AAAGCAGCATCACAATCC
AVR_{A6}A49TF	TTATTTATTCACCCAAAGAACGTATAG
AVR_{A6}A49TR	GACTTGTGTGATTCCTCG
AVR_{A6}L47FF	AGTCTTATTTTTTCGCCCAAAGAAC
AVR_{A6}L47FR	TGTGTGATTCCTCGGTTG
AVR_{A6}ME40GF	GTTCCAACCGGGGAATCACAC
AVR_{A6}ME40GR	ATTTGGGTGTTTATCTATGTTTTCTAC
AVR_{A6}MF27LR	CACAATCCATATTCAAAACC
AVR_{A6}MI31RF	TGTAGAAAACCGGGATAAACACCCAAATG
AVR_{A6}MK33EF	AAACATAGATGAACACCCAAATG
AVR_{A6}MK33ER	TCTACAAAAGCAGCATCAC
AVR_{A6}MN36RF	TAAACACCCACGAGTTCCAACCGAGG
AVR_{A6}MN36RR	TCTATGTTTTCTACAAAAGC
AVR_{A6}NBamHI	CGCGGATCCATGAACCTATATTACAAATGT
AVR_{A6}Q50KF	TTTATTCGCCAAAAGAACGTATAG
AVR_{A6}Q50KR	TAAGACTTGTGTGATTCC
AVR_{A6}Y53CF	CAAAGAACGTGTAGAGATAGAC
AVR_{A6}Y53CR	GGCGAATAAATAAGACTTG
AVR_{A7}-1_XhoIRNostop	CCGCTCGAGGGGTTTATGGAAGGGAC
AVR_{A7}-1_XhoIRstop	CCGCTCGAGTTAGGGTTTATGGAAGGGAC
AVR_{A9}-1_XhoIRNostop	CCGCTCGAGACACGGCCCGCATT
AVR_{A9}-1_XhoIRstop	CCGCTCGAGTTAACACGGCCCGCATT
AVRPM17AXhoI	CCGCTCGAGTTAGGACAGAGGGCATTCCAG
AVRPM17AXhoIno	CCGCTCGAGGGACAGAGGGCATTCCAG
AVRPM17BamHI	CGCGGATCCATGACTCAAGTCTACACTTGC
AVRPM1ABamHI	CGCGGATCCATGGCCTTCAGCTACAAGACC
AVRPM1AXhoIstop	CCGCTCGAGGGAGTGCACGGAGCA
AVRPM1AXhoInostop	CCGCTCGAG GTCGCGCAGCACCAGGGT
AVR_{PM2}BamHI	CGCGGATCCATGGAGTCCTACTGGGACTGC
AVRPM3$^{A2/F2}$BamHI	CGCGGATCCATGGGTCCTGTTGCTAACGCT

Table S2. Primers used in this study – continued.

AVRPM3$^{A2/F2}$XhoI	CCGCTCGAGTTA GTGCAGGATGATGTTCAG
AVRPM3$^{A2/F2}$XhoIno	CCGCTCGAG GTGCAGGATGATGTTCAG
AVRPM3$^{B2/C2}$BamHI	CGCGGATCCATG TACCTGTTCTACCGTTGC
AVRPM3$^{B2/C2}$XhoI	CCGCTCGAGTTA GTTAGCGTAGTAGGGCTC
AVRPM3$^{B2/C2}$XhoIno	CCGCTCGAG GTTAGCGTAGTAGGGCTC
AVRPM3^{D3}BamHI	CGCGGATCCATG GTGATCTTCGACTGCTCC
AVRPM3^{D3}XhoI	CCGCTCGAGTTA GATCACGGAGGAGGAGCA
AVRPM3^{D3}XhoIno	CCGCTCGAG GATCACGGAGGAGGAGCA
AVRPM8BamHI	CGCGGATCCATG CTGCAGTACTACAAGTGC
AVRPM8XhoI	CCGCTCGAGTTA CATCACGAAGTCCAGCAG
AVRPM8XhoIno	CCGCTCGAG CATCACGAAGTCCAGCAG
CSEP0333RemoveStop	ACGAATATCTTGCAGAGAAC
GST	ACCGTCTCCGGGAGCTGCATGTGTCAGAGG
HAVR$_{A6}$C53YF	AAAAGAGCTTATGCAAATATACC
HAVR$_{A6}$C53YR	GGTGAAAAAATAAGACTTGTG
HAVR$_{A6}$K50QF	TTTTTTCACCCAAAGAGCTTGTG
HAVR$_{A6}$K50QR	TAAGACTTGTGTGATTCC
HAVR$_{A6}$ME33KR	AAACCGGGATAAACACCCACG
HAVR$_{A6}$ME33KR	TCTGTCAAAGCACTATTACAAG
HAVR$_{A6}$MG40EF	ATTCCAACCGAGGAATCACACAAG
HAVR$_{A6}$MG40ER	TCGTGGGTGTTCATCCCG
HAVR$_{A6}$ML27FF	ATAGTGCTTTTACAGAAAACCG
HAVR$_{A6}$ML27FR	TACAAGCCTTTTTATTTACATC
HAVR$_{A6}$MN36RF	TGAACACCCAAATATTCCAACCGGG
HAVR$_{A6}$MN36RR	TCCCGGTTTTCTGTCAAAG
HAVR$_{A6}$MR31IF	GACAGAAAACATAGATGAACACCCAC
HAVR$_{A6}$MR31IR	AAAGCACTATTACAAGCC
HAVR$_{A6}$F47LF	AGTCTTATTTATTCACCAAAAGAGCTTG
HAVR$_{A6}$F47LR	TGTGTGATTCCCCGGTTG
HAVR$_{A6}$T49AF	TTATTTTTTCGCCAAAAGAGCTTG
HAVR$_{A6}$T49AR	GACTTGTGTGATTCCCCG
Hybird F	GACCCAGCTTTCTTGTAC
M1 F27L+I31R+K33E+N36R+E40GF	GTTCCAACCGGGGAATCACAC

Table S2. Primers used in this study – continued.

M1 F27L+I31R+K33E+N36R+E40GR	TCTTGGGTGTTCATCTCTG
M1 F27L+I31R+K33E+N36RF	GAACACCCAAGAGTTCCAACCG
M1 F27L+I31R+K33E+N36RR	ATCTCTGTTTTCTACCAAAG
M1 F27L+I31R+K33EF	AAACAGAGATGAACACCCAAATG
M1 F27L+I31R+K33ER	TCTACCAAAGCAGCATCAC
M1 F27L+I31RF	GTAGAAAACAGAGATAAACACC
M1 F27L+I31RR	CAAAGCAGCATCACAATC
M1 N36R+E40G F	GTTCCAACCGGGGAATCACAC
M1 N36R+E40G R	TCGTGGGTGTTTATCTATGTTTTCTAC
M1 N36R+E40G+K33E F	AAACATAGATGAACACCCACG
M1 N36R+E40G+K33E R	TCTACAAAAGCAGCATCAC
M2 47+52 F	CCAAAGAGCGTATAGAGATAG
M2 47+52 R	GCGAAAAAATAAGACTTGTGTGATTCC
M2 49+47 F	AGTCTTATTTTTTCACCCAAAGAACGTATAG
M2 49+50+53+52 F	CACCAAAAGAGCGTGTAGAGATAG
M2 49+50+53+52 R	AATAAATAAGACTTGTGTGATTC
M2 4950+47 F	AGTCTTATTTTTTCACCAAAAGAACGTATAG
M2 495052+47 F	AGTCTTATTTTTTCACCAAAAGAACGTG
M2 4953+47 F	AGTCTTATTTTTTCACCCAAAGAACGTG
M2 5053+47 F	AGTCTTATTTTTTCGCCAAAAGAACG
M2 A49T_F	TTATTTATTCACCCAAAGAACGTATAGAG
M2 A49T_R	GACTTGTGTGATTCCCCG
M2 A49T+Y53C F	CAAAGAACGTGTAGAGATAGAC
M2 A49T+Y53C R	GGTGAATAAATAAGACTTGTG
M2 Q50K+A49T F	TTATTTATTCACCAAAAGAACGTATAGAG
M2 Q50K+A49T R	GACTTGTGTGATTCCCCG
M2 Q50K+A49T+Y53C F	AAAAGAACGTGTAGAGATAGACTAG
M2 Q50K+A49T+Y53C R	GGTGAATAAATAAGACTTGTG
M2 Q50K+Y53C F	AAAAGAACGTGTAGAGATAGACTAG
M2 Q50K+Y53C R	GGCGAATAAATAAGACTTG
M2 T52A F	CGCCCAAAGAGCGTATAGAGATAG
M2 T52A R	AATAAATAAGACTTGTGTGATTCC
pGEX- 6P-1F	TTGAAACTCTCAAAGTTGATTTTCTTAGCA
PWI2BamHI	CGCGGATCCATG GGTGGTGGCTGGACCAAC
PWI2XhoIstop	CCGCTCGAGTTA CATGATGTTGCAACCCTC

Table S2. Primers used in this study – continued.

PWI2XhoInostop	CCGCTCGAG CATGATGTTGCAACCCTC
RemoveStopAVR$_{A6}$_R	ACGAATATCTTGCAGAGAAC
RemoveStoppENTR_F	GACCCAGCTTTCTTGTAC
SVRPM3$^{A1/F1}$ BamHI	CGCGGATCCATG TTCGACCTGATCGACGAC
SVRPM3$^{A1/F1}$XhoIstop	CCGCTCGAGTTA CTGACGACGGTAGGTAGC
SVRPM3$^{A1/F1}$XhoInostop	CCGCTCGAG CTGACGACGGTAGGTAGC

Dataset S1 (separate file). Raw relative luciferase measurements and statistics for protoplast assays.
Dataset S2 (separate file). Sequence alignments for RALPH subfamilies shown in Fig. 5.

Supporting References

1. S. Bauer *et al.*, The leucine-rich repeats in allelic barley MLA immune receptors define specificity towards sequence-unrelated powdery mildew avirulence effectors with a predicted common RNase-like fold. *PLoS Pathog.*. **17** (2021).
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