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Workshop on structure-guided investigation of effector function, action and recognition

10th – 12th September 2014, Bucharest, Romania

Institute of Biochemistry

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Workshop on structure-guided investigation of effector function, action and recognition
Programme

WEDNESDAY 10TH OF SEPTEMBER

8:45 – 9:00 **REGISTRATION & COFFEE**

9:00-9:45 **KEY NOTE LECTURE – JIJIE CHAI** *(Tsinghua University, China)*
Structural insight into activation of plant pattern recognition receptors

EFFECTOR AND HOST PROTEINS: IDENTIFICATION, BIOPHYSICS AND BIOCHEMISTRY OF PROTEIN ACTION AND INTERACTIONS

9:45 – 10:15 **LENNART WIRTHMUELLER** *(The Sainsbury Laboratory, United Kingdom)*
A structure-aided approach to understand downy mildew effectors and their targets

10:15 – 10:45 **ADAM ROUND** *(European Molecular Biology Laboratory, France)*
BioSAXS – Current possibilities for low resolution and functional studies under physiological conditions

10:45 – 11:05 **MAGDALENA KRZYMOWSKA** *(Institute of Biochemistry and Biophysics, Poland)*
Cellular dynamics of HOPQ1, a type III secretion effector from PSEUDOMONAS SYRINGAE

11:05 – 11:30 COFFEE BREAK

11:30 – 12:00 **MARTIN CANN** *(Durham University, United Kingdom)*
The potato NLR immune reception RX1 is a pathogen dependent DNA deforming protein

12:00 – 12:30 **FRANK TAKKEN** *(University of Amsterdam, The Netherlands)*
The *Fusarium oxysporum* effector Six8 manipulates plant immunity through association with the transcriptional co-repressors TPL and TPR1.

12:30 – 13:00 **ANDRE PADILLA** *(Centre de Biochimie Structurale, France)*
Biomolecular Nuclear Magnetic Resonance Spectroscopy: Application to M. Oryzae AVR effectors

13:00 – 13:20 **FLORENCE VIGNOLS** *(CNRS-INSB, France)*
How biochemical and biophysical studies demonstrated the reversible zinc binding and redox-dependent flexibility of the viral suppressor/activator of RNA silencing $P1_{rymv}$

13:20 – 14:30 LUNCH (CATERING)

14:30-15:00 **ISABELLE FUDAL** *(INRA, France)*
The avirulence gene AVRLM4-7 of *LEPTOSPHAERIA MACULANS*: linking crystal structure to functional and adaptive characteristics

15:00-15:20 **ALBERTO MACHO** *(The Sainsbury Laboratory, United Kingdom)*
A bacterial tyrosine phosphatase inhibits plant pattern recognition receptor activation

15:20-15:40 **HANNA RÖVENICH** *(Wageningen University, The Netherlands)*
Towards the determination of exact interaction mechanisms between the tomato immune receptor Ve1 and the fungal effector Ave1

15:40 – 16:00 **DIANA ORTIZ** *(INRA, France)*
Structural insights into the specific recognition of the *Magnaporthe oryzae* effector AVR-Pia

16:00 – 16:30 BREAK

16:30 – 20:30 POSTER SESSION COMBINED WITH WORKSHOP DINNER (ACADEMY BUILDING)

THURSDAY 11TH OF SEPTEMBER

9:00-9:30 **KEY NOTE LECTURE – BOSTJAN KOBE** *(University of Queensland, Australia)*
Structure and function of flax rust effectors

STRUCTURE-INFORMED EXPERIMENTS ON HOST PROTEINS AND EFFECTOR PROTEINS INVOLVED IN PLANT-PATHOGEN INTERACTIONS

9:30 – 10:00 **THORSTEN NÜRNBERGER** *(University of Tübingen, Germany)*
The superfamily of necrosis and ethylene-inducing peptide 1 (Nep1) like proteins (NLPs) harbors cytotoxic and non-cytotoxic, virulence-promoting members

10:00 – 10:30 **BART THOMMA** *(Wageningen University, The Netherlands)*
How LysM effectors contribute to fungal pathogenicity

10:30 – 10:50 **MATTHIEU JOOSTEN** *(Wageningen University, The Netherlands)*
The receptor-like protein (RLP) CF-4, present in a constitutive complex with SOBIR1, recruits BAK1 to mount plant immunity

10:50 – 11:10 COFFEE BREAK

11:10 – 11:40 **FRANK MENKE** *(The Sainsbury Laboratory, United Kingdom)*
Effectoromics, where proteomics joins up with cell biology

11:40 – 12:10 **MARK BANFIELD** *(The John Innes Centre, United Kingdom)*
"Seeing is believing": Structural insights into host cell manipulation by filamentous plant pathogen effectors

12:10 – 12:30 **BARBARA GERIC STARE** *(Agricultural Institute of Slovenia, Slovenia)*
3D model structures of effector expansin-like protein (EXPB2) from plant parasitic nematode *Globodera rostochiensis*

12:30 – 12:50 **ADINA MILAC** *(Institute of Biochemistry of the Romanian Academy, Romania)*
Symmetry-driven computational methods generate refined structural models of plant transmembrane proteins

12:50 – 14:00 LUNCH (CATERING)

14:00 – 14:30 **ANDREI PETRESCU** *(Institute Of Biochemistry Of The Romanian Academy, Romania)*
Modelling Structures in the Twilight Zone and Beyond.
Lessons from Resistance and Effector Gene Families.

14:30 – 15:00 **TINA JORDAN** *(University of Tübingen, Germany)*
3D modeling combined with diversity analysis reveals distinct AVR recognition sites and different evolutionary pathways in wheat *Pm3* genes from wild and domesticated species

15:00 – 15:20 **ASKA GOVERSE** *(Wageningen University, The Netherlands)*
Structural determinants involved in effector recognition by the nematode immune receptor GPA2

15:20 – 15:40 **JOHN STEELE** *(The John Innes Centre, United Kingdom)*
Structural and biochemical characterization of an NB-ARC domain from a plant disease resistant protein

16:00 – 18:00 POSTER SESSION

19:00 – CITY TOUR (TBC)

FRIDAY 12TH OF SEPTEMBER

RESOLVING PROTEIN-PROTEIN AND INTERDOMANIN INTERACTIONS BETWEEN HOST PROTEINS AND/OR EFFECTORS TO UNDERSTAND THEIR FUNCTION

9:00 – 9:30 **LIAM MCGUFFIN** *(University of Reading, United Kingdom)*
Structural Bioinformatics of *Blumeria graminis f. sp. hordei* effector candidates

9:30 – 10:00 **PIETRO SPANU** *(Imperial College London, United Kingdom)*
The cereal powdery mildew RNase-like effectors

10:00 – 10:30 **JANE PARKER** *(Max Planck Institute for Plant Breeding Research, Cologne, Germany)*
Structure-function analysis of TNL-EDSI resistance signalling in Arabidopsis

10:30 – 10:50 **THOMAS KROJ** *(INRA Montpellier, France)*
The NB-LRR immune receptors RGA4 and RGA5 interact functionally and physically to confer blast disease resistance

10:50 – 11:10 COFFEE BREAK

11:10 – 11:40 **TAKAKI MAEKAWA** *(Max Planck Institute for Plant Breeding Research, Cologne, Germany)*
Conservation of a coiled-coil type NLR-triggered immunity across plant lineages and dissection of the bifurcated signaling mechanism

11:40 – 12:00 **ERIK SLOOTWEG** *(Wageningen University, The Netherlands)*
Deconstructing a molecular switch; structure-function analysis of the CC-NB-LRR proteins RX1 and GPA2

12:00 – 12:20 **YAN MA** *(The Sainsbury Laboratory, United Kingdom)*
Unravelling the mystery of the AVRRPS4 recognition by arabidopis resistance gene pair RRS1/RPS4

12:20-14:00 LUNCH (CATERING)

14:00 VISIT TO THE INSTITUTE (OPTIONAL)

END OF THE WORKSHOP

ABSTRACTS
ORAL PRESENTATIONS & POSTERS

Structural insight into activation of plant pattern recognition receptors

Jijie Chai

School of Life Sciences, Tsinghua University, Beijing, China

Plant receptor kinases (RKs) are a large family of single transmembrane proteins that play critical roles in many biological processes including development, growth and immunity. RKs mediate plant immunity by acting as pattern recognition receptors (PRRs) to recognize pathogen-derived signature components (termed pathogen-associated patterns, PAMPs) or host-derived danger signals (danger-associated molecular patterns, DAMPs). Recognition of PAMPs/DAMPs by their cognate PRRs initiates activation of RLKs. We recently solved crystal structures of the extracellular domains derived from a few RKs (FLS2, CERK1 and PEPR1) in complex with their respective ligands. The structures define the molecular mechanisms by which the three PRRs recognize their specific ligands. More importantly, a general mechanism underlying ligand-induced activation of the RKs is formulated. Applicability of the mechanism to activation of other RKs is demonstrated by an example in which a co-receptor was identified for a known RK. I will also discuss how RKs are manipulated by bacterial pathogens.

A structure-aided approach to understand downy mildew effectors and their targets

Lennart Wirthmueller[1,3,#], Shuta Asai[1], Marie-Cècile Caillaud[1], Ghanasyam Rallapalli[1], Georgina Fabro[1], Michael Wrzaczek[2], Jaakko Kangasjärvi[2], Jan Sklenar[1], Frank Menke[1], Mark J. Banfield[3], Jonathan D. G. Jones[1]

[1]*The Sainsbury Laboratory, Norwich Research Park, Norwich, NR4 7UH, UK*
[2]*University of Helsinki, Division of Plant Biology, FIN-00014, Finland*
[3]*Dept. of Biological Chemistry, John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, UK*

Protein-protein interaction screens continue to identify host proteins that bind to effectors from filamentous plant pathogens. However, the molecular consequences of many effector/target interactions remain unknown and not all plant proteins that bind to effectors are virulence targets. Therefore the next critical step is to understand the molecular functions of select effectors and how they manipulate host proteins. We use a combination of *in planta* virulence assays, x-ray crystallography and protein biochemistry to elucidate structures, molecular functions and host targets of effectors from the Arabidopsis downy mildew pathogen *Hyaloperonospora arabidopsidis* (*Hpa*). We prioritize *Hpa* effectors that localize to the host cell nucleus and suppress defence gene expression. Several nuclear *Hpa* effectors interact with core components of the transcriptional machinery, suggesting that they directly manipulate defence gene transcription. I will present our on-going work aiming to understand how *Hpa* effectors perturb transcription of host defence genes at the molecular level.

BioSAXS – Current possibilities for low resolution and functional studies under physiological conditions

Adam Round,[a,b]

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[b]*Unit for Virus Host-Cell Interactions, Univ. Grenoble Alpes-EMBL-CNRS, France,*

Small-angle X-ray scattering (SAXS) of macromolecules in solution is in increasing demand by an ever more diverse research community, both academic and industrial. Experiments to investigate the conformational state under physiological and functional conditions are greatly improved with the advent of dedicated experimental facilities such as the ESRF BioSAXS beamline, BM29 [1], Fully-automated data collection is provided by the sample changer developed at EMBL Grenoble as part of a trilateral collaboration with ESRF and EMBL Hamburg. Additionally, online size exclusion chromatography is integrated immediately prior to SAXS data collection to enable separation of species from dynamic mixtures. Additional biophysical characterisation is also available using DLS, RALS, UV and refractive index measurements which provide independent measures of the MM and the hydrodynamic radius of the purified species. User friendly operation is a priority at all stages of the experiment from preparation to data analysis. The extension of the ISPyB database [2] provides user oriented feedback (via an intuitive web interface) on sample requirements during preparation, sample quality during data acquisition and data quality during analysis. Such automated systems with integrated online feedback are enabling more sensitive and elaborate BioSAXS experiments to be undertaken with confidence even by those new to the technique.

Cellular dynamics of HOPQ1, a type III secretion effector from *PSEUDOMONAS SYRINGAE*

Fabian Giska[1], Marcin Piechocki[1], Michal Taube[2], Malgorzata Lichocka[1], Rafal Hoser[1], Maciej Kozak[2], Jacek Hennig[1] & **Magdalena Krzymowska[1]**

[1]*Laboratory of Plant Pathogenesis, Institute of Biochemistry and Biophysics, PAS, Warsaw, Poland;*
[2]*Department of Macromolecular Physics, Adam Mickiewicz University, Poznan, Poland*

HopQ1 (for Hrp outer protein Q), a TTSS effector secreted by *Pseudomonas syringae* pv. *phaseolicola*, promotes the development of halo blight in bean. We found that after expression *in planta*, HopQ1 co-purified with host 14-3-3 proteins. The physical interaction between HopQ1 and 14-3-3a was confirmed using FRET-FLIM and thermophoresis techniques. Mass spectrometric analyses detected specific phosphorylation of the canonical 14-3-3 binding site present in HopQ1. Substitution within this motif (S51A) abrogated the interaction and affected HopQ1 properties. Size exclusion chromatography coupled to MALS (Multi-Angle Light Scattering) revealed that HopQ1 formed *in vitro* monomers, dimers and trimers. Mutations in either one of both HopQ1 cysteines abolished oligomerization. Consistently, treatment with reducing agent dithiothreitol (DTT) converted HopQ1 oligomers into monomers indicating that HopQ1 oligomeric state depends on disulfide bridge formations. However, after calcium depletion HopQ1 monomers were reversibly converted to dimers. These results were corroborated by SAXS (Small Angle X-ray Scattering) analyses. Importantly, mutations in the predicted calcium binding motif of HopQ1 (HopQ1-D107A_D108A) produced HopQ1 dimers *in vitro* and *in planta*.
While wild-type HopQ1 localized primarily to the cytoplasm, HopQ1-S51A accumulated in the nucleus, HopQ1-D107A_D108A was distributed between these two cellular compartments. Therefore, we propose a model in which cellular dynamics of HopQ1 is determined by phosphorylation-dependent interaction with 14-3-3s and calcium-dependent oligomerization of the effector.

The potato NLR immune receptor RX1 is a pathogen dependent DNA deforming protein

Martin Cann

Department of Biological Sciences, Durham University, United Kingdom

Plant NLR proteins confer pathogen recognition specificity upon the immune system and several act in the nucleus following activation. However, conserved nuclear targets for NLRs that support their role in immune signalling are unknown. We observed a structural homology between the Rx1 NLR of potato and DNA replication origin-binding Cdc6/Orc1 proteins. Consistent with this, Rx1 binds DNA *in vitro* and *in vivo*. Rx1 induces ATP-dependent DNA bending and melts DNA dependent upon an intact P-loop motif. Facets of this biochemistry are conserved in I-2 of tomato indicating a mechanism generic to plant immunity. DNA binding is further conserved among NLRs from diverse monocot and dicot species. The cognate Rx1 elicitor, the coat protein of potato virus X, triggers Rx1 DNA binding *in vivo*. The data demonstrates DNA distortion as central to Rx1 immune signalling and generally defines DNA as the first conserved molecular target for plant NLRs upon immune activation.

The *Fusarium oxysporum* effector Six8 manipulates plant immunity through association with the transcriptional co-repressors TPL and TPR1.

Fleur Gawehns, Mara de Sain, Hanna Richter, Petra Houterman, Guido van den Ackervecken, Martijn Rep, Harrold van den Burg and **Frank Takken**

Swammerdam Institute for Life Sciences, University of Amsterdam, Netherlands

Fusarium oxysporum f. sp. *lycopersici* (Fol) is the causal agent of tomato wilt disease. During infection of the host the fungus secretes many small proteins in the xylem sap. We are studying the role of these proposed effector proteins during infection. For Six8 we found a potential host target. Using pull-down assays a member of the TOPLESS (TPL) family was identified. The Six8-TPL interaction was confirmed in yeast two-hybrid assays. TPLs belong to a group of co-repressors interacting with transcription factors that are involved in plant development, hormone signalling, and in the "*SNC1*-mediated" defence mechanism[1]. SNC1 is a NB-LRR plant resistance protein, providing a direct link between Six8 and host defence signalling. Transgenic Arabidopsis plants expressing *SIX8* exhibit a temperature-dependent dwarf phenotype and induction of defence gene (PR1 and PR2) expression, suggesting a direct link to *SNC1*-mediated defences.

Arabidopsis T-DNA insertion lines lacking either *SNC1* or components of the SNC1- mediated defence pathway (i.e. *tpl, tpr1, tpr3, eds1, pad4*, and NahG) have been transformed with SIX8 to identify the pathways affected by Six8. In addition, bioassays on *SIX8*-containing Arabidopsis plants using *Pseudomonas syringae* and *Hyaloperonospora parasitica* have been performed to assess potential perturbation of other NB-LRR resistance proteins belonging to either the CC- or TIR-NB-LRR family. Together, the obtained data make TPL a likely candidate for a genuine Six8 effector target. A possible mechanism of how Six8 triggers SNC1-mediated immune signalling will be presented.

Biomolecular Nuclear Magnetic Resonance Spectroscopy: Application to M. Oryzae AVR effectors

André Padilla

Centre de Biochimie Structurale Montpellier, France

Nuclear magnetic resonance spectroscopy (NMR) has become a preeminent technique for determining the structure of biological molecules in the solution phase. NMR has several unique advantages, as it is non-destructive, allowing samples to be regenerated and versatile, being used to determine concentrations, dynamics, folding, interactions and structures of a wide variety of molecules. NMR experiments are typically performed in liquids, which may resemble the cellular environment, allowing biologically relevant states to be observed. In contrast to most other methods NMR spectroscopy deals with chemical properties by studying individual nuclei. This is the power but sometimes also the weakness of the methods. It is the purpose of this presentation to introduce descriptions and applications of the methods of NMR spectroscopy most commonly applied in studies of biological macromolecules, in particular proteins. The structures of AVR effectors will be presented showing the application of some of the NMR methods.

How biochemical and biophysical studies demonstrated the reversible zinc binding and redox-dependent flexibility of the viral suppressor/activator of RNA silencing $P1_{rymv}$

V. Poignavent[(1)], F-X. Gillet[(1)], S. Petiot-Becard[(3)], F. Delalande[(3)], F. Hoh[(2)], Y. Yang[(2)], G. Terral, D. Cattoni[(2)], E. Lacombe, Jh. Kim, C. Brugidou[(1)], S. Sanglier-Cianferani[(3)], H. Demene[(2)], **F. Vignols**[(1)]

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[(2)] Centre de Biochimie Structurale, UMR CNRS-5048, INSERM-U554, & Université de Montpellier, Montpellier-France;
[(3)] Laboratoire de Spectrométrie de Masse BioOrganique, Institut Hubert Curien, UMR CNRS-Université de Strasbourg 7178, Strasbourg-France.

Viral suppressors of RNA interference (VSRs) are remarkable multifunctional proteins that play important roles in the viral cycle and in the host cells. In particular, VSRs are essential components of viral machineries for counteracting host innate immune responses by targeting different components of the host gene antiviral silencing pathways [(1,2)]. However, the mechanisms by which VSRs target components of the host cell and ensure several functions remain poorly understood. We have analyzed the biochemical and biophysical properties of $P1_{RYMV}$, a model cysteine-rich VSR encoded by the *rice yellow mottle* virus. $P1_{RYMV}$ is a multifunctional protein, acting as a dual suppressor/activator of RNAi[(3)], capable of RNA- and protein-binding activities, and required for viral replication. Using SDS-PAGE redox shift assays coupled to native LC-MS, we demonstrated that $P1_{RYMV}$ as a monomer binds two zinc atoms in a reducing environment, while $P1_{RYMV}$ oxidation leads to disulfide bond formation along with zinc release and oligomerization[(4)]. These redox-dependent conformational changes were confirmed using MALDI-Tof, circular dichroism and fluorescence anisotropy. Strikingly, zinc release from oxidized $P1_{RYMV}$ is reversible upon reduction, a redox-dependent switch never reported for cysteine-rich VSRs to date. Analysis of truncated $P1_{RYMV}$ isomers using X-ray crystallography allowed us to delineate zinc binding domains and to identify structural determinants for redox flexibility. Interestingly, oxidized oligomeric forms of $P1_{RYMV}$ evolving throughout infection by RYMV were detected in rice. To establish a relationship between $P1_{RYMV}$ redox-dependent flexibility and its functions (cellular movement, RNA binding, viral replication), we currently analyze a collection of $P1_{RYMV}$ structural mutants in planta.

The avirulence gene AVRLM4-7 of *LEPTOSPHAERIA MACULANS*: linking crystal structure to functional and adaptive characteristics

Karine Blondeau[1], Françoise Blaise[2], Marc Graille[1], Shiv D. Kale[3], Juliette Linglin[2], Bénédicte Ollivier[2], Audrey Labarde[1], Noureddine Lazar[1], Dannielle H.Y. Choi[3], Brett M. Tyler[4], Thierry Rouxel[2], Herman van Tilbeurgh[1] and **Isabelle Fudal[2]**

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The avirulence gene *AvrLm4-7* of *Leptosphaeria maculans*, the causal agent of stem canker of oilseed rape, encodes a small cysteine-rich secreted protein. *AvrLm4-7* confers a dual specificity of recognition by two resistance genes (*Rlm4* and *Rlm7*) and is strongly involved in fungal fitness. In order to elucidate the biological function of AvrLm4-7 and understand the specificity of recognition by *Rlm4* and *Rlm7*, the AvrLm4-7 protein was produced in the heterologous system *Pichia pastoris* and its crystal structure determined. It revealed the presence of four disulfide bridges and no close structural analogs could be identified. Translocation assays in oilseed rape roots and transient expression in tobacco leaves suggested that AvrLm4-7 is translocated into plant cells in the absence of the pathogen and targeted to the cytoplasm and nucleus. Translocation of AvrLm4-7 into plant cells was found to necessitate the presence of a RAWG motif located in a loop as part of a positively charged region and also the presence of a well-conserved stretch of amino acids (R/N)(Y/F)(R/S)E(F/W) in the C-terminal part of the protein. Using bombardment assays, we determined that recognition by Rlm4 and Rlm7 occurred into the cytoplasm of plant cells. Loss of recognition of AvrLm4-7 by Rlm4 is due to mutation of a single glycine to arginine residue located in a loop of the protein. Loss of recognition by Rlm7 is governed by more complex mutation patterns, some of which are associated with gene loss while others probably result in drastic modifications of the protein structure. Three point mutations on an a4A7 allele targeting residues either located in the (R/N)(Y/F)(R/S)E(F/W) conserved motif or close to the glycine involved in Rlm4-mediated recognition resulted in a loss of Rlm7-mediated recognition.

A bacterial tyrosine phosphatase inhibits plant pattern recognition receptor activation

Alberto P. Macho[1#], Benjamin Schwessinger[1§#], Vardis Ntoukakis[1¶#], Alexandre Brutus[2], Cécile Segonzac[1†], Sonali Roy[1‡], Yasuhiro Kadota[1], Man-Ho Oh[4,5], Jan Sklenar[1], Paul Derbyshire[1], Rosa Lozano-Durán[1], Frederikke Gro Malinovsky[1∞], Jacqueline Monaghan[1], Frank L. Menke[1], Steven C. Huber[4], Sheng Yang He[2,3] and Cyril Zipfel[1]

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[#] *These authors contributed equally to this work*

The first active layer of plant innate immunity relies on the perception of pathogen-associated molecular patterns (PAMPs) by surface-localised pattern-recognition receptors (PRRs). Many known plant PRRs are receptor kinases, which are annotated as serine/threonine kinases. However, the exact phosphorylation events that lead to receptor activation and initiation of PAMP-triggered immune signaling remain unknown. Here, we report that the Arabidopsis receptor kinase EFR, which perceives bacterial EF-Tu (or the derived peptide elf18), is phosphorylated on tyrosine residues and that this modification is critical for EFR activation upon ligand binding. We identify a single tyrosine residue required for EFR activation, downstream responses and immunity to the phytopathogenic bacterium *Pseudomonas syringae*. Pathogenic bacteria employ type-III secreted effectors to suppress PAMP-triggered immunity and cause disease. The effector HopAO1 is important for the virulence of *Pseudomonas syringae* and possesses tyrosine phosphatase catalytic activity, but its plant targets were still unknown. We found that HopAO1 directly interacts with EFR and FLS2, and blocks elf18-induced EFR activation and immune responses, revealing that HopOA1 targets tyrosine phosphorylation of plant PRR to block their activation. Our results shed light on a novel regulatory mechanism controlling plant immune signaling and highlight a host-pathogen battle to take control of PRR tyrosine phosphorylation that is critical for anti-bacterial immunity.

Towards the determination of exact interaction mechanisms between the tomato immune receptor Ve1 and the fungal effector Ave1

Hanna Rovenich, Zhao Zhang, Yin Song, Bart P.H.J. Thomma

Laboratory of Phytopathology, Wageningen University, The Netherlands

Recognition of pathogen effectors, small secreted molecules that facilitate host colonization, represents the basis for plant resistance against well-adapted pathogens. In tomato, the *Ve1* gene confers resistance against race 1 strains of the vascular wilt pathogen *V. dahliae*. *Ve1* encodes a surface-localized leucine-rich repeat (LRR) receptor-like protein (RLP), which recognizes the recently identified secreted *V. dahliae* effector Ave1. Immunopurification of affinity-tagged Ave1 transiently co-expressed with HA-tagged Ve1 in tobacco showed that the receptor protein co-purifies with Ave1, indicating an interaction between the pathogen effector and the immune receptor. A mutational screen of extracellular solvent-exposed residues across the LRR domains within the C1 region of Ve1 showed that stable Ve1 mutant alleles of the two consecutive LRR regions LRR3-LRR8 and LRR20-LRR23 were compromised in their HR-inducing activity when co-expressed with Ave1. Accordingly, when challenged with race1 *V. dahliae*, transgenic *Arabidopsis* plants carrying the non-functional *Ve1* alleles displayed symptoms similar to inoculated non-transgenic controls. These results suggest that Ve1 functionality is determined by two distinct clusters of LRR domains that may be involved Ave1 perception. Despite the fact that further mutational analyses in combination with biochemical methodologies can be employed to further elucidate the interaction between the receptor and the effector proteins, structural information will greatly enhance our ability to improve those strategies.

Structural insights into the specific recognition of the *Magnaporthe oryzae* effector AVR-Pia

Diana Ortiz[1], Karine de Guillen[2], Véronique Chalvon[1], Andre Padilla[2], Thomas Kroj[1]

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Plant immunity relies on direct or indirect recognition of pathogen effectors by plant resistance (R) proteins. This recognition activates disease-resistance signaling pathways leading to the inhibition of pathogen growth and the induction of a localized programmed cell death called the hypersensitive response (HR). To gain a better understanding of the molecular mechanisms governing effector recognition in plants, we study the translocated effectors Avr-Pia and AVR1-CO39 from the rice blast fungus *Magnaporthe oryzae* and their recognition by the rice nucleotide-binding and leucine-rich repeat domain (NB-LRR) proteins RGA4 and RGA5. Yeast two-hybrid and co-immunoprecipitation experiments revealed physical interaction of AVR-Pia and AVR1-CO39 to an unconventional domain in RGA5 related to the yeast copper chaperone ATX1 (RATX1 domain). This suggests that Avr recognition occurs by direct binding and that RGA5 acts as an effector receptor. This hypothesis is supported by the finding that a polymorphic site, present in a natural allele of AVR-Pia, abolishes Avr activity and RGA5-binding. A three dimensional structure model of AVR-Pia was generated using nuclear magnetic resonance spectroscopy (NMR) and the polymorphic site was mapped on the model identifying a region which may be important for RGA5-binding and resistance induction. Point mutations were introduced in this region and other sites of AVR-Pia by site-directed mutagenesis and analyzed by yeast two hybrid and transient expression in *Nicothiana benthamiana.* By this, six additional amino acids, crucial for interaction with RGA5 and activation of defense responses were identified. They are located either in the previously identified region or on other surfaces of the molecule suggesting that different parts of AVR-Pia are engaged in physical interaction with RGA5.

Structure and function of flax rust effectors

Bostjan Kobe[1], Thomas Ve[1], Xiaoxiao Zhang[1], Li Wan[1], Maud Bernoux[2], Jeffrey G Ellis[2], Peter N Dodds[2], Simon Williams[1]

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[2]CSIRO Plant Industry, Canberra, Australia

Effector-triggered plant immunity (ETI) is initiated through the recognition of a pathogen effector (avirulence) protein by a plant resistance (R) protein, leading to the activation of plant defenses and a localized cell death response. The effectors usually have roles in virulence and are structurally diverse. We have used the fungal pathogen flax rust interaction with flax as a model system to characterize the structures and functions of fungal effector proteins and the molecular basis of ETI. A number of flax rust effector-flax R proteins pairs have been identified. Many flax rust effectors show no sequence similarities to proteins of known function, and their transport mechanism into the plant cell is unknown. We determined the crystal structures of three flax rust effectors, AvrL567 (1), AvrM (2) and AvrP (unpublished). The three proteins reveal structures unrelated to each other and with little structural similarity to other proteins with known structure. We showed that AvrL567 (3) and AvrM (4) interact directly with the corresponding R proteins L6 and M, respectively. Our work reveals insights into the virulence functions of flax rust effector proteins, into their interactions with R proteins, and into their mechanisms of transport into plant cells.

The superfamily of necrosis and ethylene-inducing peptide 1 (Nep1) like proteins (NLPs) harbors cytotoxic and non-cytotoxic, virulence-promoting members

T Nürnberger*[1], I Albert[1], H Böhm[1], G Anderluh[2], Vesna Hodnik[2], Marjetka Podobnik[2], Tea Lenarcic[2], C Oecking[1], Steve Whisson[3], Bart Thomma[4], Guido van den Ackerveken[5].

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Members of the superfamily of necrosis and ethylene-inducing peptide 1 (Nep1) like proteins (NLPs) are widely found in bacteria, fungi and oomycetes. A subset of these proteins causes leaf necrosis on dicot, but not on monocot plants. NLP cytotoxicity was shown to be crucial for microbial virulence and a necrotrophic lifestyle of the producing microbe. X-ray crystallography-based analyses of two microbial NLPs revealed substantial fold conservation of these proteins with cytolytic toxins produced by marine organisms (actinoporins). Actinoporins bind to animal host sphingomyelin prior to membrane pore formation and cytolysis. While plants do not produce sphingomyelins, we show that the target site for NLP toxins is most likely of lipid nature and resides in the outer layer of the plasma membrane of dicot plants. Membrane binding and phytotoxicity requires the presence of a coordinately bound calcium cation within an electrophilic cavity on NLPs, suggesting that the plant docking site is negatively charged. In binding assays, NLPs preferentially bind to phosphorylated phosphatidylinositols (PIP), and incubation of NLPs with PIPs inhibits the cytotoxic activities of these proteins. Thus, NLP susceptibility of plant membranes is determined by its interaction with yet unknown PIP-like lipid structures that might define a biologically significant difference in the composition of plasma membranes from monocot and dicot plants. Recently, the production by various oomycetes and fungi of non-cytotoxic members of the NLP superfamily was shown and the 3D-structure of a non-cytolytic NLP was solved. The possible mode of action of these proteins and their biological activity will be discussed.

How LysM effectors contribute to fungal pathogenicity

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While multicellular organisms activate immunity upon recognition of pathogen-associated molecular patterns (PAMPs), successful microbial pathogens deliver effector proteins to deregulate PAMP-triggered host immunity and to establish infection. Chitin is the major component of fungal cell walls, and chitin oligosaccharides act as PAMPs in plant cells that are perceived by LysM domain-containing cell surface receptors. We have previously show that the LysM domain-containing effector protein Ecp6 of the fungal plant pathogen *Cladosporium fulvum* mediates virulence through sequestration of chitin oligosaccharides that would otherwise activate chitin-triggered immunity. However, the mechanism by which Ecp6 can compete for chitin binding with host immune receptors remained unclear. Based on structural analysis of Ecp6 we reveal a novel mechanism for chitin binding by intrachain LysM dimerization, leading to a chitin binding groove that is deeply buried in the Ecp6 effector protein. Isothermal titration calorimetry experiments reveal that the ligand-induced composite binding site mediates chitin binding with ultra-high (pM) affinity. Intriguingly, a third, singular, LysM domain of Ecp6 binds chitin with low micromolar affinity but can nevertheless still perturb chitin-triggered immunity. Conceivably, the perturbation by LysM2 is not established through chitin sequestration.

The receptor-like protein (RLP) CF-4, present in a constitutive complex with SOBIR1, recruits BAK1 to mount plant immunity

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Plants perceive microbial patterns by cell surface receptors that are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs), usually containing extracellular leucine-rich repeats (LRRs). However, RLPs lack an intracellular kinase domain for activation of downstream signaling upon ligand perception. Recently, we showed that tomato (*Solanum lycopersicum*, *Sl*) Cf-4, an LRR-RLP mediating resistance to *Avr4*-expressing strains of the fungal pathogen *Cladosporium fulvum*, constitutively interacts with the RLK SOBIR1. SOBIR1 is unrelated to the RLK BRI1-Associated Kinase (BAK1), which is an essential regulatory LRR-RLK for pattern recognition receptors (PRRs), such as the LRR-RLK FLS2. In collaboration with the group of Dr. Silke Robatzek (TSL, Norwich, UK), we found that the Cf-4/SOBIR1 complex localizes at the plasma membrane, from where it is mobilised to ARA7/ARA6-positive late endosomes upon activation with Avr4. Similar trafficking takes place for FLS2 upon its activation by flg22, a process that is BAK1-dependent. Strikingly, we discovered that the Cf-4/SOBIR1 complex also recruits BAK1 upon its activation by Avr4, an interaction that is required for both Avr4-triggered HR and Cf-4/SOBIR1 endocytosis. Thus, Cf-4 immune signalling is initiated by the formation of at least a tripartite receptor complex involving Cf-4, SOBIR1 and BAK1, showing that RLPs, in a complex with SOBIR1, function like *bona fide* PRRs. Recently, the structure of the flg22/FLS2/BAK1 complex was resolved by the group of Dr. Jijie Chai (Tsinghua University, China) and we are now collaborating with Dr. Chai with the aim to decipher the structure of the constitutive Cf-4/SOBIR1 complex. Furthermore, we want to determine whether Cf-4 indeed acts as a receptor by directly binding the Avr4 protein and we aim to identify what changes occur in the Cf-4/SOBIR1 complex that allow it to recruit BAK1 upon its activation by Avr4.

Effectoromics, where proteomics joins up with cell biology

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The availability of genome sequences has revolutionized biology and in particular proteomics. In most cases protein function can be inferred from sequence data. However evolutionary pressure from the host over millions of years has shaped microbial effectors into small proteins with few functionally recognizable domains. This makes it difficult to identify effectors based on genome sequences or proteomics data alone. With the use of bioinformatics on genomics and proteomics data sets and filtering for specific characteristics it has been possible to identify putative microbial effectors. At the Sainsbury Laboratory (TSL) several different hybrid approaches have been used to identify effectors, their targets and effector function. Follow up studies on these putative effector required cell biological approaches as well as rounds of immunoprecipitation coupled with LC-MS/MS, to verify localization and identify interacting proteins respectively. In addition to being a tool of discovery, proteomics also has a role to play in delineating function and host targets of putative microbial effectors. Targeted quantitative proteomic approaches such as selected reaction monitoring (SRM) are ideally suited to analyze effector function. We have successfully used shotgun proteomics followed by SRM to characterize effector function. I'll discuss these hybrid approaches by showing some examples from the TSL groups we collaborate with.

“Seeing is believing”: Structural insights into host cell manipulation by filamentous plant pathogen effectors

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A mechanistic understanding of how plant pathogens re-program their hosts to enable colonisation, and how plants respond to attack, may provide novel genetic or chemical opportunities to interfere with the development of disease. Filamentous plant pathogens, fungi and oomycetes, secrete effector proteins both outside of and into plant cells to suppress host defences and manipulate other cellular processes to the benefit of the pathogen. We have taken a structure-led approach to the investigation of filamentous plant pathogen effector function. The structures of multiple RXLR-type effectors from *Phytophthora* have revealed that these proteins are frequently built from a conserved, but versatile protein fold. This fold can adopt monomeric and different oligomeric states, in addition to being repeated within individual coding sequences. We hypothesised that this fold is highly suited to effector function by underpinning functional diversity (the ability to develop new adaptive functions of relevance to virulence, or escaping detection by the plant immune system). However, in isolation, the functional insights delivered by structures of effectors can be somewhat limited. Recently, we have extended our studies to exploit biophysical, structural and *in planta* approaches to investigate the interactions of filamentous plant pathogen effectors with their host cell targets. In particular, structural studies of filamentous plant pathogen effectors in complex with their targets are lacking. Here, I will present our latest work on structure/function studies of effector/target complexes.

3D model structures of effector expansin-like protein (EXPB2) from plant parasitic nematode *Globodera rostochiensis*

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Infective larvae (J2) of potato cyst nematodes secrete effector proteins which facilitate successful infection of the host plants. Expansins are one group of the effectors, which help in degradation of plant cell wall by loosening non-covalent interactions between components of the plant cell wall. Molecular variability of *exp*B2 gene was shown in diverse populations of the *Globodera rostochiensis* (Geric Stare et al., 2012, Physiol Mol Plant P). The *in planta* expression and activity of GR-EXPB2 type protein (GenBank acc. no.: GCAC84564.1) has been demonstrated in *Nicotiana benthamiana, Solanum lycopersicum* and *S. tuberosum* leaves. However, *in planta* functional analysis of the GR-EXPB2 variant proteins induced no visible symptoms (Dr. Shawkat Ali, personal communication). Previously we have determined models of GR-EXPB2 type protein based on barely seed protein (PDB: 1bw3A) and the second model on PHL P 1, a major Timothy grass pollenallergen (PDB: 1n10A) (Geric Stare et al., 2013, Acta Biol Slov). Recently we have determined a more probable 3D model structure for GR-EXPB2 type protein based on a determined crystal structure of EXPB1 from maize (PDB: 2hczX) (Geric Stare et al., unpublished). All three AA changed in the variant proteins are positioned on the outer surface of the protein model. Small changes (1 or 2 AA change compared to type protein GrEXPB2) resulted in inactive EXPB2 protein variants. These changes probably affect the interaction with polymers of the cell wall and the proteins function as well as the 3D structure of the protein (one variant possibly affecting a disulphide bond).

Symmetry-driven computational methods generate refined structural models of plant transmembrane proteins

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Transmembrane proteins (TMPs) are involved in all plant vital processes including defense against pathogens. While structural knowledge of TMPs is crucial for a detailed understanding of their function and interactions, experimental structure determination is extremely difficult due to challenges encountered during protein expression, purification and crystallisation. Moreover, available TMPs structures do not have plant origin, the only method for structural analysis of plant proteins remaining similarity-based computational prediction. However, building homology models (HMs) is challenging due to large protein size, distant target-template relationship and low resolution of most available templates. Fortunately, often crystallized homo-oligomeric TMPs are symmetric, which decreases conformational space and facilitates building HMs. However, symmetry is lost during refinement through molecular dynamics (MD) simulations and models accumulate thermal defects, which affects their quality. We assessed the ability of symmetry constrained MD simulations to improve HMs accuracy, using an approach similar to CASP (Critical Assessment of techniques for protein Structure Prediction) competition: build HMs of ion channels with known structure and evaluate the efficiency of proposed methods in improving HMs accuracy (measured as deviation from experimental structure). Results indicate that unrestrained MD does not improve accuracy of HMs, instantaneous symmetrization improves HMs accuracy but not stability during subsequent unrestrained MD, while gradually imposing symmetry constraints improves both HMs accuracy and stability. Moreover, accuracy and stability are strongly correlated, making stability a reliable criterion in predicting accuracy of new HMs. Although our method was tested on ion channels, it is a useful tool applicable to any symmetric protein structure.

Modelling Structures in the Twilight Zone and Beyond. Lessons from Resistance and Effector Gene Families

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Probabilistic modeling of a protein structure starting from sequence became lately increasingly popular in the absence of crystallographic or NMR based models.
Especially when combined with computer experiments such as Molecular Dynamics simulation (MD) and docking such structural models provide a wealth of information on the flexibility and interaction potential of a protein - useful in understanding function, predicting mutational effects and designing further experiments.
In probabilistic modelling the model accuracy is nevertheless very sensitive to the homology level of the new, target sequence with possible templates of known 3D structure. This is a critical factor that should be taken into account when using such models as an input in structure informed work. The common practice is to consider as reliable those models that are based on templates showing above 30% identity.
Unfortunately in most cases of interest, including resistance or effector gene families, the identity is significantly lower than this threshold, bringing us into what is known as the "Twilight modelling zone" - where special remote-homology techniques have to be used; and going even beyond random noise identity - where only ab-inito modelling can be applied, provided that adequte bioinformatic or

experimental bounds might be found.
We critically review here results obtained by our laboratory in the past couple of years using such techniques, especially in the realm of resistance and effector gene products modelling

3D modeling combined with diversity analysis reveals distinct AVR recognition sites and different evolutionary pathways in wheat *Pm3* genes from wild and domesticated species

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The *Pm3* gene confers resistance against wheat powdery mildew. In our study southern populations of wild emmer wheat from Israel were studied extensively to reveal novel *Pm3* alleles that are absent from the cultivated gene pool. Known and newly identified *Pm3* genes were subjected to variation analysis and polymorphic amino acid residues were superimposed on a 3D model of PM3. The region of highest inter-species diversity between *T. aestivum* and *T. dicoccoides* lies in LRRs 19-24, whereas most intra-species diversity in *T. aestivum* is located in LRRs 25-28. Interestingly these two regions are separated by one large LRR whose propensity for flexibility facilitates the conformation of the PM3 LRR domain into two differently structured models. The combination of evolutionary and protein 3D structure analysis revealed that *Pm3* genes in wild and domesticated wheat show different evolutionary histories which might have been triggered through different interactions with the powdery mildew pathogen.

Structural determinants involved in effector recognition by the nematode immune receptor GPA2

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The potato cyst nematode resistance gene *Gpa2* confers resistance to *Globodera pallida* and is located on a complex locus together with the closely related Potato Virus X (PVX) resistance gene *Rx1*. They encode NB-LRR immune receptors, which are 88% identical at the amino acid level. Yet, they confer resistance to completely unrelated pathogens. Gpa2 recognizes the secreted nematode effector RBP1, whereas Rx1 detects the PVX coat protein. This makes Gpa2 and Rx1 an excellent model system to investigate how the recognition specificity is determined in NB-LRR proteins and how pathogen effectors evade recognition. To investigate the structural determinants involved in RBP1 recognition by Gpa2, an integrated approach was used combining bioinformatics, remote homology modeling and functional assays. First, Gpa2-specific residues were identified in a sequence alignment of 35 closely related Rx1/Gpa2 homologues derived from wild Solanum species. Six residues were located in the C-terminal region of the LRR domain of Gpa2 involved in RBP1 induced activation of the protein. Next, systematic exchange of these polymorphic residues between Gpa2 and Rx1 revealed that a single amino acid residue in the C-terminal end of the LRR domain is required for RBP1 recognition. This mutant was still signalling competent, as it was able to induce a constitutive cell death response *in trans* when combined with the D460V mutation in the CC-NB-ARC domain. The fact that this residue

maps on the LRR surface and is subject to diversifying selection further supports its role as specificity determinant of Gpa2. Evasion of RBP1 recognition by Gpa2 is also dependent on a single amino acid substitution (S/P), which resides in the hypervariable surface of RBP1. Computational modeling of the 3D structure suggests that this substitution causes a change in the loop region, which might explain the observed differences in Gpa2 recognition. In addition, a structure-informed approach was used to investigate the contribution of other structural motifs present in RBP1 to better understand the underlying mechanism of RBP1-mediated activation of Gpa2.

Structural and biochemical characterisation of an NB-ARC domain from a plant disease resistance protein

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Despite their long record of use in agriculture to produce disease-resistant cultivars, there is very little biochemical information regarding the regulation of plant-disease proteins (R-proteins). Numerous molecular and genetic studies have been used to produce generalised models for *in planta* behaviour of these proteins, however the fine details of regulation from repression of signalling to activation have not been elucidated through these *in vivo* experiments. Investigations into the *in vitro* behaviour of R-proteins have been limited due to the challenge of obtaining sufficient yields of native, folded protein at sufficient yields for analysis. We have screened multiple R-proteins and R-protein domains to obtain suitable material for structural and biochemical investigations. One domain successfully expressed in a soluble form was the NB-ARC domain of a tomato (*Solanum lycopersicum*) R-protein. *In planta* and homology models suggest this domain acts as a switch determining the activation state of R-proteins via intramolecular interactions, ATP binding and hydrolysis. We have demonstrated that this natively-folded NB-ARC domain is able to facilitate ATP turnover, however we have seen no indications of interactions with its cognate coiled-coil (CC) domain when these proteins are mixed *in vitro*. Finally, we have been successful in obtaining the crystal structure of this plant NB-ARC domain. In future studies, we will build on this preliminary biochemical and structural knowledge to better understand how this “switch” domain functions to regulate plant disease resistance.

Structural Bioinformatics of *Blumeria graminis f. sp. hordei* effector candidates

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Predicting 3D models of proteins allows us to bridge the sequence-structure gap and cope with the sequence data deluge. Computational methods for predicting protein structures are fast and inexpensive and they allow us to study proteins that are problematic to resolve experimentally, for example, those with long regions of intrinsic disorder or those with membrane spanning regions. In the majority of cases, accurate 3D models of proteins can be generated, which may be used to inform and direct experimental work. Furthermore, predicted protein structures may allow you to infer function and help you to understand more about protein evolution.

We have developed an integrated suite of structural bioinformatics tools, IntFOLD, for protein fold recognition, 3D model quality assessment, intrinsic disorder prediction, domain prediction and

ligand binding site prediction. We applied the IntFOLD web server in two related studies to investigate the structure and function of candidate effectors from *Blumeria graminis f. sp. hordei.* We found that a large proportion of effector proteins may represent novel protein folds or are or highly distantly related to known structures. Analysis of the predicted 3D models shows overall similarity to known fungal effectors, but also highlights unexpected structural similarities to ribonucleases throughout the entire effector super-family.

When the amino acid residues under positive selection are mapped on the high confidence models, they are often found in exposed loop-regions and thus available for interactions with other proteins as part of their effector functions. We speculate that some of these effectors may be involved in interactions with host RNAs and perhaps modulate host immunity via this route. Alternatively, as extracellular ribonucleases are highly resistant to proteolytic degradation, the effectors may have evolved a similar stable scaffold while allowing high variability in loop-regions.

The cereal powdery mildew RNase-like effectors

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The fungi that cause powdery mildews in cereals (*Blumeria graminis*) devote over 7% of their genomes' protein coding capacity to effector genes. Most of these genes are highly expressed and up-regulated in the haustoria. The structures of a large number (118) of these effectors are predicted to resemble that of fungal RNAses. In fact, they also appear to have evolved from a single ancestral RNAse gene. A screen for functionally active effectors in the barley powdery mildew fungus *B. graminis* f. sp. *hordei* led to the identification of BEC1011 and BEC1054: two closely related RNAse-like effectors. The experimentally derived structure of BEC1054 resembles that of fungal RNAses. However, like most of these powdery mildew genes, the amino acids required for RNAse activity are not conserved. We propose that the mode of action of these proteins necessary for full pathogenic development involves interactions with nucleic acids. Here, I report on the evidence for this activity. Comparative sequence analysis of the RNAse-like genes and proteins suggests that they also interact with host proteins. We have identified candidate host interactors by LC-MS/MS and have confirmed some of these by yeast-2-hybrid assays. I propose models of how these central effector genes may be involved in modulating host immunity in the powdery mildews by interacting with nucleic acids and proteins.

Structure-function analysis of TNL-EDS1 resistance signalling in Arabidopsis

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We're interested in understanding how plants connect pathogen effector recognition by intracellular NLR receptors to activation of downstream resistance responses in effector-triggered immunity (ETI). For this, we are examining ETI conferred by the Arabidopsis TNL resistance pair RPS4/RRS1

recognizing two bacterial effectors, AvrRps4 and PopP2, respectively from leaf-infecting *Pseudomonas syringae* and root-colonizing *Ralstonia solanacearum*. Like other characterized TNL receptors, RPS4/RRS1 recruit the nucleo-cytoplasmic basal resistance regulator EDS1 for transcriptional reprogramming of cells. Our results show that EDS1 interacts both with the effectors and the TNL receptors inside nuclei. We are testing a working model that a sub-pool of EDS1 resides at the chromatin with the RPS4/RRS1 receptor complex and that effector recognition produces molecular reorientation of the receptor complex to a signalling-active form, potentially releasing EDS1 to interact with its signalling partners, PAD4 and SAG101, and other components. Our functional analysis of the EDS1 crystal structure as a heterodimer with SAG101, and a derived heterodimer model of EDS1 with PAD4, is helping to ascertain the sequence of molecular events linking TNL-effector recognition to transcriptional changes. We will report on a protein structure-function dissection of EDS1 in interactions with its direct partners, PAD4 and SAG101, the TNL receptors, the TNL-recognized effectors, and the chromatin.

The NB-LRR immune receptors RGA4 and RGA5 interact functionally and physically to confer blast disease resistance

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Plant resistance proteins of the class of nucleotide-binding and leucine-rich repeat domain proteins (NB-LRRs) are immune sensors which recognize pathogen-derived molecules termed avirulence (AVR) proteins. RGA4 and RGA5, two NB-LRRs from rice, interact functionally and physically to mediate resistance to the fungal pathogen *Magnaporthe oryzae* and accomplish different functions in AVR recognition. RGA4 triggers an AVR-independent cell death that is repressed in the presence of RGA5 in both rice protoplasts and *Nicotiana benthamiana*. Upon recognition of the pathogen effector AVR-Pia by direct binding to RGA5, repression is relieved and cell death occurs. RGA4 and RGA5 form homo- and hetero-complexes and interact through their coiled-coil domains. These results establish a model for the interaction of hetero pairs of NB-LRRs in plants: RGA4 mediates cell death activation while RGA5 acts as a repressor of RGA4 and as an AVR-receptor.

Conservation of a coiled-coil type NLR-triggered immunity across plant lineages and dissection of the bifurcated signaling mechanism

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The NLR family of intracellular sensors acts as regulatory signal transduction switches and is activated upon perception of non-self molecules derived from microbial pathogens. The polymorphic barley mildew A (MLA) locus encodes coiled-coil (CC) type NLRs, conferring isolate-specific resistance against the pathogenic powdery mildew fungus. Intriguingly, the invariant CC domain encoded by the MLA locus forms a rod-shaped homodimer in the crystal structure. MLA receptors also self-associate *in planta*, but self-association appears to be independent of receptor activation.

MLA CC mutants that fail to self-interact impair *in planta* cell death activity triggered by the CC domain alone and by an autoactive full-length MLA receptor that mimics its ATP-bound state. Thus, CC domain-dependent dimerization of the immune sensor defines a minimal functional unit and implies a role for the dimeric CC module in downstream immune signaling. Our previous work revealed evidence for bifurcating and compartment-specific MLA signaling pathways, in which nuclear and cytoplasmic receptor pools initiate disease resistance signaling through transcriptional reprogramming and host cell death activation, respectively. We recently demonstrated that the barley MLA is fully functional in dicotyledonous *Arabidopsis thaliana*. The established heterologous system provides unique opportunities to explore the underlying resistance mechanism. Since the MLA-mediated signaling in both compartments is predominantly initiated through the N-terminal CC domain, we have generated stable *Arabidopsis* transgenic lines expressing the N-terminus fused with various epitope tags under chemically-inducible promoters. Upon expression of the MLA CC, these lines exhibit a phenotype resembling the induction of authentic immune responses (e.g., stunted growth and cell death). To examine the nuclear receptor activity of MLA, we have conducted ChIP-seq experiments in *Arabidopsis*. We found that the N-terminus binds directly or indirectly at promoter regions, where a novel 14-mer palindromic motif is consistently present. In *A. thaliana* and *A. lyrata*, this motif is well conserved and enriched at promoter regions. We are currently examining how this palindromic motif connects NLRs to the transcriptional output. In parallel to the chromatin study, we have used these transgenics for a genetic suppressor screen that yielded 21 candidates. These do not carry mutations in known loci required for NLR function (e.g., *NDR1* and *RAR1*). Our data illustrate a mechanism how a single NLR could mediate robust immune responses through multiple downstream targets.

Deconstructing a molecular switch; structure-function analysis of the CC-NB-LRR proteins RX1 and GPA2

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Many plant immune receptors have a modular nucleotide-binding-leucine-rich repeat (NB-LRR) architecture in which a nucleotide-binding switch domain, NB-ARC, is tethered to a coiled coil (CC) and a LRR sensor domain. The cooperation between the CC, switch and sensor domains, which regulates the activation of these proteins, is poorly understood.
Through targeted mutagenesis, interaction studies and structural modelling we investigate the way the domains of the potato resistance proteins Rx1 or Gpa2 work together. We demonstrated that the correct cooperation between the CC-NB-ARC and the LRR is focussed on a region in the ARC2 subdomain of the NB-ARC and the N-terminal repeats of the LRR. A mismatch leads either to autoactivation or loss-of-function. Complementary charged surface patches in the ARC2 and LRR are key determinants of the physical interaction between the domains, but do not appear to be involved in signalling. Mutagenesis of specific residues in the ARC2 region affected either elicitor-dependent activation or autoactivation. Furthermore alanine-substitution of several aromatic residues in the CC domain shows that distinct surfaces are required for the interaction of the CC with the NB-ARC-LRR domains or with RanGAP2. The CC domain of Rx1 consists of 4 helices and mutagenesis of the hydrophobic residues required for their interaction revealed that distinct parts of the CC are involved in either the cell death or PVX resistance signaling by Rx1.
Currently we are characterising how the binding of RanGAP2 and the recognition of specific elicitors affects the conformation and subcellular localisation of the resistance protein in the cell via fluorescence lifetime imaging.

Unravelling the mystery of AVRRPS4 recognition by arabidopsis resistance gene pair *RRS1/RPS4*

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Plants are often attacked by pathogens. Successful pathogens produce effectors that suppress plant immunity and facilitate infection. Plants have evolved disease resistance (R) proteins to recognize effectors and activate effector-triggered immunity (ETI). *Arabidopsis* TIR-NB-LRR R proteins RPS4 and RRS1 confer resistance to *Pseudomonas syringae* carrying AvrRps4, and to *Ralstonia solanacearum* carrying PopP2. Our understanding of how this dual R protein-effector recognition system operates remains limited.
RPS4 and RRS1 associate in a complex. Structural-guided studies of RPS4 and RRS1 TIR domains revealed the importance of TIR/TIR heterodimer in complex formation, and RPS4 TIR/TIR homodimer in defence signaling (Williams *et al.* Science, 2014). Thus, the two components of the complex may play distinct roles in recognition and signaling. The recognition component RRS1 carries a WRKY DNA-binding domain, which appears to be the common target of the effectors AvrRps4 and PopP2. The direct interaction between AvrRps4 and RRS1-WRKY domain is required for recognition. When AvrRps4 is mutated in the predicted AvrRps4/RRS1-WRKY interface, it loses RRS1 binding activity, resulting in the loss of recognition. Likewise the acetylation of RRS1-WRKY by PopP2 is required for recognition. Remarkably, when RRS1 is acetylated by PopP2 it can no longer interact with, or recognize, AvrRps4. We postulate that RRS1-WRKY mimics the host WRKY transcription factors implicated in plant defence for effector perception, while RRS1-WRKY is guarded by RPS4 for defence activation. As the structure of AvrRps4 is available, either co-crystalization with RRS1-WRKY domain or modeling AvrRps4-RRS1-WRKY complex in future studies will better elucidate the recognition mechanism of RRS1/RPS4.

ABSTRACTS
POSTERS

Unveiling and exploiting *P. capsici* nuclear effector functions

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Plant pathogens are a major agricultural concern hampering food production worldwide. The highly destructive oomycete *Phythophtora capsici* threatens a diverse range of economically important crops such as pepper, tomato, eggplant, snap, lima beans as well as many cucurbits. *Phythophtora capsici* secretes a vast arsenal of proteins (effectors) that are thought to be crucial for its virulence. Work in our lab has identified a suite of *P. capsici* effectors that localise to the host nucleus, nonetheless little is known about the nuclear processes they target.
The aim of my project is then to unveil the activities of *P. capsici* effectors responsible for reprogramming the host nucleus during infection. An interdisciplinary approach that combines Yeast two hybrid (Y2H), proteomics, transcriptomics and functional analyses will be employed to study the functions of effectors and their targets *in vivo*.
By Y2H screening two protein interactors of Crinkler 83_152 (CRN83_152), a nuclear effector from *P. capsici*, were identified: SUMO ligase and endonuclease. Here I will present my results regarding the confirmation and characterization of CRN83_152 interactions.

A Structure-guided mutagenesis study identifies one amino acid that is crucial for the recognition specificity of the potato cyst nematode resistance gene Gpa2

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The potato cyst nematode resistance gene *Gpa2* confers resistance to *Globodera pallida*. *Gpa2* belongs to the class of CC-NB-LRR resistance genes and is located on a complex locus that also harbours the closely related Potato Virus X (PVX) resistance gene *Rx1*. Rx1 and Gpa2 are 88% identical at the amino acid level, yet they confer resistance to completely unrelated pathogens. For both R proteins, the pathogen elicitor that triggers the resistance response is known. Gpa2 recognizes the secreted *Globodera pallida* protein RBP1, whereas Rx1 detects the PVX coat protein. This it an excellent model system to investigate how the recognition specificity is determined in NB-LRR proteins.
To determine which amino acids may play a role in Gpa2 specificity, we combined sequence information of 35 closely related homologues derived from wild Solanum species, functional data and a consensus *in sillico* 3D model. Two residues (M1 and M2) were detected that are located in the region that is crucial for elicitor recognition. In the 3D model, they map together on the LRR surface, suggesting a potential role in pathogen recognition.
Functional validation was performed by mutagenesis of these candidate amino acids. Only mutation of M2 resulted in a loss of function upon agroinfiltration in *N. benthaminana*. Western blotting showed that this was not due to protein instability. Mutation of M2 in an auto-active mutant of Gpa2 showed that signal transduction was not inhibited. Therefore, we conclude that M2 is crucial for the recognition specificity of Gpa2.

Avirulence and Virulence Functions of the *Fusarium oxysporum f.sp. lycopersici* effector pair Avr2 and Six5

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Fusarium oxysporum f.sp. *lycopersici* (*Fol*) is the causal agent of vascular wilt disease in tomato. The *I-2* resistance gene of tomato confers resistance to *Fusarium* by recognizing a pair of effector proteins; Avr2 and Six5. The effector gene pair shares its promoter region and deletion of either gene breaks I-2 mediated resistance. Besides breaking resistance, loss of either effector compromises *Fol* virulence on susceptible tomato cultivars. To study the virulence function *Avr2* and *Six5* transgenic *Arabidopsis* have been generated and bioassays revealed enhanced susceptibility to Fo5176 an *Arabidopsis*-infecting *Fusarium* strain. Transient expression of cytosolic edition of fluorescently tagged RFP-dspAvr2 or GFP-dspSix5 in *N. benthamiana* revealed a distinct localisation pattern for each protein. Whereas dspAvr2 localizes in both the cytoplasm and the nucleus, dspSix5 is excluded from the nucleus and could only be detected in the cytoplasm. Using bimolecular fluorescence complementation (BiFC) Avr2 and Six5 were found to interact *in planta*, which is in agreement with their interaction in yeast-two-hybrid assays. Notably, Avr2 and Six5 do not only interact in the cytoplasm but a strong BiFC signal is also found in spots along the cell periphery. These spots colocalise with YFP-LYM2, an Arabidopsis plasmodesmatal resident protein, suggesting a function of these effectors at the plasmodesma. A working model of this activity in relation to the observed virulence function of these effectors will be presented.

The effector Sad1 of *Sporisorium reilianum* increases secondary branching in mono- and dicotyledonous plants.

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The biotrophic fungus *Sporisorium reilianum* causes head smut of maize. Maize infection leads to changes in the development of the inflorescences. In addition to the formation of spores in male and female inflorescences, infected maize plants do not show apical dominance in ear branches, which leads to an increased number of developing ears. We could identify two genes of *S. reilianum* responsible for the outgrowth of additional ears on inflorescence branches that we called s*uppressor of apical dominance1*/2 (*sad1, sad2*). Due to the more prominent effect on the outgrowth of additional ears triggered by Sad1 we continued investigating this protein. Sad1 is a small secreted protein lacking homology to other known proteins. When expressed heterologously in *Arabidopsis thaliana* an increase in secondary branching can be detected. A C-terminally tagged version of Sad1 (Sad1-GFP) expressed by *S. reilianum* localizes around the fungal hyphae in infected tissues. However, this tagged version is non-functional. The addition of smaller C-terminal HA- or N-terminal myc-tags also results in non-functional Sad1 proteins. This suggests that free N- and C-termini are obligate for Sad1 function. Sad1 shows no homology to any known protein or predicted domains. To be able to decipher the function of Sad1 we want to use X-ray crystallography to resolve its 3D structure and check for homologous proteins at the level of the tertiary structure. Currently, we are overexpessing and purifying the protein from *E. coli*. Structure determination will help to shed light onto the function of Sad1.

Structural analysis of *H. schachtii* putative secretory proteins

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Beet cyst nematode *Heterodera schachtii* (BCN) is one of the biotrophic sedentary endoparasite nematodes, which depends on a specific, multinucleate, syncytial feeding structure. The nematode secretes effector proteins into the affected root cell in order to induce and maintain the syncytium. We analyzed BCN transcriptome aiming to identify its secretome and functionally annotate it based on their structure. Meanwhile, the same approach was applied on known transcripts of other sedentary nematodes. We identified a set of homologous putative secretory proteins (PSP). We queried the Biana Interlog Prediction server (BIPs) to identify putative interacting partners within different host plants by each of selected PSP. We identified 40 proteins that are predicted to have a putative interacting protein partner(s) within *A. thaliana* (At_Partners). Interestingly, some of the predicted At_Partners was found to play role within pathways of enriched metabolites and/or differentially expressed genes of *A. thaliana* after *H. schachtii* infection. Confirming the nature of our genes of interest to be considered as PSPs, we analyzed their level of expression and localization by qPCR and in situ hybridization, respectively. In our study, we aim to identify and compare structures of nematode PSP and their putative interacting partner within their host plant.

Identification of fungal proteases responsible for proteolytic cleavage of tomato chitinases

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Plants defend themselves against fungal pathogens by secreting enzymes with antifungal activities such as chitinases that degrade the fungal cell wall. In response, fungal pathogens secrete chitin-binding proteins such as Avr4 which can protect them against plant chitinases. This protection might be incomplete, so released chitin oligomers may still be detected by plant recognition receptors and induce defence. Fungi like *C. fulvum* secrete Ecp6 which can sequester chitin oligomers preventing them to be detected. Previously, degradation of PR proteins has been proposed as a component of virulence of some plant pathogens. Here, we employed a combined biochemical, proteomics, bioinformatics and mass spectrometry approach to identify the chitinase modifying protein(s). We have produced four chitin binding tomato chitinases (CBD-tomato chitinases) in *Pichia pastoris* and analysed eight tomato fungal pathogens for their ability to degrade their CBD-. We showed that enzymes present in culture filtrate of some of these tomato pathogens can degrade two of the four CBD-tomato chitinases. Culture filtrates with proteolytic activity were fractionated on Superdex 75 and analysed. Mass spectrometry confirmed the presence of at least two novel secreted serine proteases in the active fractions. We also performed expression profiling for the fungal proteases and CBD-tomato chitinases during infection of susceptible and resistant tomato cultivars. In addition, we are testing whether the two serine proteases play a role in virulence.

Molecular analysis of the AvrPm3-Pm3 interaction in the wheat powdery mildew pathosystem: mapping and identification of the effector partner of the *Pm3f* resistance gene

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The powdery mildew *Blumeria graminis* f. sp. *tritici* (*Bgt*) is an obligate biotrophic fungal pathogen of wheat. The wheat powdery mildew resistance gene *Pm3* occurs in an allelic series, hypothetically corresponding to a series of specific avirulence (*Avr*) genes from *Bgt*. Segregation patterns observed in our mapping population revealed the presence of two genetic loci required for avirulence on *Pm3f*. Several approaches were used to map the second locus of *AvrPm3f*: i) genome-wide SNP genotyping, ii) whole genome sequencing of bulked segregant DNA, iii) comparative genomics of *Bgt* and barley powdery mildew, and iv) *de novo* prediction of effector families. Genetic analysis of 151 progeny with 228 SNP markers yielded a genetic map containing *AvrPm3f2* in a genetic interval of 20.2 cM. Fine mapping using CAPS markers reduced the genetic interval to 5.3 cM. Both *Bgt/Bgh* colinearity and bulk segregant analysis using 23 avirulent progeny identified the specific contigs containing sequences within the candidate region. Fine mapping reduced the genetic interval to 2.2 cM, or approximately 500 kb. *De novo* gene prediction within the candidate region yielded an effector family of 22 candidate secreted effector proteins (CSEPs). One member, *AvrPm3f2_7*, cosegregates with *AvrPm3f2*, and exhibits the characteristics of a promising candidate: i) its expression is supported by RNA sequence data, ii) it has the typical *Bgt* CSEP structure, and iii) there is one conservative, and one radical amino

acid substitution in the virulent vs. avirulent allele. Functional validation of the promising candidate *AvrPm3f2_7* is currently in progress

Identification of *xanthomonas campestris* pv. *Vesicatoria* type iii effectors that suppress pti signaling

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The Gram-negative bacterium *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*), is the causal agent of spot disease in pepper and tomato plants. *Xcv* pathogenicity depends on a type III secretion system (T3S) that translocates effector proteins into the plant cell. Collectively T3S effectors suppress plant PAMP-triggered immunity (PTI), alter metabolism and gene expression for the benefit of the pathogen. About thirty T3S effectors are encoded in the genome of the *Xcv* strain 85-10 and nine of them were previously implicated in PTI suppression. However, little is known about their molecular functions and plant targets. We used an *Arabidopsis thaliana* pathogen-free protoplast assay to identify a comprehensive set of *Xcv* 85-10 effectors that manipulate early PTI response signaling. Of 34 tested effectors, 18 suppressed flg22-dependent activation of a reporter gene under control of a typical PAMP-inducible promoter (*pFRK1-Luc*). Among them, seven effectors also suppressed ABA-dependent activation of a reporter gene driven by an ABA-inducible promoter, while eleven effectors were specific PTI suppressors. PTI signaling includes activation of mitogen-activated protein kinase (MAPK) pathways and phosphorylation of the MPK3 and MPK6 MAP kinases. Interestingly, expression of effectors with PTI-suppressing activity did not affect flg22-mediated phosphorylation of MPK3 and MPK6 indicating that all eighteen effectors interfere with PTI signaling downstream to MAPK3/6.

Tumor progression in leaves in *Ustilago maydis* – maize interaction is modulated by an organ specific effector protein

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Ustilago maydis infects all aerial organs of its host plant maize. Disease progression goes along with comprehensive reprogramming of the plant tissue which ultimately results in formation of tumors. This tumor induction is triggered by small secreted proteins by the fungus, so called effectors. Due to varied fundamental differences between the different maize organs that are colonized by *U. maydis*, the fungus deploys organ specific effectors to manipulate physiology and development of specific host tissues. To investigate the role of individual organ specific effectors in modulating biotrophy, we in present study identified a novel secreted protein, termed See1 (**S**eedling **e**fficient **e**ffector 1) that is strongly induced in seedling leaves but only weakly expressed in floral tissues. *U. maydis* deletion mutants for *see1* show a strong reduction of tumor formation only in maize seedlings. Laser confocal microscopy shows that the mutant might be blocked in mesophyll cell layer which is specific to leaf. Moreover, *in vivo* cell division approach shows that maize seedling colonized by Δ*see1* do not show

mitotic activity during infection, while cell division in leaves is specifically induced in wildtype infected host cells. To understand its role for organ specific function for *U. maydis* virulence, See1 interaction partners have been identified using a yeast two hybrid screen. The effector is translocated to the plant nucleus and might be acting after post translation modification. We hypothesize that See1 might be involved in eliciting plant cell cycle progression to form tumors in seedlings. The presented approach provides novel insight into tissue specific virulence strategies of a biotrophic plant pathogen.

Peptide aptamer-mediated resistance to barley powdery mildew

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Barley powdery mildew, *Blumeria graminis* f. sp. *hordei* (*Bgh*) is a biotrophic fungus that develops feeding structures (haustoria) in the living plant cells. In order to control the host plant, fungi secrete effector proteins that are believed to inhibit host defence mechanisms. Approximately 500 barley powdery mildew effector candidate proteins (CSEPs) have been identified, showing no homology to any known proteins outside powdery mildew fungi (Pedersen et al., 2012). The only common feature of 63% of those putative effectors is YxC-motif in the N-terminal of the mature protein (Godfrey et al., 2010). This feature makes it a very interesting target in engineering disease resistance. Peptide aptamers are an excellent tool for this purpose, because of their small size, high recognition specificity and high binding affinity to their target.

In this study, we applied yeast two-hybrid screen to identify peptide aptamers targeting the YxC motif of CSEPs. This interaction was confirmed by bimolecular fluorescence complementation assay in *Nicotiana benthamiana* and has been showed to be YxC-motif specific, which suggests that the identified peptide aptamers should be able to target the majority of CSEPs. Transient overexpression of selected peptide aptamers in barley epidermal cells resulted in lower susceptibility to barley powdery mildew. Therefore, in the next part of this study we would like to focus on the functionality of the YxC motif within an effector structure. We believe that this motif might be involved in the correct folding of the effector by making disulfide bond with the cysteine at the C-terminus. Hence, different point mutations within the motif will be tested.

Modelling protein-DNA complexes using FRET constraints

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FRET experiments are useful in both building and validating structural models. Distances derived from FRET are bounds that significantly reduce the configurational searching space in generating a model and in the same time could be used to confirm model based predictions.

We present here an example of using FRET in modelling the DNA bending and interactions with proteins involved in the so called V(D)J recombination. This is essential in generating the hyper variability of IG light and heavy chains and involves the formation of a large complex mainly between two DNA fragments: 12RSS and 23RSS, and RAG1 protein.

By attaching FAM and TAMRA fluorophores to T, C as donors and G, T, C/A as acceptors respectively, distances between various positions in the two strands of these two DNA fragments could be measured and based on these models of 12RSS/23RSS, bending in the complex could be derived by a two-step procedure consisting in a coarse grained bending imposed by the maximal FRET distance followed by model refinement involving introducing successively the more localized constraints via MD simulations. Furthermore measurement of a number of inter DNA distances allowed a drastic reduction of the configuration space probed during the overall DNA-protein complex modeling which consisted in constrained molecular docking of RAG1 and the bended models of 12/23RSSs.

As discussed, FRET was also used here to validate the models of 12/23RSSs bending by measuring distances in given DNA locations and comparing them with model based predictions.

NRB1, a NB-LRR protein required for multiple immune receptors in solanaceous plants

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Most plant resistance genes encode nucleotide-binding leucine-rich repeat (NB-LRR) immune receptors that recognize effectors secreted by pathogens. NB-LRR mediated effector recognition can provide valuable disease resistance against important crop pathogens, and thus understanding how NB-LRRs function is of great interest. However, our basic knowledge of NB-LRR function is mostly limited to Arabidopsis, with little information about NB-LRRs involved in important crop pathosystems. *Phytophthora infestans* is a notorious oomycete plant pathogen that causes late blight on potato and tomato. Rpi-blb2, an NB-LRR protein from the wild potato *Solanum bulbocastanum*, recognizes the RXLR type effector AVRblb2 from *P. infestans*, and confers late blight resistance in potato and the model solanaceous plant *Nicotiana benthamiana*. In this study, we found that an additional NB-LRR protein, we termed NRB1, is required for the function of Rpi-blb2 as well as some other immune receptors. Using immunoprecipitation and mass spectrometry, we identified that Rpi-blb2 associates with NRB1 in *N. benthamiana* leaf lysates. The association between Rpi-blb2 and NRB1 was validated by *in planta* co-immunoprecipitation. Furthermore, by using gene silencing, we showed that NRB1 is genetically required for Rpi-blb2-mediated resistance and cell death. Remarkably, NRB1 is also required for the activities of two additional NB-LRR proteins, potato blight resistance protein R1 and nematode resistance protein Mi. We conclude that NRB1 is a signaling hub for a subset of NB-LRR immune receptors in solanaceous plants. Further structure-function analysis will be carried out to understand how does NRB1 mediate the signaling from up stream NB-LRR immune receptors

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