Title:
Binding of the AVR4 elicitor of Cladosporium fulvum to chitotriose units is facilitated by positive allosteric protein-protein interactions*

Subtitle:
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

Running title:
Chitin-binding site of the AVR4 elicitor

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ABSTRACT

The attack of fungal cell walls by plant chitinases is an important plant defense response to fungal infection. Anti-fungal activity of plant chitinases is largely restricted to chitinases that contain a non-catalytic, plant-specific chitin-binding domain (ChBD) (also called Hevein domain). Current data confirm that the race-specific elicitor AVR4 of the tomato pathogen Cladosporium fulvum can protect fungi against plant chitinases, which is based on the presence of a novel type of ChBD in AVR4 that was first identified in invertebrates. Although these two classes of ChBDs (Hevein and invertebrate) are sequentially unrelated, they share structural homology. Here, we show that the chitin-binding sites of these two classes of ChBDs have different topologies and characteristics. The $K_D$, $\Delta H$, and $\Delta S$ values obtained for the interaction between AVR4 and chito-oligomers are comparable to those obtained for Hevein. However, the binding site of AVR4 is larger than that of Hevein, i.e. AVR4 interacts strictly with chitotriose, while Hevein can also interact with the monomer N-acetyl-glucosamine. Moreover, binding of additional AVR4 molecules to chitin occurs through positive cooperative protein-protein interactions. By this mechanism AVR4 is likely to effectively shield chitin on the fungal cell wall, preventing the cell wall from being degraded by plant chitinases.
INTRODUCTION

Binding and conversion of carbohydrates by proteins is of fundamental importance in numerous biological processes, including (self and non-self) cell-cell recognition, cell adhesion, and carbohydrate turnover. Recently, protein domains responsible for this interaction have been reclassified into distinct Carbohydrate-Binding Modules (CBMs) (1). CBMs are often present in carbohydrate-degrading enzymes, where they appear to mediate a prolonged and more intimate contact between the catalytic domain and insoluble carbohydrate polymers (2,3). Lectins, on the other hand, are carbohydrate binding proteins that lack enzymatic activity but often contain tandem repeats of CBMs.

Chitin, a polymer consisting of β-1,4-linked N-acetyl-D-glucosamine (GlcNAc) residues, is a major component of crustacean shells, insect exoskeletons, and fungal cell walls, but is absent in plants. In higher organisms, two CBMs predominantly confer binding of proteins to chitin, i.e. the Hevein domain (hereafter denoted as CBM18) (4) and the invertebrate chitin-binding domain (CBM14) (5). CBM18 is nearly exclusively found in plants (to date one additional member of CBM18 has been identified in *Streptomyces griseus*, ref. 6), while CBM14 is commonly found in the genomes of baculoviridae, invertebrates, and mammals, but absent in plants (5). Both ChBDs are typical CBMs, i.e. lectins with tandem repeats are known for both [e.g. wheat germ agglutinin (WGA) (7) and peritrophin-44 (8)] and both domains can be found in chitinases. However, CBM18 is only fused to the plant-specific family-19 catalytic domain, while chitinases of mammals and invertebrates utilize CBM14 in combination with the family-18 catalytic domain. Sequence homology is missing between the two motifs, but the 3D structure of Tachycitin revealed that CBM14 and CBM18 partially share their tertiary structure (9).

The race-specific elicitor AVR4 of the tomato pathogen *Cladosporium fulvum* is hitherto the only fungal protein containing a CBM14 (10,11). AVR4 binds specifically to chitin (10,11) and appears to have a high affinity for crude fungal components that resist harsh treatments such as heating and treatment with proteinase K (12). Originally, AVR4 was identified as an extracellular race-specific elicitor of *C. fulvum* that induces plant defense responses in tomato plants carrying the complementary *Cf-4* resistance gene. Recognition of AVR4 is sufficient for
induction of complete resistance in tomato against isolates of the fungus *C. fulvum* that carry the AVR4 encoding gene (13,14). The natural isolates of *C. fulvum* that were found to evade Cf-4 mediated resistance were reported to secrete protease sensitive isoforms of AVR4, whereas native mature AVR4 (86 amino acids) is insensitive to these proteases (10,14). The corresponding *avr4* alleles in these isolates all contain single nucleotide polymorphisms causing in all but one case single amino acid substitutions (13). These mutations appear to have no direct effect on the chitin-binding properties of the isoforms as was shown for a set of Cys-to-Tyr mutations (10).

Amongst the Cf-4/Avr4 triggered plant defense responses are the hypersensitive response (*i.e.* a plant-specific programmed cell death response) and the accumulation of plant pathogenesis-related proteins (PR-proteins) (15,16), which includes different types of chitinases and chitinase-like lectins (PR-3, PR-4, PR-8, and PR-11) (17-19). The precursors of two well-studied plant lectins, *i.e.* Hevein of *Hevea brasiliensis* and *Urtica dioica* agglutinin (UDA), are also chitinase-like proteins (PR-4), but their inactive catalytic domain is cleaved off during maturation leaving only the ChBD (7,20). Anti-fungal activity has been well established for these chitinases (21-23) and lectins (24-26), and a substantial number of transgenic plants that constitutively express plant chitinases has now been reported to be less susceptible to a wide set of pathogenic fungi (27).

*C. fulvum* is reported to be insensitive to a combination of tomato chitinases and β-1,3-glucanases, at least under *in vitro* conditions (28). Studies using two other fungi, *i.e.* *Trichoderma viride* and *Fusarium solani* f.sp. *phaseoli*, showed that AVR4 can protect these two fungi against anti-fungal activity of PR-3 chitinases (11). The protective effect was further substantiated by the observation that AVR4 binds to chitin present in the cell walls of these two fungi.

To better understand the role of AVR4 during infection of tomato, we here examined the binding properties of AVR4 to chitin using soluble chito-oligomers. This system allows for a detailed comparison between AVR4 and CBM18 lectins (*e.g.* Hevein, Prohevein, UDA, and WGA). For the CBM18 lectins, the use of chito-oligomers has provided a detailed description of the chitin-binding site. In CBM18 lectins, the binding site consists of three binding subsites (a subsite is defined as all amino acids that interact with one sugar residue). Subsite +1 is
formed by the residues S19, W23, and Y30, while W21 is involved in subsite +2 and +3 (29-36). A hallmark of the CBM18 lectins is that they already interact with one GlcNAc residue. Here we show that binding of AVR4 requires at least a stretch of three GlcNAc residues. Using NMR, we identified several residues in AVR4, which are important for ligand binding. These residues are indeed positioned in the structural motif shared by CBM14 and CBM18, but they appear to highlight different binding sites rather than overlapping binding sites as compared with CBM18 (9).
EXPERIMENTAL PROCEDURES

**Materials.** AVR4 was produced in culture by the methylotrophic yeast *Pichia pastoris* and purified from culture fluids (37). AVR9 was obtained by solid-phase synthesis followed by oxidative-folding (38,39). Ribonuclease A was obtained from Sigma. Chito-oligomers (N-acetyl-D-glucosamine (GlcNAc), N-N'-diacetylchitobiose (chitobiose), N,N',N''-triacetylchitotriose etc.) were purchased from Seikagaku (Tokyo). All solvents and chemicals used were of the highest grade available. Concentrations of AVR4 were determined by UV absorbance with $\varepsilon_{280}(\text{AVR4}) = 1.50 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$.

**Isothermal Titration Calorimetry (ITC).** ITC measurements were performed at 298 K following standard procedures using a MCS Microcal titration calorimeter (40). The reaction cell (with a volume of ~1.35 mL) containing the AVR4 protein sample was continuously stirred while successive aliquots of ligand solution were added (final volume of the additions was 250 µL). Ligand and protein were dissolved in the same buffer. The AVR4 concentration in the cell was in the range of 90-360 µM depending on degree of polymerisation (DP) of the chito-oligomer added (see legend Fig. 1). The chito-oligomer concentrations used were 23 mM, 20 mM, 3.2 mM, and 2.0 mM for DP=3, 4, 5, and 6, respectively. The integrated heat effects after correction for heat of dilution were analyzed using standard software provided by Microcal Inc. The cumulative heat effect (Q) during the titration process for a simple set of binding sites is given by the following equations:

$$Q = M_t V_o n \nu \Delta H$$  \hspace{1cm} (a)

where $M_t$ is the macromolecule concentration in the calorimetric cell, characterized by the volume ($V_o$), $n$ the number of binding sites with a binding enthalpy of $\Delta H$, and $\nu$ the fractional saturation of the binding sites, which can be related to the apparent association constant ($K_A$) and to the total ligand concentration ($L_T$).

$$K_A = \frac{\nu}{[(1-\nu)L_T]}$$  \hspace{1cm} (b)

$$L_T = L_f + M_t n \nu$$  \hspace{1cm} (c)
where $L_f$ is the concentration of free ligand. Other thermodynamic parameters were calculated using the standard thermodynamic equation:

$$-RT \ln K_A = \Delta G = \Delta H - T \Delta S$$  \hspace{1cm} (d)

_Tryptophan fluorescence quenching._ Fluorescence measurements were performed with a Varian Cary Eclipse thermostated at 293 K. The excitation wavelength was 295 nm with an excitation slit of 2.5 nm. Emission intensities were collected over the wavelength range of 315-400 nm with an emission slit of 5 nm. Spectra were the average of 3 scans and corrected for the effect of dilution, buffer and chito-oligomer additions. Quantitative binding experiments were performed in a volume of 3 mL to which aliquots of a ligand solution (5-30 μL) were added under continuous stirring. AVR4 was dissolved at a protein concentration of 3.6 μM in 20 mM potassium phosphate buffer pH 7.0 containing 50 mM sodium chloride. Chito-oligomers were dissolved in the same buffer at a concentration of 38 mM, 30 mM, 16 mM, and 2.0 mM for a DP=3, 4, 5, and 6, respectively. The maximum change in volume due to the ligand additions was less than 5%. The fluorescence quenching at full saturation of binding ($F_0$) was estimated by plotting $1/(F_0 - F)$ versus $1/[S]$, and extrapolating to the $y$-axis, where $F_0$ is the fluorescence intensity of AVR4 without ligand and $F$ is the fluorescence intensity of AVR4 at the chito-oligomer concentration [S]. Association constants ($K_A$) were estimated using two methods: the fluorescence quenching titration equivalent of the Hill Plot (i.e. $\log (F_0 - F/F_0)$ versus $\log [S]$) (41), and Scatchard plot analysis (i.e. $\nu/L_f$ versus $\nu$) (42).

_Size-exclusion chromatography._ pH-dependent size-exclusion chromatography was performed at 293 K using a Superdex-75 (HR 10/30; Amersham) column operated at a flow rate of 0.5 ml/min. The apparent molecular mass ($M_w$) of the oligomeric/complexed state of AVR4 (25 μM in 50 μL injection volume) was estimated from a standard curve produced at different pH values (5.0, 7.0 and 8.6) in buffer containing 50 mM potassium phosphate and 150 mM potassium chloride. Standard curves were obtained by plotting the log molecular mass of protein standards (aprotinin, insulin, ubiquitin, Ribonuclease A, serum albumin (all
bovine), horse myoglobin, chicken albumin, and blue dextran) versus $K_{av}$. The $K_{av}$ is defined as:

$$K_{av} = \frac{(V_E - V_V)}{(V_B - V_V)}$$  \hspace{1cm} (e)

where $V_E$ is the elution volume, $V_V$ is the void volume, and $V_B$ is the bed volume of the column matrix.

**Mass spectrometry.** ESI-MS was performed with a Q-Tof *Ultima* Global mass spectrometer (Waters Corporation, MS Technologies Centre, UK). AVR4 and the chito-oligomers were dissolved in 10 mM ammonium acetate / acetic acid (pH range 5.0-8.6). The sample infusion flow rate was 10 $\mu$L/min. Instrument settings were: capillary potential, 3 kV; cone voltage, 100 V; desolvation gas flow rate, 150 L/h; source temperature, 90 °C; radio frequency 1 (RF1), 225 kHz; and the MALDI strip was positioned at 3600 arbitrary units resulting in an elevated intermediate pressure of 4.35 mbar. The instrument was operated under standard ESI conditions. Calibration of the TOF analyzer was performed with a CsI solution of 2 mg/mL in isopropanol/water (50:50, v/v) over the mass range 800-7100 Da.

**Nuclear magnetic resonance spectroscopy.** The NMR samples contained typically 1.5 mM $^{13}$C/$^{15}$N-AVR4 dissolved in 20 mM acetate-$d_4$ pH 4.6 and 50 mM sodium chloride. Isotopic labeling and purification of AVR4 was performed as described (37). All NMR samples were prepared in a mixture of 95%/5% (v/v) H$_2$O/D$_2$O and contained trace amounts of sodium azide as preservative. All NMR spectra were acquired at 298 K on Varian *Inova* 500, 600, or 800 MHz, and Bruker AMX500 spectrometers. Triple and double-resonance hetero-nuclear NMR experiments performed to obtain backbone and side-chain assignments of AVR4 included 3D HNCA, HN(CO)CA, CBCA(CO)NH, (H)CCH-TOCSY and HC(C)H-TOCSY (Protein pack, Varian Inc.). The assignment was performed using the standard assignment procedures based on triple and double resonance NMR spectra: First, $^{15}$N HSQC spectra were used to obtain a set of $^1$H-$^{15}$N resonance frequencies. Sequential assignment was then performed using these shift-pairs in combination with HNCA, HN(CO)CA, HNCAcb, and CBCA(CO)NH spectra. Assignment of the non-aromatic side chain resonances were obtained by means of (H)CCH-TOCSY and HC(C)H-TOCSY spectra. A $^{15}$N-NOESY-HSQC (43) spectrum was used.
for NOE assignments of the backbone $^1$HN and tryptophan side chain HE1 protons. All data processing and analysis was done using the programs NMRPipe (44) and XEASY (45), respectively. The chemical shifts of Tachycitin were retrieved from BioMagResBank (46).

**NMR titration experiments.** Binding of chito-oligomers to AVR4 was followed by recording $^1$H-$^15$N-HSQC spectra at five different temperatures. Thereto, temperature in the NMR tube was carefully calibrated by referencing the water resonance to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The protein concentration was kept constant during the titration (1 mM), while the ligand concentration was increased in successive steps (30). As buffer we used 20 mM Acetate-d$_4$ pH 6.0 and 150 mM NaCl. Final concentrations of the ligands were 50 mM GlcNAc, 35 mM (GlcNAc)$_2$, 27.2 mM (GlcNAc)$_3$, and 3.5 mM (GlcNAc)$_6$. The association constants ($K_a$) were estimated using a NMR derivative of the Scatchard plot:

$$\frac{\Delta/\Delta o}{[L]} = -K_a n + K_a \Delta/\Delta o$$

where $\Delta = \delta_{\text{observed}} - \delta_{\text{free}}$ and $\Delta o = \delta_{\text{saturated}} - \delta_{\text{free}}$. Thermodynamic parameters ($\Delta H$ and $\Delta S$) were estimated from a van’t Hoff plot based on a set of $K_a$s obtained from a set of backbone resonances.
RESULTS AND DISCUSSION

*Ligand binding studies.* Recent studies using affinity precipitation showed that AVR4 specifically binds to chitin and not to other polysaccharides (11). These studies included, besides chitin, the polymers cellulose, xylan, curdlan (β-1,3-glucan), lichenan, and chitosan (i.e. deacetylated chitin).

*Calorimetric titrations.* Isothermal titration calorimetry (ITC) was used to determine the affinity of AVR4 for linear soluble chitin fragments. No heat-of-binding was detected when 50 mM N-acetyl-D-glucosamine (GlcNAc) or 50 mM (GlcNAc)₂ (chitiobiose) was added to AVR4. The addition of longer chito-oligomers resulted, however, in a substantial heat-of-binding (Table 1). The binding curves obtained for (GlcNAc)₃, (GlcNAc)₄, and (GlcNAc)₅ could be fitted assuming a one-binding site model (Fig. 1A-1C). The binding curve obtained for (GlcNAc)₆ deviated from a one-binding site model. However, a model with two AVR4 binding sites per one (GlcNAc)₆ molecule described accurately the binding event (Fig 1D). This second AVR4 binding site was not observed for (GlcNAc)₅ (Fig. 1C). Together, these results suggest that AVR4 exclusive interacts with (GlcNAc)₃ repeats. In contrast, CBM18 lectins already bind to one GlcNAc molecule (33,42,47).

The dissociation constant ($K_D$) found for the binding of (GlcNAc)₆ to AVR4 is ~45 times lower than the $K_D$ observed for (GlcNAc)₅ and ~200 times lower than the $K_D$ observed for (GlcNAc)₄ (Table 1). This decreased $K_D$ for (GlcNAc)₆ originates from a steep decrease in $\Delta H$ as compared to $\Delta H$ for (GlcNAc)₅ (concomitantly, a small negative contribution to binding comes from a slight decrease in the $\Delta S$). The substantially decreased $\Delta H$ could indicate that positive allosteric interactions occur between the two AVR4 molecules that bind to one (GlcNAc)₆. Additionally, a substantial part of the decreased $\Delta H$ could originate from a decrease in the solvent-exposed area of the two bound AVR4 molecules. The existence of such a protein-protein interaction is supported by the fact that the number of binding sites (n) appears to be pH-dependent with an apparent $pK_A$ of ~4 (Table 2). This implies protonation of an acidic residue (i.e. Asp or Glu), which could effectively disrupt an interaction at the protein-protein interface.
The slight decrease of $\Delta S$ found for the binding of (GlcNAc)$_6$ to AVR4 as compared to (GlcNAc)$_5$ and (GlcNAc)$_4$ suggests that an increased number of translations and rotations in the sugar chain is restrained upon binding of AVR4 to (GlcNAc)$_6$. Potentially each of the GlcNAc residues is restrained due to the interaction with the two AVR4 molecules leading to an overall reduced flexibility of (GlcNAc)$_6$. Correspondingly, a certain degree-of-freedom should remain in the chito-oligomer chain for (GlcNAc)$_4$ and (GlcNAc)$_5$ when bound to AVR4. Indeed, $\Delta S$ is increased for both (GlcNAc)$_4$ and (GlcNAc)$_5$ as compared to (GlcNAc)$_3$. Hevein differs from the foregoing situation in that two Hevein molecules already bind to (GlcNAc)$_5$ rather than (GlcNAc)$_6$ (33). The corresponding $K_D$ is $\sim$45 times lower than the $K_D$ for (GlcNAc)$_4$. In this case, $\Delta S$ is the main contributor to the decreased $K_D$ (Fig. 2), which originates from the existence of several Hevein-(GlcNAc)$_5$ complexes with different stoichiometries (1:1 and 2:1 protein ligand complexes) (30,33).

This cost of restraining translations and rotations of the GlcNAc chains has been given as one explanation for the “enthalpy-entropy” compensation generally observed for lectin-sugar interactions, which results in $K_D$s in the micro- to millimolar range (48) (see also supplementary data). The correspondingly small and negative $\Delta H$ and $\Delta S$, as obtained here for AVR4 and as reported for other lectins such as Hevein (30,33), Prohevein (32), and UDA (42) (Fig. 2), points out that these interactions are enthalpically driven. Moreover, this sign and this order of magnitude of $\Delta H$ indicates that hydrogen bonds, CH-π interactions, and Vander-Waals forces are the principal forces stabilizing the complex (49-52). However, this conclusion does not extend beyond small soluble chito-oligomers, as binding to insoluble polysaccharides was shown to be entropically driven (53).

The fact that the binding energies (i.e. $\Delta H$ and $\Delta S$) of AVR4 cluster with those reported for Hevein and UDA but not with those of WGA (see supplementary figures), suggests that the binding site of AVR4 resembles to some extend the binding site of Hevein and UDA, but not that of WGA. The 3D structures of Hevein (33) and UDA (35) in complex with (GlcNAc)$_3$ showed that both have a surface-exposed binding site, while the binding site of WGA (29) is completely solvent-buried at the interface of the WGA dimer. This more solvent-buried binding site is reflected in a three-fold increased $\Delta H$ for WGA, but it is also compensated by a more negative $\Delta S$, so that for WGA $\Delta G$ does not differ from the $\Delta G$ obtained for the other plant.
chitin-binding lectins (Fig. 2). In overall conclusion, the ITC data support a model where the binding site of AVR4 is solvent-exposed and AVR4 exclusively interacts with (GlcNAc)₃ repeats.

*Tryptophan fluorescence quenching.* Surface-exposed tryptophans are often involved in protein-carbohydrate interactions forming CH-π interactions (52). We used Trp fluorescence quenching (41) to study the role of the two Trp residues in AVR4 (W63 and W71). The addition of GlcNAc or (GlcNAc)₂ (up to 50 mM) did not result in quenching or a blue shift of the Trp fluorescence (Table 3). However, when longer chito-oligomers were added to AVR4, the Trp fluorescence was significantly quenched, which was accompanied by a small blue shift from 354 nm to 348 nm (Fig. 3). This blue shift indicates that one of the Trp residues becomes more solvent-buried upon complexation, which is most likely W71 as the corresponding residue in Tachycitin is solvent-exposed (9). In contrast, W63 would remain solvent-buried as W63 is involved in a hydrophobic interaction in the core of the protein with Y38 (again based on the structure of Tachycitin) (9). This hydrophobic interaction is strictly conserved based on the high degree of conservation of both aromatic residues in the CBM14 family.

Irrespective of the length of the ligand, Trp fluorescence quenching was always ~50% at full saturation of binding (Fₐ) (Table 3). The fact that we observed no difference in quenching between (GlcNAc)₆ and the smaller ligands suggests that both Trp residues are distant from the protein-protein interface. Second, these data give no further indication for additional interactions between AVR4 and the ligand as the length of the ligand increases. These data corroborate, therefore, that AVR4 exclusively interacts with (GlcNAc)₃ repeats. In contrast, it is known for CBM18 lectins (*e.g.*, Hevein) that additional interactions occur for longer ligands such as (GlcNAc)₄ and (GlcNAc)₅ (33).

The fluorescence quenching experiments were used as a second method (besides ITC) to determine *K₀* values for the interaction between AVR4 and chito-oligomers (Table 3). Estimates for the *K₀* were obtained from both Scatchard (Fig. 3B) and Hill plot analyses (supplementary data). These *K₀* values are in good agreement with the *K₀* values obtained by ITC. The slopes obtained for the corresponding Hill plots approached unity for all chito-oligomers, including (GlcNAc)₆. However, in the case of (GlcNAc)₆ the Scatchard plot was...
clearly curved (Fig. 3B), while smaller chito-oligomers showed a perfect linear regression (as expected for a single binding event). This confirms the presence of two AVR4 binding sites at (GlcNAc)_6. Moreover, the curved Scatchard plot provides a second indication that positive cooperativity contributes to binding of AVR4 to chitin. A re-examination of the ITC data obtained for (GlcNAc)_6 using now a model with two dependent binding sites gave, however, no statistically significant improvement of the data fit. Therefore, we only present one $K_D$ for (GlcNAc)_6, but this $K_D^{ITC}$ is only an apparent value.

Trp fluorescence quenching experiments have also been reported for peritrophin-44, a CBM14 lectin containing four ChBD repeats (8). In this case, Trp fluorescence quenching was ~16% at full saturation for (GlcNAc)_3, whereas peritrophin-44 only contains one Trp residue in one of the four CBM14 repeats. Strikingly, similar experiments with UDA and WGA (both CBM18) resulted in enhanced fluorescence in the presence of the chito-oligomers, i.e. up by 27% for UDA (42) and ~36% for WGA (41). The increased fluorescence for CBM18 as compared to fluorescence quenching for CBM14 indicates that the Trp residues have a different topology in regard to the ligand in the two types of ChBDs.

Analytical size-exclusion chromatography. To exclude that non-specific aggregation of AVR4 occurred under any of the tested conditions, we performed analytical size-exclusion chromatography. AVR4 eluted from the column as a monomer at acidic to neutral pH (Table 4), whereas at pH 8.6 higher order complexes were observed (the estimated pI of AVR4 is 8.6). The formation of higher order complexes (dimer, trimer, etc.) proved to be reversible as the equilibrium shifted to monomer when the pH was decreased again. The monomer of AVR4 eluted at an apparent molecular mass (Mw) of 10.3 kDa at pH 7.0 (Table 4), which is 8% higher than the mass determined by MALDI-TOF mass spectrometry (37). This supports that AVR4 behaves like a globular protein on the column. Subsequently, the column was equilibrated with either 35 mM (GlcNAc)_5 or 25 mM (GlcNAc)_6. Under these conditions, the apparent Mw of AVR4 increased significantly. When the column was equilibrated with (GlcNAc)_5, a protein complex eluted at an apparent Mw corresponding to the Mw of AVR4 plus one (GlcNAc)_5 molecule. However, in the case of (GlcNAc)_6, a protein complex eluted at an apparent Mw that was 60% higher than expected for AVR4 alone. Apparently, we observed an continuous equilibrium between one and two AVR4 molecules that bind to
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(GlcNAc)$_6$. Similar experiments with 35 mM GlcNAc or 35 mM (GlcNac)$_2$ caused no change in the elution time of AVR4 as compared to a buffer-equilibrated column. Attempts with more increased concentrations of the chito-oligomers were found to be impossible as the column pressure increased above the column operation conditions recommended by the manufacturer.

**Mass Spectrometry.** Electrospray ionization mass spectrometry (ESI-MS) is increasingly used for the detection of non-covalent complexes over an extended range of $K_D$ values from $10^{-6}$ to $10^{-14}$ M (54,55). Despite the $K_D$ values in the order of $10^{-2}$-$10^{-6}$ M for the chito-oligomers, we investigated whether we could specifically detect non-covalent complexes between AVR4 and chito-oligomers using ESI-MS. Care was taken to optimize the instrument settings, such as source temperature, cone potential, and the desolvation gas flow rate, which all have been reported to influence the detection of non-covalent complexes (56,57). Instrument settings were optimized using the mass peak of the tetramer of yeast alcohol dehydrogenase (ADH) in 10 mM ammonium acetate at pH 7.0. Subsequently, ESI MS was performed on a sample of 20 μM AVR4 in the presence of 20 μM (GlcNAc)$_6$ (both dissolved in 10 mM ammonium acetate at pH 7) (Fig. 4). Under these conditions, three distinct entities were observed in the mass spectrum, i.e. AVR4, (GlcNAc)$_6$ and a non-covalent complex between AVR4 and (GlcNAc)$_6$ with a 1:1 stoichiometry (with a charge state distribution ranging from 3+ to 8+ with the dominance of the 6+ charge state for AVR4).

A primary concern for the detection of complexes with ESI-MS is the specificity of the observed complex. First of all, the observed 1:1 stoichiometry for the complex in the gas-phase is regarded as a good indicator for specificity (54). A surplus of AVR4 (100 μM) gave a relative reduction in the intensity of the mass peaks corresponding to the 1:1 complex (as expected). On the other hand, a five times surplus of the ligand (100 μM), as tested for (GlcNAc)$_5$ and (GlcNAc)$_6$, gave non-specific aggregates that contained one AVR4 molecule and two, three or even four chito-oligomers. Next, we performed a survey over the pH range 3.5-8.5 (with 10 mM ammonium acetate as buffer). Higher order “hybrid” assemblies were not observed over this pH range, but the mass peak corresponding to the 1:1 complex was best observed at neutral pH. This agreed with the ITC data, which already had shown that the interaction weakens towards acidic pH (Table 2). Finally, when we compared the relative
peak intensities for the different AVR4-GlcNAc complexes (all 5+ charge state), we noted that
the relative peak intensity of the non-covalent complex displayed a positive correlation with
increasing length of the chito-oligomer (Fig. 4B). No complex could be observed for GlcNAc or (GlcNac)₂. Control experiments under identical conditions with the proteins AVR9 of C. fulvum (3.3 kDa) and bovine Ribonuclease A (13.5 kDa) showed that these proteins interacted in a none-specific manner with the chito-oligomers, i.e. the peak intensity of the complex was always less than 5% and it appeared to be independent of the length of the chito-oligomer (Fig. 4B). These two proteins were chosen for three reasons, i.e. they have a relatively small size, a basic pI, and no known affinities for carbohydrates.

Altogether, the MS data appear to reflect our previous \( K_d \) values, as obtained with ITC and fluorescence quenching, and therefore, the detected complexes would in fact be specific complexes. Our initial idea was, however, that we would be able to detect complexes consisting of two AVR4 molecules and one (GlcNAc)₆ molecule. None of our conditions (including variations of the instrument settings) resulted in mass peaks that would correspond with such a complex. A possible explanation could be the gas phase itself, which is known to affect the stability of complexes.

**Nuclear magnetic resonance.** The \(^1\text{H}, ^{13}\text{C}, ^{15}\text{N} \) backbone and side chain resonances of AVR4 were assigned using common NMR protocols. In general, the backbone amides were well-resolved and dispersed in the \(^{15}\text{N}-\text{HSQC} \) spectrum, indicative of a folded protein (37). Of the 72 expected amide cross-peaks in the \(^{15}\text{N}-\text{HSQC} \), the peaks C57, G68 and C72 could not be assigned, presumably due to unfavorable chemical exchange processes. Determination of the solution structure of AVR4 was impaired by the presence of 14 prolines and the overlap of their side chain \(^1\text{H} \) and \(^{13}\text{C} \) resonances. We obtained, however, information about the secondary structure of AVR4. First, the NOE patterns in the \(^{15}\text{N}-\text{NOESY-HSQC} \) spectrum clearly indicated an \( \alpha \)-helix (58) for the residues 14-22. \(^{13}\text{CA} \) chemical shift index (CSI) analysis (59) confirmed the \( \alpha \)-helical character of these residues (Fig. 5A). Notably, these residues form a sequence insertion in AVR4, which is connected to the core of the protein by an additional disulfide bond, C21-C27 (10). Second, the \(^1\text{HA} \) chemical shift of the residues 25-46 and 58-80 follows closely the \(^1\text{HA} \) shift of the corresponding residues in Tachycitin (Fig. 6). These two stretches of residues form the consensus of CBM14 motif (5) and are in the core of
the protein fold of Tachycitin, i.e. the two anti-parallel β-sheets in Tachycitin excluding the loop regions. The 13^CA CSI plot of AVR4 shows also two long stretches with β-sheet propensity, which overlap with the β-sheets in Tachycitin. Correspondingly, we noted strong dαN(i+1) NOE contacts for residues in these two stretches indicative for β-sheet (data not shown). Six Cys residues are conserved in the CBM14 motif and we showed recently that the corresponding Cys residues in AVR4 are indeed involved in a similar disulfide bond pattern as found in Tachycitin (10). Based on these facts, we conclude that the protein fold of AVR4 is similar to the fold of Tachycitin with the exception that we found one additional α-helix that comprises the residues 14-22.

NMR studies of the AVR4-chito-oligomer complex. Residues in AVR4 that interact with chitin were identified from changes in chemical shifts of the 1^HN and 15^N resonances of AVR4 induced by adding chito-oligomers. When aliquots of (GlcNAc)_3 were added to AVR4, a ligand concentration dependent change in chemical shift was noted for a set of 1^HN resonances without substantial line broadening (see supplementary data). Addition of GlcNAc or (GlcNAc)_2 did not induce such changes in the NMR spectrum. The continuous change in chemical shift is characteristic for fast exchange on the NMR time scale (60). Residues D73 and Y74 showed the largest concentration dependent changes in chemical shift upon binding (Fig. 5B) and were used to derive binding constants using the NMR-derivative of the Scatchard plot. For D73 and Y74, respectively, K_D values of 5.3 mM and 5.4 mM (at 298 K) were obtained in close agreement with the ITC and fluorescence quenching data. Using the van ’t Hoff analysis (i.e. $-R \ln(1/K_D)$ versus $1/T$), we were able to estimate $\Delta H$ and $\Delta S$ using the average $K_D$ of D73 and Y74 (supplementary data). This gave a slope that corresponds with $\Delta H^o_{\text{vth}} = -7.78 \text{ Kcal/mol}$ yielding $\Delta S = -15.7 \text{ cal mol}^{-1} \text{ K}^{-1}$. These numbers are in the same order as our ITC data. Nevertheless, these numbers should be regarded as qualitative rather than quantitative since the derivation of thermodynamic parameters from a van ’t Hoff plot assumes that $\Delta H^o$ is independent of the heat capacity $\Delta C_p$ ($=\delta \Delta H / \delta T$). However, a small, but negative contribution of $\Delta C_p$ to $\Delta H^o_{\text{cal}}$ is generally observed for lectin-sugar interactions, but is not included in $\Delta H^o_{\text{vth}}$ (51,61,62).

The titration of AVR4 with (GlcNAc)_6 gave two discrete phenomena for the NMR resonances. At low ligand concentrations line broadening occurred for only a subset of the
\(^1\)HN resonances, while at increased concentrations (> 0.5 mM) almost all \(^1\)HN resonances experienced increased line widths. The set of broadened resonances overlapped largely with the set of resonances that experienced shifts with (GlcNAc)_3. Conclusively, for the initial additions of ligand we observed binding of AVR4 to (GlcNAc)_6, but the exchange between bound- and free AVR4 had changed to the intermediate regime as a consequence of the decreased dissociation constant. As a result, we were not able to estimate the \(K_D\) for (GlcNAc)_6 from our NMR data. The fact that at increased concentrations of (GlcNAc)_6 the entire spectrum was affected by line broadening, points to an increased rotational correlation time \(\tau_c\). As the \(\tau_c\) reflects the apparent size of AVR4, higher order complexes must have been present, most likely complexes with a 2:1 protein-ligand stoichiometry (as seen with the ITC and fluorescence experiments).

**Residues involved in Chitin Binding.** Studies with CBM18 lectins have shown that both the \(^1\)HN and HA resonances can be used as indicators for residues involved in ligand-binding, i.e. the resonances important for binding show shifts exceeding 0.1 ppm at full saturation, while shifts <0.1 ppm are apparently caused by a reorientation of the aromatic side chains influencing other residues as well (30,31,33,34). In addition, large conformational changes were never found for these types of protein-lectin interactions. Performing similar experiments for AVR4, we found that the \(^1\)HN resonances of N64, D65, N66, D73, and Y74 experienced large shifts in the presence of (GlcNAc)_3 (Fig. 5B). These five residues are located near the predicted chitin-binding site of Tachycitin (Fig. 7), i.e. the second \(\beta\)-sheet that shows structural similarities with Hevein (9). Figure 7B shows a ribbon structure of both Tachycitin and Hevein with the residues involved in binding shown in red. First conclusion is that the residues involved in binding in CBM14 and CBM18 have only a limited overlap. N64 and N66 would align with S19 (subsite +1 in Hevein) and W21 (subsite +2) (Fig. 7A). N64 is highly conserved in the CBM14 family, which adds to a role in chitin-binding (90% similarity: Asn, Asp, and less often Ser). However, N66 is not conserved in the CBM14 family. The residues D73 and Y74, which experienced the largest shift upon binding of AVR4 to (GlcNAc)_3, are highly conserved in the CBM14 family (based on the 233 annotated sequences in the Pfam protein database), but not in the CBM18 family (Fig. 7A). Based on the structural similarities between Tachycitin and Hevein it was, previously, proposed that the
chitin-binding site in Tachycitin would overlap with the binding site in Hevein (11). However, Fig. 7B shows that D73 and Y74 are not situated near the putative binding site. Moreover, our data indicate a novel binding site on the folding scaffold shared between Tachycitin and Hevein. This binding site is solvent-exposed as suggested by the ITC data, but perhaps more interestingly these residues appear to form a stretch of residues at the surface of Tachycitin (Fig. 7B). This extended binding site could explain why AVR4 exclusively interacts with (GlcNAc)₃ repeats. In contrast, for Hevein a small binding pocket is seen in the form of of subsite +1, which seems to provide enough contacts to sustain an interaction with GlcNAc alone (Fig. 7B). Remarkably, Hemmi et al. (63) reported recently that the 3D structure of the antifungal peptide scarabaecin from the coconut beetle Orycetes rhinoceros shares also a significant structural similarity with Hevein and Tachycitin. Again, this peptide has no overall sequence similarity with either one of the two ChBDs, but a structural comparison of the region of the putative chitin binding site indicates that binding of chitin by scarabaecin is likely to occur in a similar fashion as in Hevein, i.e. all three residues that form subsite +1 in Hevein are conserved in scarabaecin (i.e. N25, F27, F35) (63), while no residues appear to correspond with D73 and Y74 (Fig 7A). Nevertheless, those results provide additional evidence for the idea of convergent evolution between the CBM14 and CBM18.

The role of W71 in the binding-site of AVR4 is more elusive. W71 would structurally align with W23 in Hevein, which is an important residue in subsite +1 of the CBM18 lectins. W71 appears not to be required for binding, as a large set of CBM14 members do not contain an aromatic residue at this position. Nevertheless, our NMR-titration data showed effectively that the side chain of W71 experiences a shift upon binding of (GlcNAc)₃ to AVR4 (supplementary data). Likewise, low concentrations of (GlcNAc)₆ caused significant line broadening of the side chain of W71 (data not shown). Thus, W71 is affected by the interaction, but is not necessarily required for binding for all CBM14 members. The side chain of W71 becomes solvent buried upon binding, confirming our initial conclusions based on the fluorescence quenching experiments. In addition, the NMR data confirm that W63 is not directly involved in binding, as both the backbone amide and the side chain of W63 experienced only a subtle effect in the presence of (GlcNAc)₃ and low concentrations of (GlcNAc)₆ (supplementary data).
In conclusion. Binding of AVR4 to chitin appears to be limited to an interaction with repeats of three GlcNAc residues. Our experiments did not indicate that additional interactions occur with GlcNAc residues situated outside this repeat. However, we detected positive allosteric interactions between AVR4 molecules that bind to \((\text{GlcNAc})_6\). Positive cooperativity has not been reported for any of the CBM18 lectins. This raises an interesting point. The ‘interlocking’ process of the AVR4 molecules during binding suggests that binding of chitin by AVR4 will be very effective and that it tends to reach saturation of binding (Fig. 8). This would explain why AVR4 effectively protects the cell wall of the fungi *T. viride* and *F. solani* f.sp. *phaseoli* against anti-fungal activity by basic PR-3 chitinases (10,11). Westerink *et al.* (12) reported recently that AVR4 binds to crude fungal components with a binding affinity in the order of nanomolar. This could indicate that the ‘interlocking’ process would be even more effective than suspected on the basis of the here presented data. Thermodynamically, the affinity of AVR4 for the substrate will only be increased in the case of chitin as compared to the chito-oligomers due to the inherent reduced flexibility of chitin. In addition, a further decrease of \(\Delta H\) is expected when additional AVR4 molecules interact with chitin, as this will further reduce the solvent-exposed area of the bound AVR4 molecules. Conclusively, our data support a model where AVR4 effectively protects the chitin in the cell wall from degradation in favour of fungal growth and sustaining cell wall formation at the hyphal tip, potentially even in a hostile environment containing increased concentrations of plant chitinases due to host responses.
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1 The abbreviations used are: Avr, avirulence; CBM, carbohydrate-binding module; ChBD, chitin-binding domain; DP, degree-of-polymerization; ESI, electrospray ionization; $\Delta H^\text{calc}$, calorimetric enthalpy; $\Delta H^\text{vH}$, van ’t Hoff enthalpy; GlcNAc, N-acetyl-D-glucosamine; inv, invertebrate; ITC, isothermal titration calorimetry; MS, mass spectrometry; Mw, molecular mass; NOE, nuclear Overhauser effect; PR, pathogenesis-related; $\tau_c$, rotational correlation time; inv, invertebrates
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### Table 1. Thermodynamics of AVR4 Binding to Chito-oligomers using Isothermal Titration Calorimetry

| DP | $K_A \times 10^{-3}$ | $K_D$ | $\Delta G$ | $\Delta H$ | $T \Delta S$ | $\Delta S$ | $1/n$ |
|----|----------------------|-------|------------|------------|--------------|-----------|------|
| 1  | ND                   | ND    | ND         | ND         | ND           | ND        | ND   |
| 2  |                      |       |            |            |              |           | ND   |
| 3  | 0.16±0.01            | 6.3 ± 0.23 | -3.0±0.02 | -6.5±0.18 | -3.5±0.20    | -11.8±0.67| 1.00 |
| 4  | 0.76±0.02            | 1.3 ± 0.04 | -3.9±0.02 | -6.6±0.11 | -2.7±0.13    | -8.8±0.44 | 1.00 |
| 5  | 3.6±0.34             | 0.28 ± 0.24 | -4.8±0.06 | -7.4±0.13 | -2.6±0.19    | -8.7±0.64 | 1.00 |
| 6  | 160±6.2              | 6.3 x 10^{-3} ± 0.23 | -7.1±0.05 | -10.3±0.12 | -3.2±0.17    | -10.7±0.57| 0.50 |

a DP, degree-of-polymerization of GlcNAc; b n is the number of AVR4 binding sites at the chito-oligomer, fixed value; c ND, no binding detected; All measurements in 50 mM potassium phosphate, 150 mM sodium chloride pH 7.0. The average result of at least three independent experiments is shown.

### Table 2. Thermodynamics of AVR4 Binding to (GlcNAc)$_n$ over a pH range using Isothermal Titration Calorimetry

| pH | $K_A \times 10^{-3}$ | $K_D$ | $\Delta G$ | $\Delta H$ | $T \Delta S$ | $\Delta S$ | $1/n$ |
|----|----------------------|-------|------------|------------|--------------|-----------|------|
| 3.4 | 74±7                | 13.5±1.2 | -6.6±0.06 | -8.8±0.20 | -2.2±0.26    | -7.4±0.87 | 1.05±0.03 |
| 4.4 | 104±8               | 9.6±0.7  | -6.8±0.05 | -10.3±0.04 | -3.5±0.09    | -11.7±0.30| 0.65±0.04 |
| 5.3 | 121±11              | 8.2±0.7  | -6.9±0.05 | -10.4±0.05 | -3.5±0.10    | -11.7±0.34| 0.73±0.03 |
| 5.9 | 149±13              | 6.7±0.5  | -7.1±0.05 | -10.0±0.05 | -2.9±0.10    | -9.7±0.34 | 0.62±0.03 |
| 6.9 | 155±11              | 6.4±0.4  | -7.1±0.05 | -10.3±0.03 | -3.2±0.08    | -10.7±0.27| 0.54±0.04 |
| 7.9 | 136±9               | 7.3±0.4  | -7.0±0.05 | -9.3±0.04 | -2.3±0.09    | -7.7±0.30 | 0.63±0.02 |

a n is the number of AVR4 binding sites at the chito-oligomer, not fixed. b in 50 mM potassium acetate buffer, 150 mM sodium chloride; c in 50 mM potassium phosphate buffer, 150 mM sodium chloride; The average result of at least three independent experiments is shown.
### Table 3 Association constants of AVR4 binding to chito-oligomers determined by tryptophan fluorescence quenching

| Dp | $K_A \times 10^{-3}$ (M⁻¹) | $K_D$ (mM) | $K_A \times 10^{-3}$ (M⁻¹) | $K_D$ (mM) | Maximum % quenching of $F_n$ | $n^f$ |
|----|---------------------------|------------|---------------------------|------------|-------------------------------|------|
| 1  | ND                        | ND         | ND                        | ND         | 50                            | 1    |
| 2  | 0.4                       | 2.9        | 0.2                       | 4.8        |                              |      |
| 3  | 1.1                       | 0.91       | 1.1                       | 0.91       | 52                            | 1    |
| 4  | 4.2                       | 0.24       | 3.5                       | 0.29       | 52                            | 1    |
| 5  |                           |            |                           |            |                               |      |
| 6  | 110                       | 9.1 x 10⁻³ | 6.1 and 42e               | 0.16 and 23 x 10⁻³ | 50                            | 2    |

| a DP, degree-of-polymerization; b intercept x-axis of log ($F-F_0$) / ($F_n-F$) vs. log $L_f$ c Scatchard plot of $v / L_f$ versus $v$; d ND, no binding detected; e apparent $K_A$ values estimated from the upper and lower segment of the Scatchard plot. f number of AVR4 binding sites at the chito-oligomer. Buffer used was 20 mM potassium phosphate, 50 mM sodium chloride pH 7.0.

### Table 4 Analytical size-exclusion chromatography of AVR4 in the presence of chito-oligomers

|          | Apparent Mw (kDa) | Calculated Mw (kDa) | Stoichiometry$^a$ |
|----------|-------------------|---------------------|-------------------|
| AVR4     | 10.3              | 9.551               | 1.08              |
| AVR4+(GlcNAc)$_5$ | 11.7              | 1.10                |
| AVR4+(GlcNAc)$_6$ | 16.7              | 1.55                |
| (GlcNAc)$_5$ | 2.5               | 1.050               |
| (GlcNAc)$_6$ | 2.5               | 1.243               |

| a Apparent MW of the Complex / Calculated MW of the monomer complex $[AVR4]= 25 \mu M$; b 50 \mu l chito-oligomer (25 \mu M) loaded. All experiments are the average of two injections.
FIGURE LEGENDS

FIGURE 1. ITC titration (top) and the corresponding integrated heat (bottom) released by the binding of AVR4 to different chito-oligomers. The concentration of AVR4 in the sample cell was (A) 350 μM, (B) 180 μM, (C) 120 μM, and (D) 100 μM. The concentration of the chito-oligomers in the syringe was (A) 23 mM (GlcNac)₃, (B) 20 mM (GlcNac)₄, (C) 3.2 mM (GlcNac)₅, and (D) 2.0 mM (GlcNac)₆. The data shown was a typical result of at least three independent replicate experiments.

FIGURE 2. Comparison of the thermodynamic parameters obtained for AVR4 with those reported for well-studied plant chitin-binding lectins. (A) The change in free energy (ΔG), (B) enthalpy (ΔH), (C) and entropy (-TΔS) for binding of chito-oligomers with increasing length (degree-of-polymerization) to either AVR4 (●), Hevein (○; Ref. 33), UDA (◇; Ref. 42), or WGA (△; Ref. 47).

FIGURE 3. Tryptophan fluorescence quenching of AVR4 in the presence of (GlcNAc)₅ and (GlcNAc)₆. (A) Emission spectrum of AVR4 (3.6 μM) without ligand (——) and in the presence of 77 μM (GlcNac)₆ (full saturation; ---; and the difference spectrum, • • •). (B) The corresponding Scatchard plots for (GlcNac)₅ (DP=5) and (GlcNac)₆ (DP=6) indicate that only (GlcNac)₅ binds to AVR4 (i.e. linear regression with an intercept at y-axis at v=1.0), while two AVR4 molecules bind to (GlcNac)₆ (DP=6). The second binding events shows positive cooperativity. Binding of smaller ligands (i.e. GlcNAc₃ and (GlcNac)₄) gave also a linear regression like for (GlcNac)₅ (not shown). Lf, concentration of free ligand; v, fraction of occupied binding sites.

FIGURE 4. Detection of the AVR4/chito-oligomer complex using ESI mass spectrometry. (A) ESI mass spectruim of AVR4 in the presence of (GlcNac)₆. The insert shows an enlargement of m/z 1850-2150 Da showing two mass peaks that correspond to the non-complexed AVR4 (Λ₅⁺) and AVR4 bound to (GlcNac)₆ (Λ₁₅⁺). (B) The detected complexes are not an artifact caused by ESI MS as the relative mass peak intensities obtained for the different AVR4-chito-oligomer complexes (black bar) correlate with the
binding affinities (Table 1). No complex is observed for GlcNAc and (GlcNAc)_2. Similar measurements with AVR9 (white bar), and Ribonuclease A (hatched bar) are shown as control experiments. The peak intensities were normalized using the mass peaks that corresponded to free protein with the same charge state. Both protein and ligand were used at a concentration of 20 μM.

FIGURE 5. **NMR data indicate that five residues in the second β-sheet of AVR4 are important for binding of chitin.** (A) The proposed secondary structure of AVR4 based on the chemical shift index (CSI), NOE contacts (not shown) and the homology between AVR4 and Tachycitin (see main text). The disulfide bonds are reported in Ref. 10. (B) Induced changes in the amide (1HN) backbone chemical shifts of AVR4 (1 mM) due to binding of 27.3 mM (GlcNAc)_3 (black bars). Open bars indicate proline residues in the AVR4 sequence for which no data was obtained.

FIGURE 6. **A comparison of the 1HA chemical shifts of AVR4 (●) and Tachycitin (○) indicates that two conserved regions in the proteins are likely to have a similar fold.** The chemical shift data of Tachycitin was retrieved from BioMagResBank databank (Nr 4290). The residue numbers correspond to residues in AVR4. Gaps in the alignment show up as an interruption of the line. Asn30 could not be assigned for AVR4.

FIGURE 7. **Three-dimensional representation of the chitin-binding sites of Tachycitin/AVR4 and Hevein.** (A) Sequence alignment of the residues involved in binding in CBM18 (top) and CBM14 (bottom). The residues in black are important for binding in the shared structural scaffold. The cysteines involved in a structurally conserved disulfide bond are shown in gray. (B) Ribbon model of the 3D structure of CBM14 (i.e. Tachycitin) and CBM18 (i.e., Hevein) (33). The side chains are shown for those residues that are important for chitin binding. The NMR data here reported for AVR4 (Fig. 5) is superimposed on the structure of Tachycitin. The color scheme corresponds with the change of the chemical shift caused by ligand binding. Data for Hevein was taken from ref. 33.
FIGURE 8. Proposed binding model for the chitin-binding domains CBM14 and CBM18 (i.e. AVR4 and Hevein). (I) AVR4 interacts with a ligand with a DP of three or more, while Hevein already interacts with N-acetyl-D-glucosamine. (II) A second binding site becomes only available for AVR4 when the ligand is six sugar residues long, while for Hevein a second binding site is available at (GlcNAc)$_5$. For AVR4 the second binding event is accompanied by positive cooperativity. In the case of Hevein several complexes with a 1:1 and a 1:2 stoichiometry were noted, which does not support positive cooperativity for Hevein.
FIGURE LEGENDS (supplementary figures)

FIGURE S1. Enthalpy-entropy compensation for the interaction between chito-oligomers of different degree-of-polymerization and chitin-binding lectins. AVR4 (●), Hevein (○; taken from (33)), UDA (●; taken from (42)), and WGA (Δ; taken from (47)).

FIGURE S2. Hill plots that correspond to the Trp fluorescent quenching experiments. Degree-of-polymerization (DP): 3 (○), 4 (●), 5 (●), and 6 (Δ).

FIGURE S3. Addition of (GlcNAc)₃ to AVR4 induced shifts in the ¹HN resonances, while the addition of (GlcNAc)₆ affected the line width. In the presence of 27 mM (GlcNAc)₃ (red) a subset of the backbone amides of AVR4 shift (→), while in the presence of 3.5 mM (GlcNAc)₆ the line width is increased (black, the affected peaks are marked by #). The spectrum prior to the addition is shown in green. Lowest depicted contour levels are 30% above signal-to-noise intensity.

FIGURE S4. Van ‘t Hoff plot obtained for ¹HN resonances of Y74 (○) and D73 (○) for the binding of (GlcNAc)₃ to AVR4.

FIGURE S5. Chemical shift changes of the backbone (H N) and side chain (H E₁) of the two Trp residues (W63 and W71) in AVR4. Bars indicate the chemical shift change induced upon binding of (GlcNAc)₃ [W63, ¹⁵N/¹HN=122.8/8.57 ppm, ¹⁵NE₁/¹HE₁=127.09/10.50 ppm; W71 (¹⁵N/¹HN =111.5/6.55, ¹⁵NE₁/¹HE₁=127.7/9.758 ppm].

FIGURE S6. Temperature study on the change of the ¹HN chemical shift of residue D73 in AVR4 as a function of the concentration of (GlcNAc)₃. The sample contained 20 mM sodium acetate, 150 mM NaCl pH 6.0. Temperature was stepwise increased from 293 K to 313 K (K_D=3.8, 5.3, 7.2, 7.9, 9.1 mM, respectively). Protein concentration was 1.0 mM.
Figure 1
Figure 2

A

\[ -\Delta G \]

\[ \text{kcal/mol} \]

Degree-of-polymerization

---

B

\[ -\Delta H \]

\[ \text{kcal/mol} \]

Degree-of-polymerization

---

C

\[ -T\Delta S \]

\[ \text{kcal/mol} \]

Degree-of-polymerization
Figure 3
Figure 4

A

B

Degree of polymerization

Relative intensity

m/z

Relative intensity

m/z

1000 1500 2000 2500 3000 3500

1850 1950 2050 2150 2250

0 5 10 15 20 25 30

0 20 40 60 80 100

1000 1500 2000 2500 3000 3500

1909.6

2156.9

1850 1950 2050 2150 2250

A6 +

A5 +

A4 +

A3 +

AH 6 +

AH 5 +

AH 4 +

AH 5 +

AH 4 +

AH 3 +

H1 +

A5 +

A4 +

A3 +

H1 +
Figure 5

A

CSI $^{15}$CA

B

Proline

Residue number

shift HN resonance

0.3

0.25

0.2

0.15

0.1

0.05

5 15 25 35 45 55 65 75

D64

N65

D66

D73

Y74

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Figure 6
Figure 7

A

| Plant           | Subsite: | +1 | +2 | +1 | +1 |
|-----------------|----------|----|----|----|----|
| Hevein          |          |    |    |    |    |
| Prohevein       |          |    |    |    |    |
| Ac-AMP2         |          |    |    |    |    |
| UDA ChBD 1      |          |    |    |    |    |
| UDA ChBD 2      |          |    |    |    |    |
| Invertebrate    |          |    |    |    |    |
| Scarabaeacin    |          |    |    |    |    |
| AVR4            |          |    |    |    |    |
| Tachycitin      |          |    |    |    |    |
| Peritrophin-44  |          |    |    |    |    |
| Chitinase-Pj     |          |    |    |    |    |

|            | 19 | 21 | 23 | 30 |
|------------|----|----|----|----|

B

Tachycitin

Hevein
### Figure 8

|   |   |   |
|---|---|---|
| **I** | DP=5 | DP=3 |
|     | ![AVR4](image1) | ![Hevein](image2) |
| **II** | DP=6 | DP=5 |
|     | ![AVR4](image1) | ![Hevein](image2) |
Figure S1

Intercept y-axis = 5.0 Kcal/mol
Slope=1.03
r=0.97
Figure S2
Figure S6
Binding of the AVR4 elicitor of Cladosporium fulvum to chitotriose units is facilitated by positive allosteric protein-protein interactions
Harrold A. van den Burg, Christian A. E. M. Spronk, Sjef Boeren, Matthew A. Kennedy, Johannes P. C. Vissers, Geerten W. Vuister, Pierre J. G. M. de Wit and Jacques Vervoort

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