Nucleotide-binding and molecular interactions of plant disease resistance proteins

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CHAPTER 6

General discussion

Our main objective was to unravel the molecular mechanism by which R proteins activate plant defence. Defence resistance signaling is activated upon direct or indirect recognition of an effector protein by an R protein. Effector recognition is proposed to induce dynamic changes in R complex-composition and –conformation that will lead to initiation of down-stream defence signaling. We wanted to understand how R proteins exert their function in these resistance complexes and to identify new members of the R protein signalosome. In addition, we wanted to identify the correlation between nucleotide binding and the dynamic interplay between subdomains in switching between the off/on activation states of an R protein. Heading for these goals we analyzed inter- and intramolecular interactions in relation to the nucleotide- binding status of various R proteins. As models we focused on four R proteins: the potato Rx (conferring resistance to potato virus X (PVX) (Bendahmane et al., 1999)); the tomato Mi-1.2 (triggering defenses against nematodes, aphids, psyllids and white flies (Milligan et al., 1998; Rossi et al., 1998; Vos et al., 1998; Nombela et al., 2003; Casteel et al., 2006) the tomato I-2 (mediating resistance to *F. oxysporum* (Simons et al., 1998; Houterman et al., 2009)) and the barley MLA27 (conferring resistance to powdery mildew (Seeholzer et al., 2010)). All four proteins are members of the CNL (Coiled Coil-NB-ARC-LRR) class, which belongs to the larger family of NB-LRR (nucleotide binding and leucine rich region containing) R proteins.

NUCLEOTIDE- BINDING IS ESSENTIAL FOR R PROTEIN FUNCTION

As presented in chapter 5, full-length MLA27 was found to form a stable complex with ADP. To our knowledge this is the first example in which the nucleotide-binding state of a full-length, R protein has been determined. Previously it has been shown that NB containing fragments of I-2, Mi-1.2 (Tameling et al., 2002; Tameling et al., 2006), N (Ueda et al., 2006), M-flax and L6 (Williams et al., 2011) can bind to nucleotides. Nucleotide binding for these R proteins was not unexpected as they carry a predicted nucleotide-binding domain called the: NB-ARC for nucleotide binding domain shared by Apaf-1, R proteins, Ced-4 (van der Biezen and Jones, 1998; Albrecht and Takken, 2006)}. Purified Apaf-1 (apoptotic protease activating factor 1) and Ced-4 (*C. elegans* death protein 4) were also found to co-purify with a bound nucleotide (Kim et al., 2005; Bao et al., 2007; Qi et al., 2010). Together with R proteins and AfsR-like bacterial transcription factors, Apaf-1 and Ced4 belong to the AP (apoptotic) ATPase subfamily of the STAND (Signal Transduction ATPases with numerous domains) family (Leipe et al., 2004). The nucleotide-binding domain of R proteins, Apaf-1 and Ced-4 (Tameling et al., 2002; Kim et al., 2005; Tameling et al., 2006; Bao et al., 2007; Qi et al., 2010) as well as few other members of the STAND family (for instance Monarch-1/NLRP12 (Ye et al., 2008), Cryopyrin/NALP3 (Duncan et al., 2007), MaIT (Marquenet and Richet, 2007)) is proposed to work as an NTP-exchanging/hydrolyzing switch, regulating signal transduction by conformational changes. However, the exact role of ATPase activity or nucleotide exchange for functioning of these multi-domain proteins is poorly understood and various mechanistic models have been proposed for different protein classes or even for particular members. Concerning the activation of R proteins, one model is based on the functional and biochemical analysis of I-2 variants (Takken et al., 2006; Tameling et al., 2006). This model proposes that in the absence of a pathogen, an NB-LRR R protein is in the resting state, in which the LRR domain exerts its negative role by stabilizing the ADP-bound state. The presence of a pathogen effector (Avr) triggers conformational changes that result in exchange of ADP for ATP allowing the R protein to switch to the activated state. The intrinsic ATPase activity of the protein is supposed to attenuate the signaling response and returns the protein to its resting state (Takken et al., 2006; Tameling et al., 2006). Our discovery that wild-type, thus likely representing the resting state, NB-LRR protein co-purifies with ADP is in agreement with the I-2 switch model and is consistent with data

recently obtained for the M-flax R protein (chapter 5; (Tameling et al., 2002; Takken et al., 2006; Tameling et al., 2006; Williams et al., 2011)).

Yet, not all experimental data and activation models proposed for studied R proteins fit the I-2-based switch model. Alternative models suggest that instead of ADP-, the ATP-bound state reflects the resting state and that either hydrolysis and subsequent nucleotide exchange (for N; (Ueda et al., 2006)) or independent of nucleotide exchange/hydrolysis release of negative regulation (in case of RPS2 and RPM1; (Qi et al., 2010)) triggers NB-LRR activation. The latter model is derived from that proposed for Ced-4, an animal apoptosis regulator carrying NB-ARC domain (Qi et al., 2010). However, for the other metazoan apoptotic NB-ARC protein, Apaf-1, two competing (both different from the one presented for Ced-4) activation mechanisms have been recently proposed. The first one is similar to the I-2 switch model in which only ADP-ATP exchange is required for activation and not its ATPase activity (Riedl et al., 2005; Bao et al., 2007). The second model, however, proposed that both the active and resting state of Apaf-1 are (d)ATP bound. Following Apaf-1 activation, a single round of hydrolysis is induced after which the resulting (d)ADP is exchanged for (d)ATP allowing the formation of the active signaling complex (Kim et al., 2005). It remains to be determined whether the differences between the models are based on apparent differences caused by the experimental set up used. The first model is based on the analysis of a truncated version of Apaf-1, which lacks its C-terminal WD40 repeat, and is produced in insect cells (Riedl et al., 2005; Bao et al., 2007). The second model was based on the analysis of full-length Apaf-1 protein produced in *E. coli* (Kim et al., 2005). The actual protein activity might have been affected by the lack or presence of WD40 domain or by improper protein folding in one of heterologous expression systems (chapter 5).

Interestingly, the switch model for R protein functioning is independently supported by the analysis of different R proteins produced in various expressing systems. These proteins include the CC-NB-ARC domains of I-2 and Mi-1.2, full-length MLA27 and almost full-length M-flax and L6 proteins, produced in *E. coli*, insect cells and yeast, respectively ((Tameling et al., 2002; Tameling et al., 2006; Schmidt et al., 2007; Maekawa et al., 2011; Williams et al., 2011); chapter 5). For I-2 and M-flax proteins, the biochemical properties of the mutants have been linked to a phenotype by expressing the mutated proteins *in planta* (Tameling et al., 2006; Williams et al., 2011). The activation mechanism as proposed for N

protein has not (yet) been confirmed using either full length N or by using another R proteins, while the mechanism proposed for RPS2/ RPM1 is not based on actual biochemical data. This makes the switch model at the moment the strongest supported model from all R protein activation mechanisms proposed and future experiments should challenge its validity.

To conclude, the different models proposed for various plant and metazoan NB-ARC proteins indicates that they might differ in their activation mechanism and the role (d)ATP/(d)ADP have in this process (Kim et al., 2005; Riedl et al., 2005; Takken et al., 2006; Tameling et al., 2006; Ueda et al., 2006; Bao et al., 2007; Qi et al., 2010; Williams et al., 2011). The common feature is that in all models nucleotide-binding is essential and that nucleotide exchange/ hydrolysis might be important for regulation of their activities. To identify further conserved characteristics of NB-ARC proteins functioning it will be important to clarify whether the proposed differences for R proteins, Apaf-1 and Ced-4 activation are real or reflect artifacts induced by *in vitro* analysis of truncated proteins. If indeed different activation mechanisms exist, it would be interesting to analyze whether they correlate with the distinct subclasses or groups of plant NB-LRR proteins. To investigate the mechanism underlying R protein activation one needs to produce examples of various NB-ARC proteins and analyze their nucleotide-binding properties and the ability to hydrolyze ATP.

CONFORMATIONAL CHANGES UNDERLIE R PROTEIN ACTIVATION.

I-2 Y2H interactions with *Sl*Formin and *Sl*Trax show distinct, often opposite intramolecular patterns for the different truncations and mutants (chapter 3). The mutants were selected on their ability to affect either hydrolysis or nucleotide binding and represent respectively the active, -ATP-bound state - or an inactive state of I-2 (Tameling et al., 2006). The correlation between the (in)ability to interact in the Y2H and the proposed nucleotide binding state, indicates that each state exposes different interfaces allowing I-2 to interact preferably with either partner. Since two opposite Y2H interaction patterns were observed, this implies that at least two distinct I-2 conformations must occur. This data provides direct support for hypothesis that the conformation of an R protein depends on nucleotide-binding, hence activation state.

Rationally, it is predicted that the resting state of an R protein interacts with pathogen recognition components, whereas activation will trigger a conformational change allowing an interaction or a release of down-stream signaling components. For Apaf-1 and Ced-4 the activated state allows the formation of the oligomeric apoptosome and the subsequent interaction with procaspases-9 or Ced-3 respectively. Additionally, for Ced-4 its interaction with Ced-9 is released before it can form the signaling complex (Yang et al., 1998; Kim et al., 2005; Riedl et al., 2005; Yan et al., 2005; Bao et al., 2007; Qi et al., 2010). Hence, the activated conformation of these proteins allows them to oligomerize. Oligomerisation of their NB-ARC domains generates a wheel-shaped platform that brings the N-terminal CARD domains into close proximity. Activated in this way, the protein is able to recruit and activate the down-stream signaling components required to induce apoptosis (Yan et al., 2005; Qi et al., 2010). Interestingly, recently solved crystal structures of the N-terminal domains of two R proteins, the CC (coiled coil) domain of MLA10 and the TIR (Toll- Interleukin-1 receptor) domain of L6, demonstrated that both form homodimers in solution. This, together with the *in planta* analysis of dimerization mutants, provides strong evidence that oligomerization is important for R protein function as well (Bernoux et al., 2011; Maekawa et al., 2011). Likewise, the N protein, a member of the TNL (TIR-NB-ARC-LRR) class, was found to oligomerize upon pathogen perception (Mestre and Baulcombe, 2006). Interestingly, different than Apaf-1, Ced-4 and N, CNL R proteins such as Prf, RPS5 and MLA10 form homomeric-complexes prior to their activation (Ade et al., 2007; Gutierrez et al., 2010; Maekawa et al., 2011). Another essential distinction is that Apaf-1 and Ced-4 heptamerize or octomerize (tetramers forming an asymmetric dimer) via their N-terminal CARD and NB-ARC domains (Yan et al., 2005; Qi et al., 2010), while for R proteins only their N-terminal domains have been shown to be required for dimerization and it is unclear whether the NB-ARC domain is actually involved in multimerisation *in planta*.

Hence, the number of evidences that R proteins, similar to their animal counterparts, require oligomerization for signaling activity grows. However, if this oligomerization represents the active state, like for N or rather the resting state of R proteins, such as for MLA10, Prf, or RPS5 is still unknown (Mestre and Baulcombe, 2006; Ade et al., 2007; Gutierrez et al., 2010; Maekawa et al., 2011). Alternatively, there is no conservation in the way R proteins oligomerize and the moment of oligomerization differs for the TNL and CNL class, as

suggested in (Bernoux et al., 2011). Regardless if and how the oligomerization happens, the R protein conformation certainly changes upon activation in order to expose different protein interacting surfaces for a different set of interactors. It would be interesting to solve the structure of activated and resting states of an R protein, preferably for full-length and various domain truncations to be able to learn by comparison about the role of particular domains in the formation of different conformations. Knowing the exposed interaction surfaces for each conformation should help to identify interacting proteins perhaps from recognition and signaling R proteins complexes. To answer these questions one should obtain and compare structures of separate R protein domains, full-length R proteins that are bound either to ATP or to ADP, and mutants reflecting the different activation states. If sufficient amounts of pure proteins are produced, methods such as trypthophan fluorescence spectroscopy and circular dichroism could be explored to identify conformational changes, as initiated by us for MLA27 protein (chapter 5). Without having purified proteins possibly Y2H patterns with C- or N-terminal interactors, or *in planta* analysis using FRET (fluorescence resonance energy transfer) or FLIM (fluorescence lifetime imaging) could provide more insight in conformational changes occurring in R proteins.

MINIMAL FUNCTIONAL DOMAIN REQUIRED TO TRIGGER HR

Deletion studies of Mi-1.2 revealed that the Nt2-NB-ARC-LRR variant triggers a hypersensitive response (HR) upon over-expression in *N. benthamiana.* Apparently, removal of a small N-terminal region containing the SD1 subdomain causes autoactivation for this member of the SD-CNL class. All further truncations abolished the autoactivating phenotype, indicating that a near full-length protein is required to induce HR (chapter 4). Differently, for Apaf-1 and CED-4, rather their N-terminal domains have been shown to function as signaling domains recruiting down-stream signaling partners (Yan et al., 2005; Qi et al., 2010). Likewise, the N-terminal domain which encodes either a TIR or a CC domain is the smallest functional module required for HR induction for a number of TNLs: flax L10 and Arabidopsis RPP1 and, RPS4 and one of CNLs: MLA10 (Frost et al., 2004; Swiderski et al., 2009; Krasileva et al., 2010; Maekawa et al., 2011). Yet, for Rx, another CNL protein, over-expression of its NB subdomain, rather then its CC domain is sufficient to trigger HR

(Rairdan et al., 2008). Under our experimental conditions, neither the NB subdomain nor N-terminal domains of Mi-1.2 alone mediated HR when over-expression. A separate CC domain has not been tested.

Briefly, for the few examples of studied NB-LRR proteins the minimal functional domain required to trigger HR is their N-terminal domain, however for Rx and Mi-1.2 proteins other regions induce autoactivity upon over-expression ((Frost et al., 2004; Rairdan et al., 2008; Swiderski et al., 2009; Krasileva et al., 2010; Maekawa et al., 2011); chapter 4). Thus, is there a preferential signaling domain and any exceptions could be rather due to differences in experimental procedures, or are there simply dissimilarities in between various R protein (sub)classes? Identification of domains triggering HR upon over-expression *in planta*, testing and comparing their expression phenotype for more candidates from different R protein (sub)classes and subsequently pull-downs with these domains could provide more insight into events of signaling.

INTRAMOLECULAR INTERACTIONS IN R PROTEINS

Mi-1.2 N-terminus consist of common CC domain extended for characteristic and unique for solanaceous CC-NB-LRR proteins, SD domain. Through co-expression of particular regions of this N-terminus *in trans* with specific autoactivating mutants or truncations, its functional intramolecular interactions and its role in regulating the Mi-1.2 mediated HR were tested. The analyzed mutants were selected as they affect function of diverse (sub)domains, consequently mimicking different protein activation steps for which various requirement for the LRR domain has been previously demonstrated (van Ooijen et al., 2008 a). Only one of these mutants, the Mi-1.2 T557S substitution in the NB subdomain of an NB-ARC-LRR variant restored full autoactivity when co-expressed *in trans* in *N. benthamiana* leaves with the Mi-1.2 N-terminal SD and CC domains. None of the other autoactive mutants did functionally trans-complement with Mi-1.2 N-terminus (chapter 4). These findings confirms distinct functions for the particular (sub)domains in activation of Mi-1.2. They also imply that Mi-1.2 activation is a multistep process of which some steps require, next to LRR domain, the SD-CC domains *in cis* and others do not ((van Ooijen et al., 2008 a); chapter 4).

In addition, the function of the Mi-1.2 N-terminus has been explored, showing its negative and positive regulatory role on protein functioning (chapter 4). The negative regulatory potential of first N-terminal region (Nt1) of Mi-1.2 is in agreement with the observation that exchange of the Mi-1.2 Nt1 region with the corresponding region from the non-functional homolog Mi-1.1 (MiDS3) induces protein autoactivity (Hwang et al., 2000). Series of analogous domain-swap experiments revealed that the Nt1 region regulates the signal transduction by interplay with the LRR domain, which, apart of this clear autoinhibitory function, has positive potential required for pathogen recognition (Hwang et al., 2000; Hwang and Williamson, 2003). Thus, alike the N-terminus of Mi-1.2 (chapter 4), the LRR domain of Mi-1.2 (Hwang et al., 2000; Hwang and Williamson, 2003) and many other NB-LRR proteins (Lukasik and Takken, 2009) has also a dual regulatory potential. It is imaginable that the balance in opposite regulatory potentials of these domains is essential and regulates accurate R protein functioning. This balance can be achieved by complex, functional intermolecular interactions, which has been demonstrated using for instance Mi-1.2 LRR domain or CC and LRR domains of Rx in trans-complementation and physical interaction assays (Rairdan and Moffett, 2006; Rairdan et al., 2008; van Ooijen et al., 2008 a).

To conclude, different R protein activation steps have various *in cis* and *in trans* requirements for particular domains and the protein function is controlled by intramolecular interactions that keep the domains regulatory potential in balance. How do regulatory domains function together, interact with each other and influence each other's role? To answer this question we need to obtain protein structures, but also get more insight into the dynamic interactions between the subdomains. These experiments require trans-complementation assays, domain-swap experiments, *in vitro* Y2H interactions or *in planta* pull-downs and FILM/FRET monitored interactions.

OUTLOOK/ PERSPECTIVE

To get mechanistic insight into CNL and TNL activation and signaling we need to solve their structure and expand identification of their intra- and intermolecular interactions as well as their (in)direct interactions with pathogen delivered effector proteins. Only studying the composition and conformation of the recognition and signaling complexes of many various R

proteins will allow identification of resemblances and divergences among R protein individuals and/or (sub)classes. To conduct most of the proposed experiments it is essential to produce both (sub)domains and the full-length proteins in sufficient amounts. Unfortunately, here lays the bottleneck of working with R proteins. In this final chapter I speculate on why it is so difficult to produce R proteins and indicate possible solutions to overcome these limitations.

Over-expression of various R proteins, or their subdomains, *in planta* induces programmed cell-death as part of the HR preventing their accumulation ((Frost et al., 2004; Rairdan et al., 2008; Swiderski et al., 2009; Krasileva et al., 2010; Maekawa et al., 2011); chapter 4). However, in many cases over-expression could not be achieved at all, which indicates that plants thightly control R protein levels, possibly to prevent spontaneous activation of defence signalling ((Williams et al., 2011); chapter 4). Similar control mechanisms might be present in heterologous hosts, which prevent high accumulation of the R protein and restricts its production (chapter 5). It is unknown why accumulation of R proteins would be harmful to non-plant cells. Possibly, being ATPases, their enzymatic activity interferes with ATP-ADP homeostasis in the cell. Alternatively, R proteins might have an as yet unknown biochemical property that effects targets that are evolutionary conserved among prokaryotes and eukaryotes. Currently, the identity of such targets, and even if they represent proteins, is unknown. Pull-down experiments or yeast two-hybrid screens might be instrumental to identify such protein targets. Once we know these targets, perhaps they could be modified to improve R protein production in heterologous hosts.

An alternative explanation why R proteins are so difficult to produce in heterologous systems is that despite successful expression the proteins often form inclusion bodies (chapter 5). Their tendency to aggregate indicates inherent instability of the proteins. NB-LRRs are multidomain proteins, of which some form dimers *in planta* (L6: (Bernoux et al., 2011), MLA10: (Maekawa et al., 2011); N: (Mestre and Baulcombe, 2006), RPS5: (Ade et al., 2007), Prf: (Gutierrez et al., 2010)). The only soluble R proteins successfully purified so far where monomeric: MLA27, M and L6 (Schmidt et al., 2007; Maekawa et al., 2011). Notably, for both M and L6 a part of their N-terminal TIR domains had to be deleted to allow their production in the yeast *Pichia pastoris* (Schmidt et al., 2007; Williams et al., 2011). Even though this region was not required for homodimerisation of the TIR domain alone, the full-

length protein missing this region is not able to dimerize in the yeast two-hybrid system (Bernoux et al., 2011; Bernoux et al., 2011). It is possible that disrupting the ability to dimerize aids production and purification of these flax R proteins from *Pichia* and it would be interesting to explore this possibility for other TNL R proteins.

Besides homodimerisation, many R proteins interact *in planta* with chaperones like Hsp90 and RSI-2 and co-chaperones such as Sgt1, PP5, RAR1 (Bieri et al., 2004; de la Fuente van Bentem et al., 2005; Van Ooijen et al., 2010)}. These chaperones are probably required for proper folding and stabilization of their client R proteins, as exemplified by the reduced abundance of I-2 accumulation upon silencing of either *RSI-2*, *Hsp90* or *Sgt1* (Van Ooijen et al., 2010). Moreover, they are required for R protein functioning, as shown for instance for Rx (Boter et al., 2007; Rairdan et al., 2008), MLA10 (Shen et al., 2003; Shirasu, 2009), Mi-1.2 and I-2 (Van Ooijen et al., 2010). This folding machinery, which is likely to differ in heterologous systems, might be essential to produce correctly folded R proteins. Therefore, for R proteins whose over-expression does not trigger HR or HR can be suppressed, it could be an option to use plants as a protein production system (Sainsbury and Lomonossoff, 2008). Alternatively other eukaryotic systems can be explored in which these plant-specific components have been supplemented. *In vitro* translation could provide an alternative for the production of limited amount of proteins.

Being aware of the limitations and bottlenecks of working with R proteins, successful production of ADP-co-purified, hence correctly folded, full-length MLA27 protein (chapter 5) is a big step forward. Not only it might serve as a positive control for further attempts to heterologously express and purify other R proteins, but also it opens the way to obtain more detailed conformational and biochemical analyses of the MLA27 protein. These studies would greatly contribute to confirm our observation that R protein conformation depends on its nucleotide-binding state (chapter 3) and that different protein activation steps exhibit distinct, functional, intermolecular interactions (chapter 4). We also hope that the tools described in this thesis regarding not only R protein heterologous production and biochemical assays (chapter 5); but also monitoring of conformational changes by Y2H (chapter 3); and identification of phenotypes by *in planta* over-expression and domain trans-complementation assays (chapter 4) will be instrumental to further explore general and specific features of other R protein functioning.

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