# SUPPLEMENTAL FIGURES

**A**

| | | Identity (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Pf-Avr4 | Cf-Avr4 | Mf-Avr4 | Ds-Avr4 | Cb-Avr4 | Sm-Avr4 |
| Similarity (%) | Pf-Avr4 | | 38 | 66 | 31 | 36 | 42 |
| | Cf-Avr4 | 48 | | 42 | 54 | 20 | 35 |
| | Mf-Avr4 | 72 | 49 | | 31 | 31 | 45 |
| | Ds-Avr4 | 42 | 58 | 39 | | 32 | 30 |
| | Cb-Avr4 | 46 | 25 | 42 | 38 | | 32 |
| | Sm-Avr4 | 50 | 45 | 55 | 35 | 40 | |

**B**

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                                                                                                  1
Pf-Avr4 --MLSTTAIALLATLILAATS-------DARATYVQDKTPA------------FVCPAAD 39
Cf-Avr4 MHYTTLLLSTLLVGTALAQPT----------NPPAKTPKKA--PKTQP----YNPCKPQE 44
Mf-Avr4 --MLSTTRITLLATFALATTS-------YAGTMQVRDTAPA------------FVCPSAD 39
Ds-Avr4 MQYNLFLLAALL-GSALAQPAKGSKAYKVPQNPKTLNTHKALPPKPQPEPPVYNPCQSTE 59
Cb-Avr4 --MYGLFHLAVLSATALSASA-------LPQQQEKPGVNKD------------FICPPED 39
Sm-Avr4 --MFSPT----LLLLALSTSS-------VLAYPQAGSVDTT------------FTCPAAD 35
                     *    *: .:                                  * . :
                  2     3       4     5                6              7
Pf-Avr4 IKTTKCLGPKDCLYPSPKTCNGYIQCSPADDSYLTGIIHEMPCPSGLLWND--NKKWCDW 97
Cf-Avr4 VIDTKCMGPKDCLYPNPDSCTTYIQCVPLDE-VGNAKPVVKPCPKGLQWNDNVGKKWCDY 103
Mf-Avr4 ITATKCLGPKDCLYPNPKTCNGYIQCSPADASYTTGIVHEMPCPSGLQWND--NQKWCDW 97
Ds-Avr4 VLATKCLGPKDCLYPNPASCGTFIQCVPQDE-VGNAKPVVMPCPKGLQWND--GKKWCDY 116
Cb-Avr4 MKRTHCMGPFDCLYALPGDCLGYIQCQPKDTTYETGIAYERPCRAGQWWND--EKKYCDT 97
Sm-Avr4 MMNTKCLGPKDCLYPDDNNCAGFLHCQPLDG-YQTGIAYRMDCPVGLRWND--NAKWCDY 92
        :  *:*:** ****.    *  :::* * *    ..      *  *  ***    *:**
                  8
Pf-Avr4 PENTTCGLVK-------SAGATEVDATKQ------GPTPGKYHG------- 128
Cf-Avr4 PNLSTCPVKT--------PQPKPKKGGV-------GGKKASVGHPGY---- 135
Mf-Avr4 PENSTCG----------NTGGTIYRCDET------GCDNV----------- 121
Ds-Avr4 PHLSTCPAKATTKTPANKPVAKPVKRPVKKPVKKKGGKKQGSGGTAQPE-- 165
Cb-Avr4 VEPPTCKAPV-------VPASAPAPAPAP------APAPAAPAAPASTSPC 135
Sm-Avr4 PANATCT-------------PHEVY------------------------- 104
           .**
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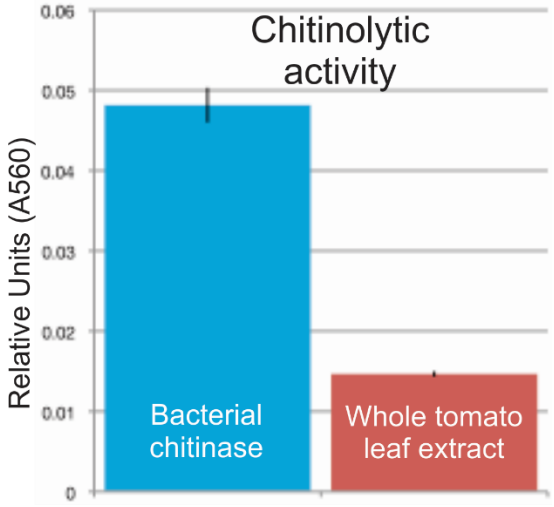
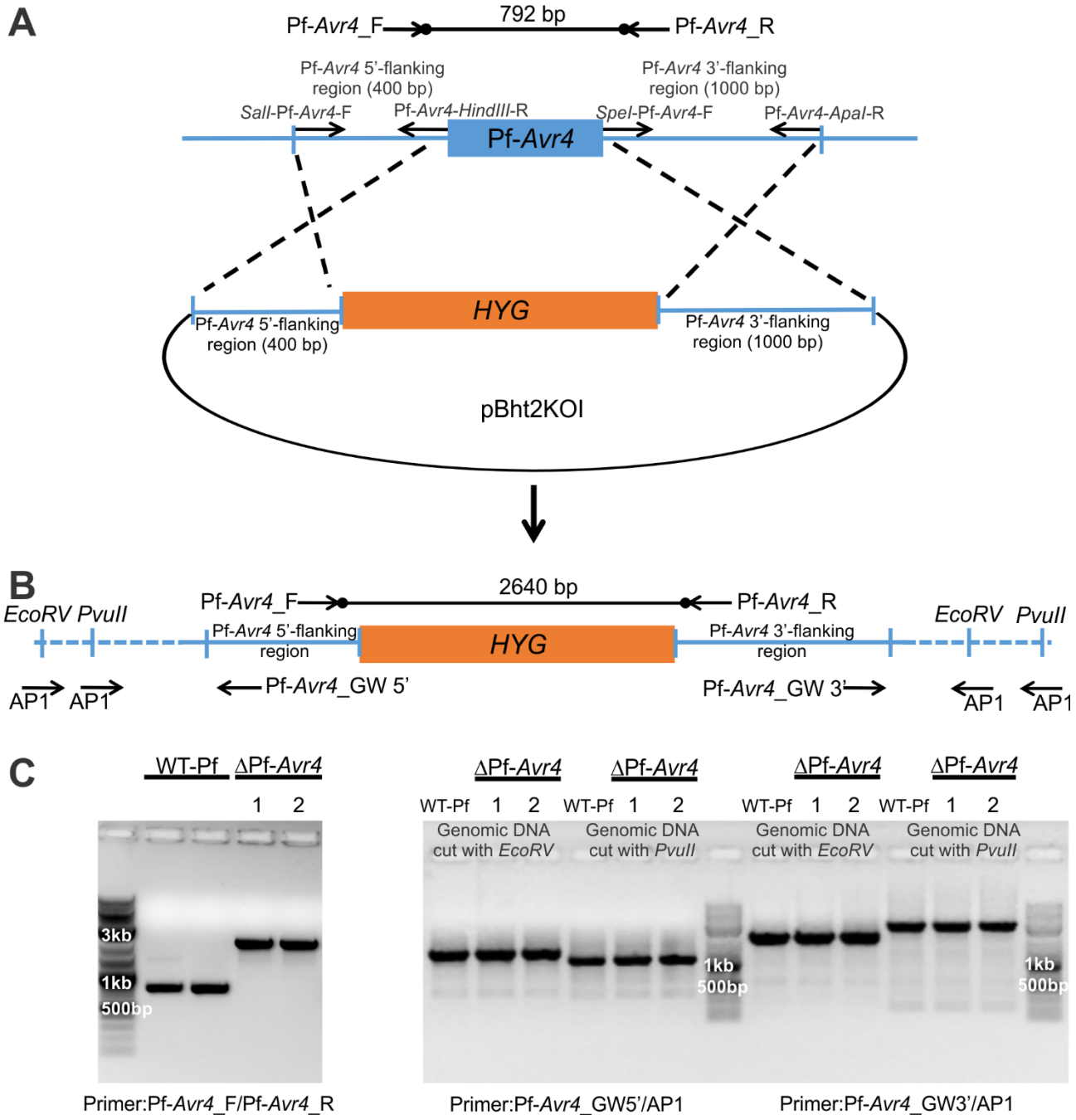
**Supplemental Figure 1.** The *Pseudocercospora fuligena* Avr4 effector protein (Pf-Avr4) is a member of the Avr4 core fungal effector family.

**(A)** % protein identity (upper triangle) and similarity (lower triangle) scores among different members of the Avr4 effector family calculated using global Needleman-Wunsch alignments. Cf-Avr4: *Cladosporium fulvum* Avr4 (Q00363), Mf-Avr4: *Mycosphaerella fijiensis* Avr4 (XP007926047), Ds-Avr4: *Dothistroma septosporum* Avr4 (EME41286), Cb-Avr4: *Cercospora beticola* Avr4 (ADE28519), Sm-Avr4: *Sphaerulina musiva* Avr4 (EMF11067).
**(B)** Alignment of Avr4 homologues from different Dothideomycete species. Alignments were produced using ClustalW. Conserved amino acid residues are indicated with an asterisk. Cysteine residues are highlighted in bold and the eight conserved cysteine are numbered above the alignment. The putative family 14 carbohydrate-binding module (CBM14: Pfam01607) is underlined and signal peptide sequences are shown in italics.




**Supplemental Figure 2.** Chitinolytic activity of the whole tomato leaf extract and of the bacterial chitinase, as measured by treatment of chitin azure at 25°C for 7.5 hours.

To measure the chitinolytic activity of the whole tomato leaf extract, chitin azure was treated with either bacterial chitinase or whole tomato leaf extract and incubated at 25°C for 7.5 hours. The chitin azure was pelleted and an absorbance reading of $A_{560}$ was taken on the supernatant of each reaction. Activity is plotted as a bar graph with standard error of the mean (SEM) indicated for each sample with a black line.

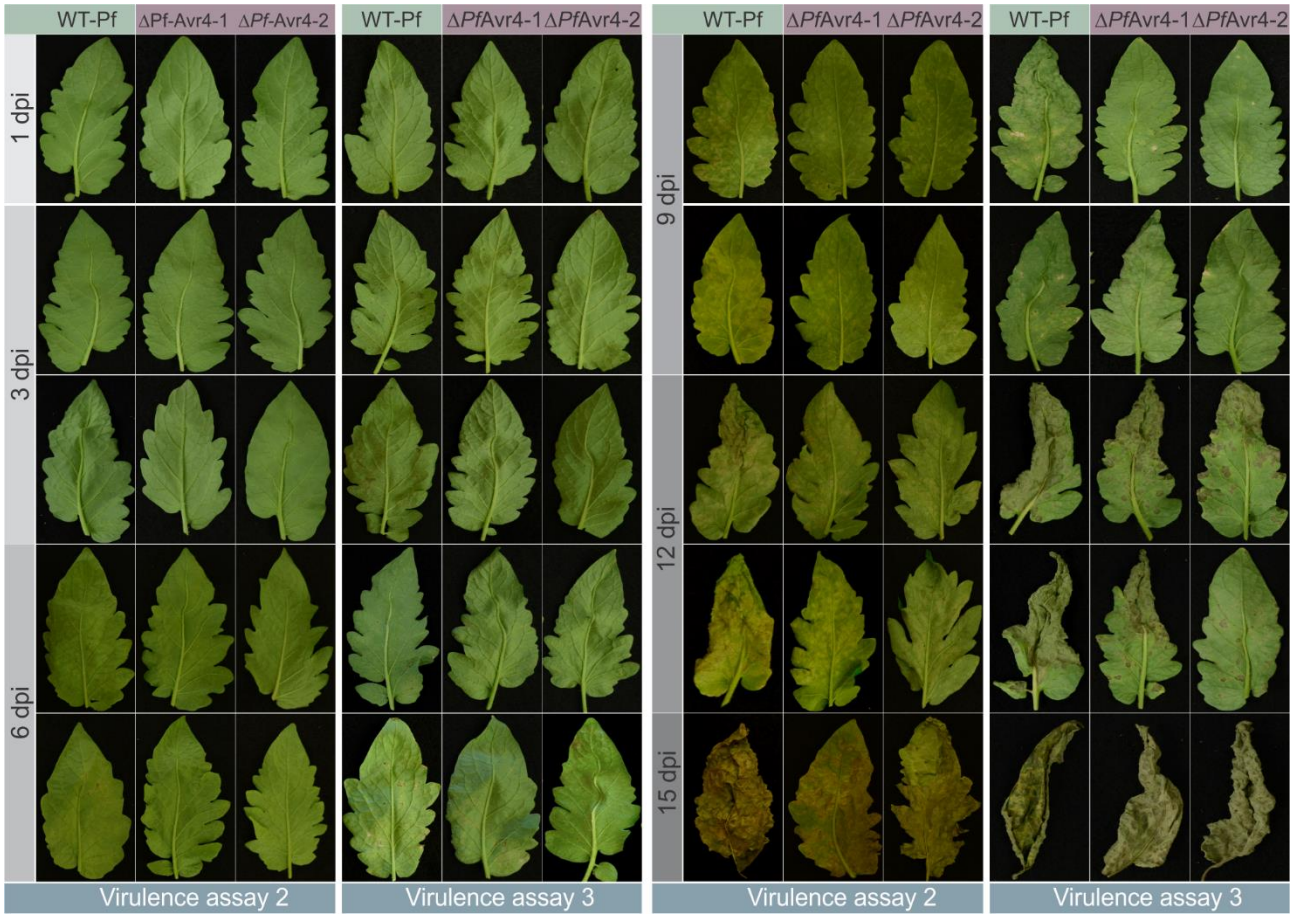



**Supplemental Figure 3.** Targeted gene replacement of Pf-*Avr4* by the hygromycin-resistance cassette in the genome of *Pseudocercospora fuligena*.

**(A)** Schematic representation of the strategy used to generate the construct for the replacement of Pf-*Avr4* by the hygromycin (*HYG*) resistance cassette. A 400 bp DNA fragment upstream of Pf-*Avr4* as well as a 1 kb DNA fragment downstream of Pf-*Avr4*, were amplified from genomic DNA of *P. fuligena* using primer pairs *SalI*-Pf-*Avr4*_F/Pf-*Avr4-HindIII*-R (for the 400 bp fragment), and *SpeI*-Pf-*Avr4*-F/Pf-*Avr4-ApaI*-R (the 1 kb fragment). The two fragments were subsequently double-digested with *SalI* and *HindIII* (the 400 bp fragment), or *SpeI* and *ApaI* (the 1 kb fragment), and cloned into pBht2KOI binary vector at the 5´-end (the 400 bp fragment) and the 3´-end (the 400 bp fragment) of *HYG*.

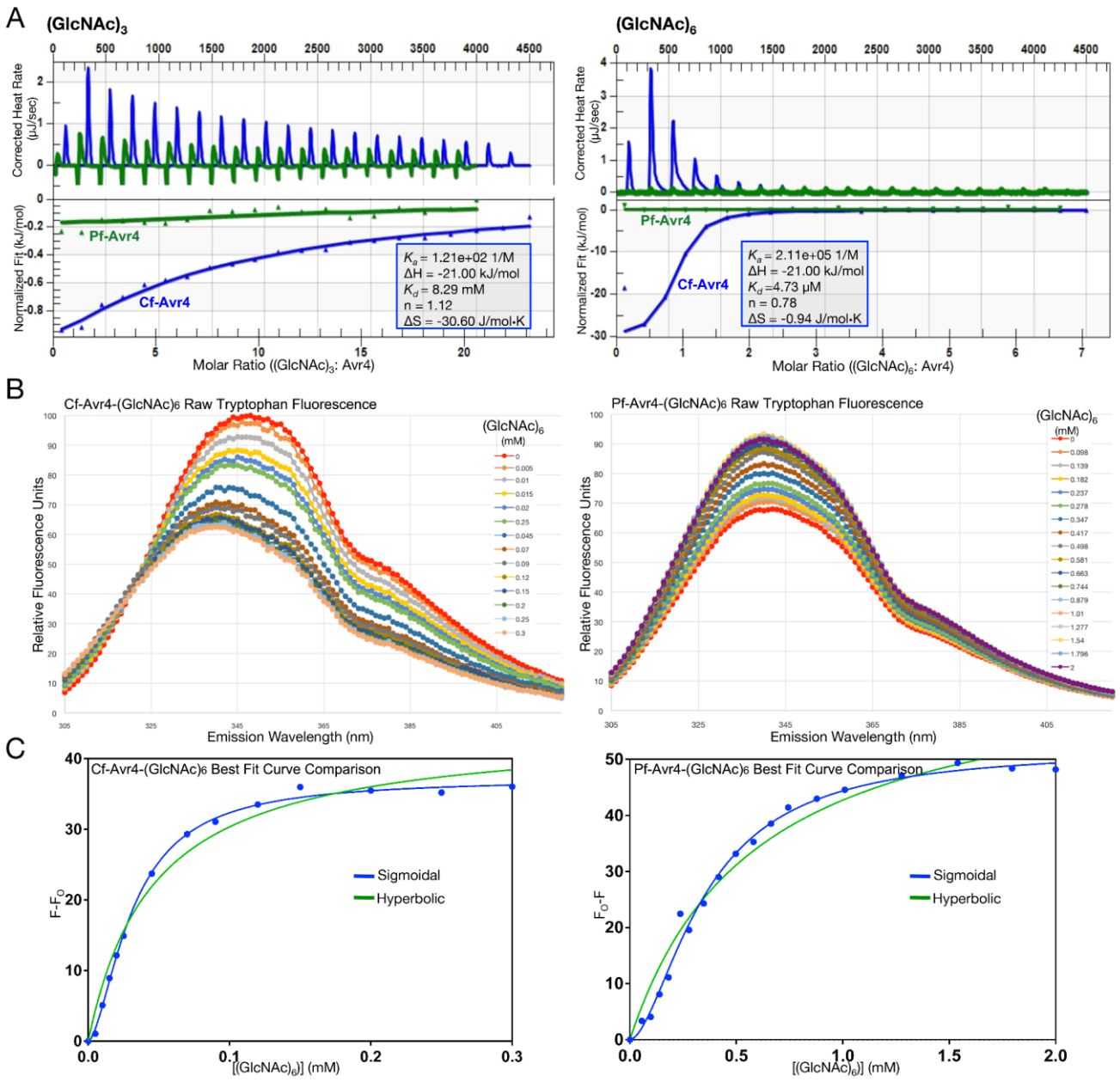
**(B)** Schematic representation of the Pf-*Avr4* locus after replacement of Pf-*Avr4* by the *HYG*-cassette (see panel **C** for further details).

**(C)** The successful integration of the *HYG*-cassette in the Pf-*Avr4* locus of two ΔPf-*Avr4* deletion mutants (ΔPf-*Avr4-1* and ΔPf-*Avr4-2*) was verified by PCR using the primer pair Pf-*Avr4*_F/Pf-*Avr4*_R (left side of panel **C**), which in case of a successful targeted Pf-*Avr4* replacement event amplifies a 2,640 bp fragment, whereas in the absence of a homologous recombination event in this gene locus it amplifies a 792 bp fragment. The absence of any ectopic integrations of the *HYG*-cassette in the background of the ΔPf-*Avr4-1* and ΔPf-*Avr4-2* mutant strains was verified by a genome walking approach. For this purpose, genomic DNA of the wild-type *P. fuligena* strain (WT-Pf) and the two ΔPf-*Avr4* mutants was digested with restriction enzymes *EcoRV* or *PvuII* and adaptors were ligated to the end of the digested DNA fragments. Subsequently, primer pairs AP1/Pf-*Avr4*_GW-5´ and AP1/Pf-*Avr4*_GW-3´ (see panel **B**) were used for amplifying DNA fragments upstream and downstream of *HYG*-cassette, respectively. Using these primers only a single PCR fragment of the same size was obtained for the WT-Pf strain and the two ΔPf-*Avr4* deletion mutants indicating a single integration event (right side of panel **C**).




**Supplemental Figure 4**. Virulence of the wild-type *Pseudocercospora fuligena* strain (WT-Pf) and two ΔPf-*Avr4* deletion mutants (ΔPf-*Avr4-1* and ΔPf-*Avr4-2*) on cv. LA3940.

Virulence assays were performed three times and shown are the results from the second and the third assay. Infected material was collected at 1, 3, 6, 9, 12, and 15 days post inoculation (dpi) from 6-9 randomly selected leaves of 2-3 plants that were sprayed at the beginning of the experiment with the fungal mycelia. For time points 6, 9, 12, and 15 dpi, the pictures from two randomly selected leaves that were inoculated with the WT-Pf strain or the two ΔPf-*Avr4-1* and ΔPf-*Avr4-2* mutants are shown. For time point 1 dpi the picture from only one leaf is shown as disease symptoms are not yet visible at this time point. The results show that ΔPf-*Avr4-1* and ΔPf-*Avr4-2* show a moderate reduction in virulence on cv. LA3940.



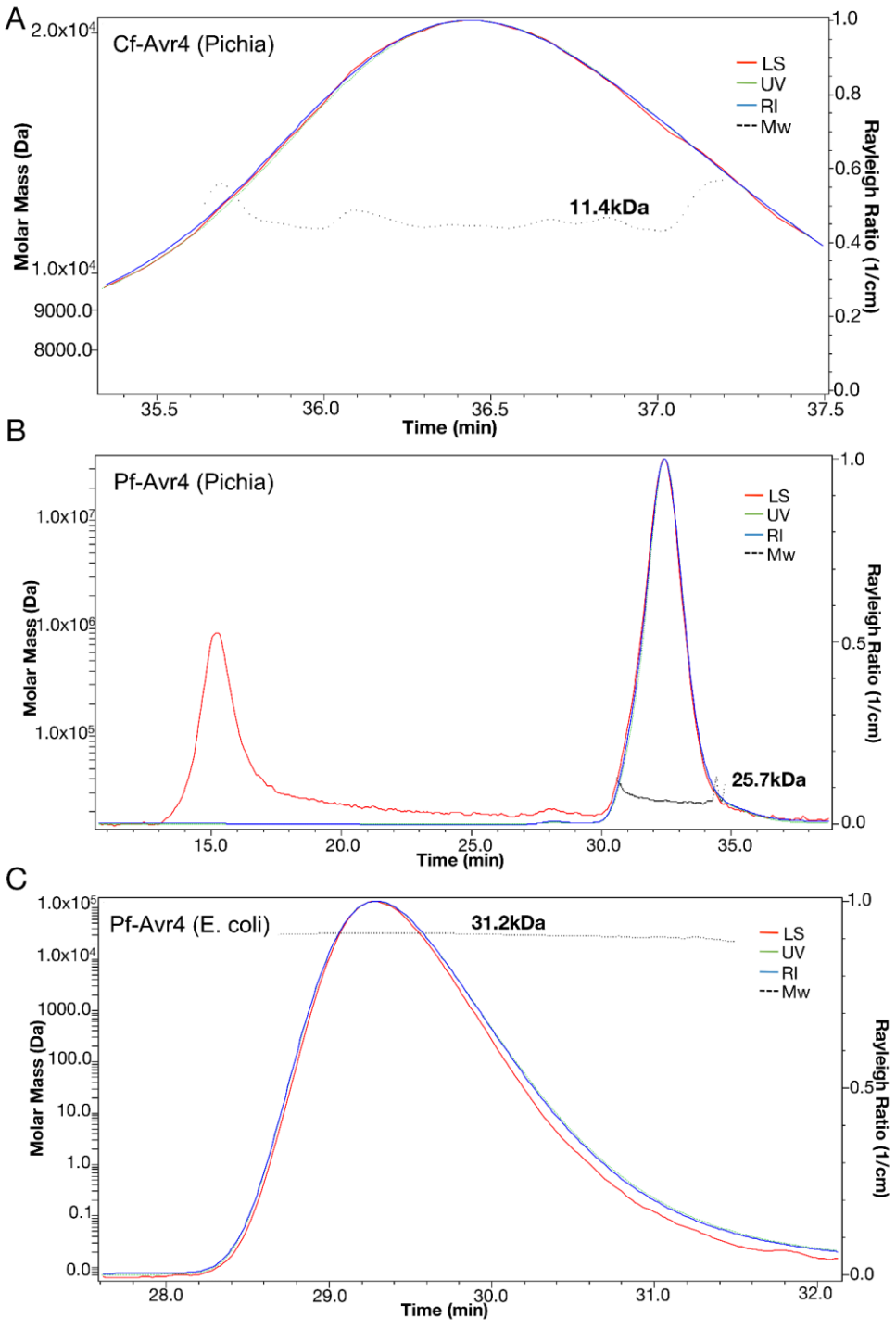

**Supplemental Figure 5.** The ITC and raw tryptophan fluorescence binding profiles for Cf-Avr4 and Pf-Avr4.

**(A)** Titration of 23 mM $(GlcNAc)_3$ into 0.35 mM of *Pichia*-produced Pf-Avr4 (green) or Cf-Avr4 (blue) resulted in no measurable heat change, or no quantifiable binding, for Pf-Avr4 and a binding interaction for Cf-Avr4 that was characterized by a $K_d$ of 6.17 mM and n=0.92. Titration of 2 mM $(GlcNAc)_6$ into 0.1 mM Pf-Avr4 (green) or Cf-Avr4 (blue) resulted in no measurable heat change, or no quantifiable binding for Pf-Avr4 and a binding interaction for Cf-Avr4 with a $K_d$ of 4.7 µM and n=0.78. Thus, although previously reported dissociation constants for Cf-Avr4 (van den Burg et al., 2004) were readily reproducible for binding to $(GlcNAc)_3$ and $(GlcNAc)_6$, binding of Pf-Avr4 to either $(GlcNAc)_3$ or $(GlcNAc)_6$ was undetectable under the testable concentrations of both Pf-Avr4 and chito-oligosaccharide, which is demonstrated by the negligible heat change detected for both the Pf-Avr4-$(GlcNAc)_3$ and Pf-Avr4-$(GlcNAc)_6$ interaction under the same conditions used for Cf-Avr4. This inability to detect binding by ITC

suggested that Pf-Avr4 had a lower affinity for $(GlcNAc)_3$ and $(GlcNAc)_6$ as compared to Cf-Avr4, thus necessitating the use of higher working protein and chito-oligosaccharide concentrations, which was not possible due to the solubility limit of Pf-Avr4 and the high viscosity of the chito-oligosaccharide at required concentrations (40 mM $(GlcNAc)_6$).

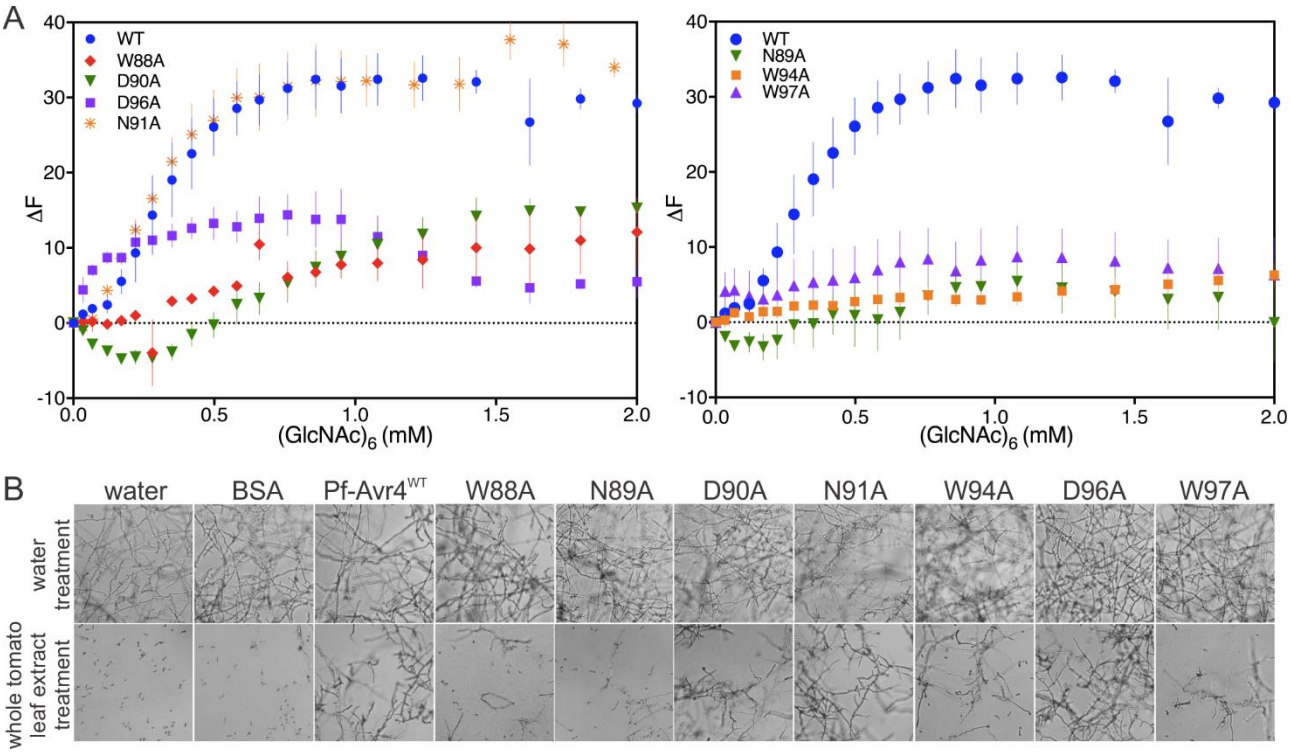
**(B)** Cf-Avr4 was titrated with $(GlcNAc)_6$ and with each addition of $(GlcNAc)_6$, the sample was excited with 295 nm and an emission spectrum was collected from 300-420 nm. Each curve on the graph correlates to one titration point, where titration point 0 mM (shown in red) corresponds to Cf-Avr4 in the absence of $(GlcNAc)_6$. The intrinsic tryptophan fluorescence of Cf-Avr4 decreases upon binding to $(GlcNAc)_6$, and exhibits a blue shift in emission wavelength. Pf-Avr4 was titrated with $(GlcNAc)_6$ following the same protocol as used for Cf-Avr4. Titration point 0 mM (red) represents Pf-Avr4 in the absence of $(GlcNAc)_6$, and upon binding to $(GlcNAc)_6$, the intrinsic tryptophan fluorescence of Pf-Avr4 increases, while the emission wavelength remains unchanged.

**(C)** The tryptophan fluorescence-based titration curves were best fit with a sigmoidal curve (blue) defined by $K_d$, $\Delta F_{max}$, and h (hill coefficient). Shown here is the fit of $(GlcNAc)_6$-binding data for Cf-Avr4 (left) and Pf-Avr4 (right). A hyperbolic fit ($K_d$, $\Delta F_{max}$) is shown for comparison in green.




**Supplemental Figure 6.** The solution behavior of Cf-Avr4 and Pf-Avr4 as determined by SEC-MALS analysis.

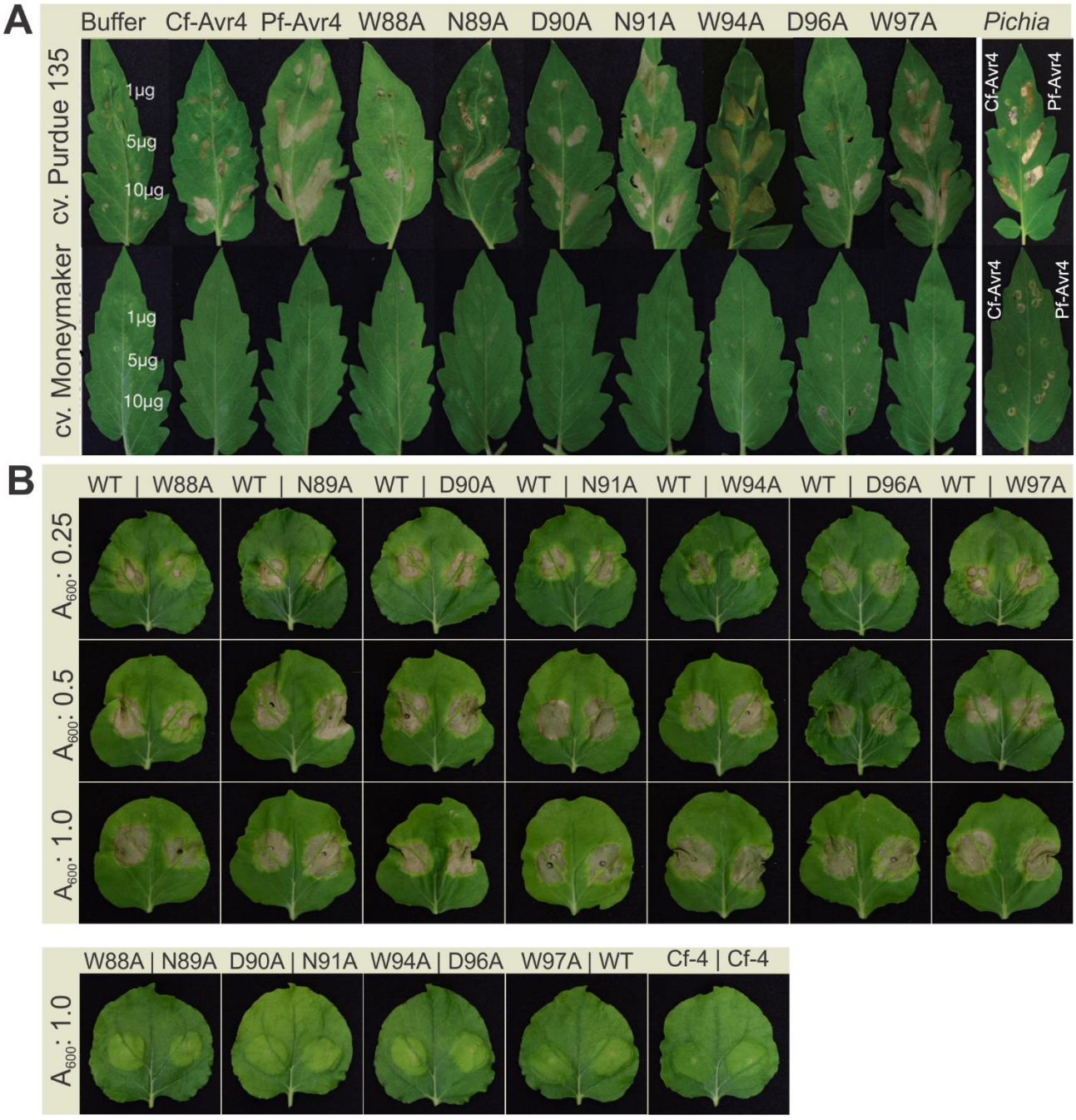
**(A)** Cf-Avr4 produced in in the methylotropic yeast *Pichia pastoris* behaves as a monomer in solution, existing as a roughly 11.4 kDa species. The predicted molecular weight of Cf-Avr4 is 11kDa.
**(B)** *Pichia*-produced Pf-Avr4 exists as a dimer in solution that is approximately 25.7 kDa in size. *Pichia*-produced Pf-Avr4 has an expected molecular weight of 13 kDa.
**(C)** *Escherichia coli*-produced Pf-Avr4 is also a dimer in solution corresponding to roughly 31.2 kDa. The *E. coli*-produced wild-type Pf-Avr4 has an expected molecular of 17 kDa.




**Supplemental Figure 7.** Affinity of wild-type (WT) Pf-Avr4 and chitin-binding domain (ChtBD) mutants for $(GlcNAc)_6$, and their ability to provide protection against plant-derived chitinases.

(**A**) The individual contribution of each predicted ChtBD residue was assessed by site-directed mutagenesis to alanine and mutant affinity for $(GlcNAc)_6$ was measured using a tryptophan fluorescence-based binding assay. Left panel, mutations that significantly reduced Pf-Avr4's affinity for $(GlcNAc)_6$. Right panel, mutations that partially reduced or altered Pf-Avr4's affinity for $(GlcNAc)_6$. Compared to Pf-Avr4$^{WT}$ and the other ChtBD mutants, Pf-Avr4$^{D90A}$ exhibits a lag phase as demonstrated by the marginally negative *ΔF* values at titrations with $(GlcNAc)_6$ concentrations below 0.5 mM, following thereafter a sigmoidal binding pattern typical of the Pf-Avr4-$(GlcNAc)_6$ interaction. The altered fluorescence behavior of Pf-Avr4$^{D90A}$ is likely due to a change in protein-protein association that occurs only when the protein is interacting with a true saccharide ligand (Supplemental Figure 10). *ΔF* represents the observed change in fluorescence upon addition of $(GlcNAc)_6$.

(**B**) The Pf-Avr4 chitin-binding domain (ChtBD) mutants offer varying degrees of protection against whole tomato leaf extracts based on their affinity for $(GlcNAc)_6$. *Trichoderma viride* germlings were treated with either water, BSA, Pf-Avr4$^{WT}$, or one of the seven ChtBD point mutants. The ability of *T. viride* to grow in the presence of the protein treatments is illustrated in the top row. To evaluate the ability of the ChtBD mutants to protect fungi from hydrolysis by plant-derived chitinases, the fungus was treated with whole tomato leaf extracts (bottom row).

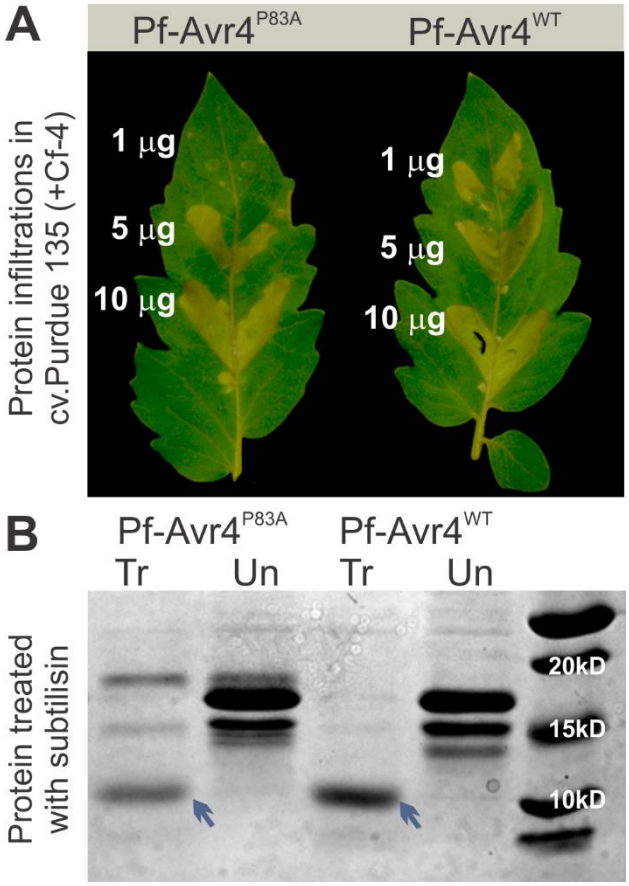



**Supplemental Figure 8.** Pf-Avr4 chitin-binding domain (ChtBD) mutants are susceptible to proteolytic cleavage under apoplastic conditions.

**(A)** Wild-type (WT) Pf-Avr4 and Pf-Avr4 ChtBD mutants were infiltrated into the tomato leaves of cultivar (cv.) Purdue 135 (+ Cf-4) and cv. Moneymaker (- Cf-4) at 1, 5, and 10 µg/mL concentrations, and the induction of a hypersensitive reaction (HR) was assessed at 5 days post-infiltration. The buffer alone and Cf-Avr4 were used as controls. Pf-Avr4$^{WT}$ (*E. coli*) elicited a strong HR in cv. Purdue 135 at all concentrations tested, while Cf-Avr4 (*E. coli*) produced strong HR at 10 µg/mL and patchy HR at lower concentrations. This HR pattern for Pf-Avr4 and Cf-Avr4 was also observed when the *Pichia*-produced proteins were infiltrated in to cv. Purdue 135 and cv. Moneymaker (far right). *Pichia*-Pf-Avr4 (right) and *Pichia*-Cf-Avr4 (left) were infiltrated into the same leaf at 1, 5, and 10 µg/mL. None of the proteins

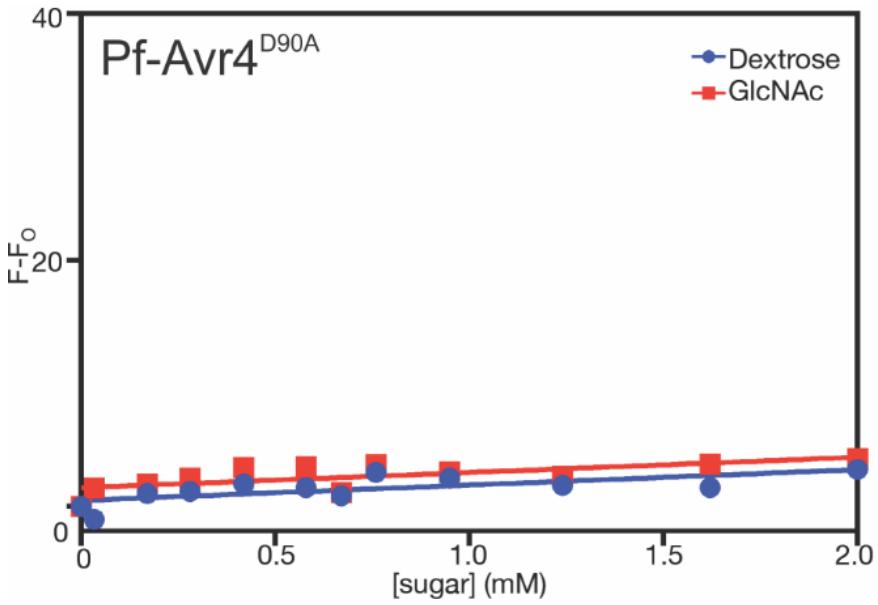
elicited HR in cv. Moneymaker, suggesting that the HR response observed in cv. Purdue 135 is mediated by the Cf-4 immune receptor.

**(B)** Pf-Avr4$^{WT}$ and ChtBD mutants were transiently co-expressed with Cf-4 in leaves of *Nicotiana benthamiana* using an *Agrobacterium tumefaciens* transient transformation assay (ATTA). Co-infiltrations at 1:2 ($A_{600}$0.5:$A_{600}$1.0), 1:1 ($A_{600}$0.5:$A_{600}$0.5), and 1:0.5 ($A_{600}$0.5:$A_{600}$0.25) ratios of Cf-4:Pf-Avr4$^{WT}$ or one of the ChtBD mutants were tested and HR was assessed seven days post-infection. Leaves were infected with Pf-Avr4$^{WT}$, left, and a ChtBD mutant, right. Control agroinfiltrations for the individual proteins are shown in the bottom row. Of the seven ChtBD mutants, only Pf-Avr4$^{W88A}$ elicited an HR different from that of Pf-Avr4$^{WT}$, exhibiting a slightly weaker and patchy HR when infiltrated at cell suspension densities of $A_{600}$0.25 and $A_{600}$0.5. Thus, all mutants are capable of being perceived by Cf-4 when both the immune receptor and effectors are highly overexpressed using the ATTA system. The differences in HR induction observed when the pure proteins are infiltrated into the leaves of Cf-4 tomato plants is likely due to a combination of factors, including the amount of Avr4 effector protein infiltrated, the amount of Cf-4 present on the cell surface, and the type and activity level of the proteases present in the tomato leaf apoplast.




**Supplemental Figure 9.** The Pf-Avr4$^{P83A}$ mutant is partially susceptible to proteolysis by subtilisin and is recognized by Cf-4.

**(A)** Wild-type (WT) Pf-Avr4 and the Pf-Avr4$^{P83A}$ mutant were infiltrated into tomato leaves of cultivar (cv.) Purdue 135 (+ Cf-4) at 1, 5, and 10 µg/mL to determine their ability to be recognized by Cf-4 and elicit a hypersensitive response (HR). Both protein isoforms were capable of inducing HR at the concentrations of 5 and 10 µg/mL but only the Pf-Avr4$^{WT}$ induced an HR at the concentration of 1 µg/mL. **(B)** To assess whether the inability of the Pf-Avr4$^{P83A}$ mutant to induce an HR at the concertation of 1 µg/mL was due to increased proteolytic vulnerability, the Pf-Avr4$^{WT}$ and the Pf-Avr4$^{P83A}$ mutant were treated with subtilisin, a non-specific protease (Un: untreated protein; Tr: subtilisin-treated). Subtilisin digested both the Pf-Avr4$^{WT}$ and the Pf-Avr4$^{P83A}$ mutant from a 17 kD protein to a 10 kD product (indicated with a blue arrow), corresponding to the full-length, mature protein sequence. However, after treatment with subtilizing, the intensity of the band corresponding to Pf-Avr4$^{P83A}$ mutant was almost half of the intensity of the band corresponding to the Pf-Avr4$^{WT}$, indicating increased proteolytic sensitivity.




**Supplemental Figure 10.** The fluorescence behavior of Pf-Avr4$^{D90A}$ is unaffected by titration of sugars to which Pf-Avr4 does not display affinity.

Pf-Avr4$^{D90A}$ was titrated with dextrose (blue) or GlcNAc (red) to a maximum of 2mM, and in the presence of either sugar, Pf-Avr4$^{D90A}$ did not exhibit a change in fluorescence. Therefore, as sugars are known to interact with and stabilize protein structures, the unique binding pattern observed with titration of Pf-Avr4$^{D90A}$ with (GlcNAc) (Supplemental Figure 7A) is likely due to a change in Pf-Avr4$^{D90A}$ tryptophan fluorescence that occurs in the presence of a true binding ligand.

## SUPPLEMENTAL REFERENCES

**van den Burg, H.A., Spronk, C.A.E.M., Boeren, S., Kennedy, M.A., Vissers, J.P.C., Vuister, G.W., de Wit, P.J.G.M., and Vervoort, J.** (2004). Binding of the AVR4 elicitor of *Cladosporium fulvum* to chitotriose units is facilitated by positive allosteric protein-protein interactions - The chitin-binding site of AVR4 represents a novel binding site on the folding scaffold shared between the invertebrate and the plant chitin-binding domain. J. Biol. Chem. **279,** 16786-16796.

# SUPPLEMENTAL TABLES

**Supplemental Table 1.** Primers used for Pf-*Avr4* gene expression analysis and targeted gene replacement.

| Primer name | Primer sequence |
|---|---|
| Pf-*Avr4*_F | 5´-CAATGCCAAGAGTCCAAACA-3´ |
| Pf-*Avr4*_R | 5´-AGAAGACTGGAGGAACTTGG-3´ |
| Pf-*Avr4*_qPCR-F | 5´-CGCCAGCAGATGACTCCTA-3´ |
| Pf-*Avr4*_qPCR-R | 5´-CAGGCCAATCACACCACTTCT-3´ |
| Pf-*Actin*-F | 5´-TTTGCTACGTTGCCCTTGAC-3´ |
| Pf-*Actin*-R | 5´-GCTCGTTGCCAATAGTGATG-3´ |
| *SalI*-Pf-*Avr4*-F | 5´-GTCGACTGCGATGTGTCTTAAAAA-3´ |
| Pf-*Avr4*-*HindIII*-R | 5´-AAGCTTATAAAGCGGCTCCTGAACTGT-3´ |
| *SpeI*-Pf-*Avr4*-F | 5´-ACTAGTAACGGCGTCATTCCAACACTG-3´ |
| Pf-*Avr4*-*ApaI*-R | 5´-GGGCCCTTCAACTCGACCGATTCGC-3´ |
| AP1 | 5´-GTAATACGACTCACTATAGGG-3´ |
| Pf-*Avr4*_GW-5´ | 5´-AAAACCGACAATGTGGACAGTTCAGG-3´ |
| AP1/Pf-*Avr4*_GW-3´ | 5´-CTAGCAGTTCTGGCAATTGCGTATCTGG-3´ |
| *RPL2*-F | 5´-ATGGGTCGTGTGATCAGAGCA-3´ |
| *RPL2*-R | 5´-ACAAGTGAATGGCTTAAGCCT-3´ |