

# An Outlook on the Localisation and Structure-Function Relationships of R Proteins in Solanum

### Potato Research

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## An Outlook on the Localisation and Structure-Function Relationships of R Proteins in *Solanum*

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**Abstract** The co-evolution of plants and plant-pathogens shaped a multi-layered defence system in plants, in which Resistance proteins (R proteins) play a significant role. A fundamental understanding of the functioning of these R proteins and their position in the broader defence system of the plant is essential. Sub-project 3 of the BIOEXPLOIT programme studies how R proteins are activated upon effector recognition and how recognition is conveyed in resistance signalling pathways, using the solanaceous R proteins Rx1 (from *S. tuberosum* spp. *andigena*; conferring extreme resistance against Potato Virus X), I-2 (from *S. lycopersicon*; mediating resistance to *Fusarium oxysporum*) and Mi-1.2 (from *S. lycopersicon*; conferring resistance to *Meloidogyne incognita*) as model systems. The results obtained in this project will serve as a model for other R proteins and will be translated to potential applications or alternative strategies for disease resistance. These include the modification of the recognition specificity of R proteins with the aim to obtain broad spectrum resistance to major pathogens in potato.

**Keywords** Co-evolution  $\cdot$  Elicitors  $\cdot$  Nematodes  $\cdot$  Potato  $\cdot$  Potato virus  $\cdot$  Resistance breeding  $\cdot$  R proteins  $\cdot$  *Solanum* 

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### Introduction

The co-evolution of plants and plant-pathogens shaped a multi-layered defence system in plants (Jones and Dangl 2006). Some of the effector proteins pathogens use to overcome the plants' non-host and generic recognition systems can be recognized in resistant plants by so-called Resistance proteins (R proteins). Most of these are large modular proteins carrying a leucine-rich repeat (LRR) domain that forms a horseshoe-shaped structure. LRR domains are specialized in specific recognition by their extended surfaces. Upon effector recognition the plant cell initiates defence responses, which in most cases culminate in local cell death, but can also stimulate a systemic acquired resistance response. For utilization of the plants' own defence mechanisms, as a natural resource for the creation of resistant crops, a fundamental understanding of the functioning of these resistance proteins and their position in the broader defence system of the plant is essential.

The exploitation of natural plant biodiversity for the pesticide-free production of food is one of the main goals of the EU 6th framework project BIOEXPLOIT. Within sub-project 3 several research approaches are pursued to study how R proteins are activated upon effector recognition and how recognition is conveyed in resistance signalling pathways. For that purpose the solanaceous R proteins Rx1, Gpa2, I-2 and Mi-1.2 serve as model systems.

In this paper an overview is given of the ongoing work on R protein mechanisms carried out at the groups participating in BIOEXPLOIT sub-project 3 in the context of the general developments in the field of research.

# The Modular Architecture and Conformational Changes of R Proteins in *Solanum*

The largest class of R proteins is formed by the so-called NB-ARC-LRR proteins (NB: nucleotide binding domain; ARC: the <u>Apaf-1, R</u> proteins, and <u>CED4</u> domain; LRR: leucine-rich repeat domain). As the overall structure of the NB-ARC-LRR proteins is well conserved, it is likely that these proteins share similar recognition and activation mechanisms. This justifies the in depth analysis of relatively few R proteins as model for understanding the general working mechanisms. Interestingly, proteins with very similar domain architectures are found to play a role in metazoan innate immunity and programmed cell death pathways: the mammalian NOD-like receptor (NLR) proteins and Apaf-1. These proteins also carry a central nucleotide-binding-ARC domain, a C-terminal recognition domain and a variable N-terminal domain similar to plant R proteins (van der Biezen and Jones 1998; Leipe et al. 2004).

The potato CC-NB-ARC-LRR R protein Rx1 confers extreme resistance against *Potato virus X*, stopping virus replication often without inducing a visible cell death phenotype (note that CC stands for coiled-coil). Rx1 has developed into a valuable model for understanding R proteins for several reasons. It was one of the first R proteins for which the elicitor was known, the PVX coat protein (CP). A change in only two amino acid residues in the CP allows PVX to escape Rx1 recognition, but at the cost of a lower virulence.

Furthermore, Rx1 is encoded in a small R gene locus on chromosome XII of potato that also contains the highly homologous R gene Gpa2 that confers resistance to a completely unrelated pathogen: the potato cyst nematode Globodera pallida (Bendahmane et al. 1999; van der Vossen et al. 2000). Exchanging sequence fragments between these two R genes provided insight on how the LRR domain determines recognition specificity (Rairdan and Moffett 2006; unpublished work by E. Slootweg et al.). Even though the Rx1 and Gpa2 proteins share more than 90% sequence identity the exchange of sequence fragments between the proteins often led to constitutively activated proteins. The autoactivation phenotype could be attributed to incompatibility between a small section of the ARC2 domain and the first half of the LRR domain. A detailed analysis of the incompatible region will provide information on how the LRR and NB-ARC domains interact and how recognition of the elicitor by the LRR can induce the shift to the active conformation of the NB-ARC domain. In collaboration with the group of A. Petrescu at the Romanian Academy both LRR and NB-ARC domains of Rx1 and Gpa2 were modelled. Based on co-evolutionary data and structural and surface characteristics a model of how the LRR and NB-ARC domains might interact has been proposed that is currently evaluated by targeted mutagenesis.

The structures determined for Apaf-1 and CED-4 were also used to model the NB-ARCs of the tomato NB-LRR R proteins I-2, mediating resistance to Fusarium oxysporum, and Mi-1 conferring resistance to the root-knot nematode Meloidogyne incognita. This resulted in a testable structural model in which the three distinguishable sub-domains are coordinated around the bound nucleotide (Takken et al. 2006). The I-2 and Mi-1.2 proteins were the first R proteins for which it was shown that they possess the predicted capacity to bind and hydrolyse ATP; this is considered a major step forward in the understanding of R protein activation (Tameling et al. 2002). Specific mutations to the phosphate binding loop (P-loop) of the NB-ARC inactivate the protein, showing that nucleotide binding is essential for R protein function. Targeted mutations that inhibit ATP hydrolysis render the R protein constitutively active and therefore it is assumed that the ATP bound conformation of the protein is the activated form and the ADP bound protein presents the inactive or resting state (Tameling et al. 2006). The NB-ARC domain can be regarded as a molecular switch, of which the transition between the different nucleotide-dependent conformations is responsive to the presence of the pathogen derived elicitor via the C-terminal LRR domain. Changes in conformation of the NB-ARC can influence the interaction with the C- and N-terminal subdomains altering the available interaction surface for downstream signalling components. For several NB-ARC containing proteins, like the tobacco N protein and Arabidopsis RPS5, it has been shown that they form oligomers. However it remains to be studied how common such mechanism is in R protein signalling (Mestre and Baulcombe 2006; Ade et al. 2007).

### Nuclear and Cytoplasmic Localisation of Rx1

An interesting characteristic of the Rx1 protein is that it remains functional when its domains are co-expressed as separate polypeptides (Moffett et al. 2002). It is possible to co-express the coiled-coil, the NB-ARC and the LRR domains in trans and still obtain a hypersensitive response to a high concentration of the avirulent PVX coat protein. This allowed us to create fluorescent constructs, not only of the full length protein, but also of the separate sub-domains. Most of these tagged constructs still form functional proteins. C- and N-terminal fusions of Green Fluorescent Protein (GFP) variants to Rx1, made it possible to study its subcellular localization in Nicotiana benthamiana cells. Contrary to our expectations we observed the presence of Rx1 in both the cytoplasm and the nucleus. Rx1 does not contain known nuclear localisation signals and the size of the protein (140 kDa including GFP) exceeds the limit for passive diffusion through the nuclear pore. Fluorescent fusions of a series of deletion constructs, CC-NB-ARC, NB-ARC, NB-ARC-LRR, CC and LRR showed three distinct patterns of sub-cellular localisation. The NB-ARC-LRR and LRR constructs have a cytoplasmic localisation and are mostly absent in the nucleus. The NB-ARC and CC-NB-ARC constructs showed equal fluorescence intensities in both the nucleus and the cytoplasm. The CC alone fused to GFP, however, seems to preferentially accumulate in the nucleus resulting in a three to four times higher fluorescence intensity in the nucleus compared to the cytoplasm. Based on secondary structure predictions a set of CC fragments was cloned as GFP fusion to test if the observed nuclear accumulation could be attributed to a particular secondary structure element or a targeting signal. No R protein coiled-coil domain 3-D structure has been solved yet. However, several secondary structure prediction programs consistently indicate that the Rx1 CC contains four alpha helices of which the second two are separated from the first two by a beta-turn. In the localisation study only the construct containing the two helices downstream of the turn gives a nuclear accumulation comparable to the complete CC. The diffusion behaviour inside the nucleus for both the complete CC and the latter CC fragment showed that their nuclear accumulation coincides with a significantly reduced nuclear diffusion as compared with non-fused GFP and the other CC fragments. This difference might point to a potential interaction between the CC and an unknown nuclear component. Several R proteins from non-Solanum species have recently been shown to be localised in the nucleus or to interact with nuclear proteins such as transcription factors, which led to new insights in R protein signalling (Burch-Smith et al. 2007; Shen et al. 2007; Wirthmueller et al. 2007).

As nucleotide binding is essential in the activation cycle of R proteins, the sub-cellular localisation of mutants that lack the ability to bind nucleotides was studied. A P-loop mutant of the full length Rx1 is mostly excluded from the nucleus in comparison to the wild type version. However, when the same mutation is made in a construct lacking the LRR domain, no change in localisation is observed between wild type and the mutated version. Both show an equal fluorescence intensity in both nucleus and cytoplasm. Apparently the nuclear exclusion that is caused by the P-loop mutation is dependent on the LRR. A similar LRR dependency can be observed when by virus induced genesilencing (VIGS) expression of the resistosome proteins SGT1 and Rar1 is knocked down, as this also results in nuclear exclusion. SGT1 and Rar1 are thought to function as chaperones involved in stabilizing R proteins. Both the silencing experiments and the P-loop mutation show that the nuclear localisation

of Rx1 is probably conformation dependent. Two approaches were followed to assess whether CP recognition or Rx1 signalling pathways were linked to a certain cellular compartment. At one hand the Rx1 protein itself or its subdomains were directed to either the nucleus or the cytoplasm by fusion to exogenous targeting signals (Nuclear Export Signals or Nuclear Localisation Signals). On the other hand the elicitor, the PVX coat protein, was directed to the nucleus or cytoplasm. The PVX coat protein is a much smaller protein and can under normal circumstances diffuse freely between the cytoplasm and the nucleus. The surprising result was that no effect was found for retargeting Rx1, but when the elicitor was targeted to the nucleus, it could not activate Rx1 anymore, indicating that recognition might have to take place in the cytoplasm.

### RanGAP2: A Component of the Rx1 and Gpa2 Resistosome in Potato

The N-terminus of the NB-ARC-LRR proteins, either TIR (Toll Interleukin Receptor) or CC, is thought to be the most likely domain to interact with downstream effectors in resistance signalling. This division of tasks within the protein between recognition (LRR), molecular switch (NB-ARC), and effector domain is also seen in structurally similar proteins such as Apaf-1. To unravel the mechanisms behind the downstream signalling of Rx1 Tameling and Baulcombe (2007) analysed which proteins interact with Rx1 by affinity-purification experiments. The Rx1 CC domain was found to specifically interact with a protein identified as NbRanGAP2 via mass spectroscopy, but not with the homologous NbRanGAP1. The interaction is specific for the CC; NbRanGAP2 binds the full length Rx1, but not a deletion construct lacking the CC domain. RanGAPs are proteins that function as activators of the small GTPase Ran that has a very low intrinsic GTPase activity (Meier 2005). Ran has a central role in the mechanism that actively cycles proteins between the nucleus and the cytoplasm. Its GTP bound form is transported to the cytoplasm where RanGAP activates GTPase activity, resulting in the GDP bound form of Ran, which is subsequently imported back into the nucleus. It is unknown if RanGAP1 and RanGAP2 are functionally divergent. Animal RanGAPs contain a sumoylation domain that mediates targeting to the nuclear envelope. In plant RanGAPs this function is fulfilled by an N-terminal WPP domain (named after the sequence Trp-Pro-Pro) (Rose and Meier 2001). This WPP domain of NbRanGAP2 is required for the association with the Rx1 CC. By VIGS of NbRanGAP2 it was shown that Rx1 mediated resistance requires the presence of NbRanGAP2. Resistance mediated by other R proteins was not affected by the absence of NbRanGAP2. The fact that a protein involved in nucleo-cytoplasmic cycling is involved in Rx1 mediated resistance is interesting in the light of the observed nuclear localization of Rx1.

One can think of several hypotheses that explain the involvement of RanGAP2 in Rx1 signalling. One hypothesis is that NbRanGAP2 has a role in the sub-cellular distribution of Rx1. Another option could be that the NbRanGAP2 protein is guarded by the Rx1 protein and upon targeting by the PVX coat protein the resistance response is initiated. The exact role RanGAP2 plays is the focus of ongoing research.

### Perspectives

In this paper, we have presented some examples of structure and function relationships, sub-cellular localisation and complex formation of the three R proteins from *Solanum*: Rx1 from *S. tuberosum* spp. *andigena* and Mi-1 and I2 from *S. lycopersicon*. These examples illustrate the work conducted in Sub-project 3 of BIOEXPLOIT. These results provided a significant contribution to our understanding of the mechanisms underlying R gene mediated resistance. An important next step will be to dissect the composition and dynamics of the recognition complexes by studying the role the corresponding pathogen effector molecules have on the localisation and complex formation of these R proteins. The results obtained in this project will serve as a model for other R proteins and will be translated to potential applications or alternative strategies for disease resistance. These include the modification of the recognition specificity of R proteins with the aim to obtain broad spectrum resistance to major pathogens in potato.

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