Nucleotide-binding and molecular interactions of plant disease resistance proteins
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CHAPTER 5

R proteins:
molecular switches
controlled by their nucleotide binding status

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Plant resistance (R) proteins recognize pathogens and induce defence responses preventing disease. Most R proteins belong to the Nucleotide Binding – Leucine-Rich Repeat (NB-LRR) family. The central NB or NB-ARC domain classifies them as a member of the STAND (Signal Transduction ATPases with Numerous Domains) proteins. The nucleotide-binding domain of STAND proteins is proposed to act as an NTP-hydrolysing switch regulating signal transduction by conformational changes. Based on phenotypic and biochemical analysis of the tomato I-2 protein, a model has been proposed in which exchange of ADP for ATP switches the protein from its resting state to its activated state. Hydrolysis of the bound ATP into ADP returns the protein into its resting state.

To examine whether this “switch” model holds for other R proteins, we undertook a biochemical analysis of the potato Rx and the barley MLA27 NB-LRR R proteins. Full-length Rx and truncated variants thereof were expressed either in E. coli or using Pichia pastoris-based expression systems, and the produced proteins were purified and analysed for their ability to hydrolyse ATP. None of the Rx variants, nor the positive I-2 control produced in this way showed specific ATPase activity that exceeded that of the negative controls. In addition, none of the produced proteins co-purified with a nucleotide, indicating that the proteins are likely mal-folded. In contrast, MLA27 produced in insect cells was found to specifically co-purify with ADP implying a correctly folded protein. The observation that the nucleotide remained bound during gelfiltration provides the first direct evidence that a full-length, wild-type NB-LRR protein can form a stable complex with ADP. A correctly folded R protein materialised the use of tryptophan fluorescence spectroscopy as a mean to monitor conformational changes in an NB-LRR protein. Although large conformational changes in the spectrum induced by either increased temperatures or treatment with a detergent were readily observed, no major changes were recorded upon prolonged incubation of the protein with or without ADP. At this stage it is therefore unclear whether MLA27 can rebind ADP and whether nucleotide binding or dissociation triggers large conformational changes in vitro. Alternative methods are required to answer this question. In conclusion, our data provide direct support for the I-2- based switch model and the techniques developed and described here provide a good basis for further biochemical studies of NB-ARC-containing R proteins.
INTRODUCTION

Defence mechanisms of a susceptible plant can be breached by specialized pathogens leading to disease. A resistant plant is able to successfully defend itself against a pathogen attack and remains healthy. After recognition of a pathogen resistant hosts rapidly activates their defences, which often include a hypersensitive response (HR) at the infection site preventing further pathogen ingress. A pathogen discloses its presence by the secretion of effector proteins, some of which are recognized by the (resistant) host. These effectors are also called avirulence proteins (AVR) and the type of resistance they trigger is referred to as Effector Triggered Immunity (ETI) (Jones and Dangl, 2006). Both pathogen recognition as well as ETI-activation is mediated by specific proteins that are called resistance proteins (R proteins). Most R proteins belong to the large family of NB-LRR proteins as these proteins contain a central Nucleotide-Binding (NB) site and a C-terminal Leucine-Rich Repeat (LRR). Some NB-LRR R proteins carry an amino-terminal domain with homology to the Toll/interleukin-receptor (TIR) whereas others often contain predicted Coiled-Coil motifs (CC) in their amino-termini. Because of the observed homology between the NBS domains of plant R proteins and the human apoptotic protease activating factor 1 (Apaf-1) and C. elegans death protein 4 (Ced-4 protein) the NBS domain is also referred to as the NB-ARC domain (Nucleotid e-Binding, shared by Apaf-1, R proteins and Ced-4) (van der Biezen and Jones, 1998). NB-ARC-LRR proteins are structurally related to the mammalian NACHT-LRR proteins, many of which function in innate immunity and regulation of programmed cell death. Both the NB-ARC-LRR and NACHT-LRR proteins are members of the STAND (Signal Transduction ATPases with Numerous Domains) family of ATPases (Leipe et al., 2004). The nucleotide-binding domain of these multi-domain proteins is proposed to work as an NTP-hydrolyzing switch, regulating signal transduction by conformational changes. How this switch functions in R proteins has been the focus of our research group over the last decade and is summarized below (Tameling et al., 2002; Albrecht and Takken, 2006; Takken et al., 2006; Tameling et al., 2006; van Ooijen et al., 2007; van Ooijen et al., 2008; Lukasik and Takken, 2009; Takken and Tameling, 2009).

Currently there are no 3D structures available for any plant NB-ARC domain, but the structure of this domain in CED4 and Apaf-1 has recently been solved. The NB-ARC domain
was found to consist of four distinct subdomains: NB, ARC1, ARC2 and ARC3 (Riedl et al., 2005; Qi et al., 2010). The NB subdomain forms a ‘classical’ NTPase fold, and is predicted to form a parallel β-sheet flanked by α-helices. In the β-sheet, the strands β1 and β3 are associated with the P-loop and Walker B motif, respectively (Vetter and Wittinghofer, 1999). The ARC1 is predicted to form a four-helix bundle, while the ARC2 adopts a winged-helix fold; the ARC3 forms a helical bundle. Apaf1 and Ced4 both form a stable complex with ATP and ADP, respectively. The nucleotide is bound at the interface between the subdomains stabilizing their interactions and structuring the protein (Kim et al., 2005; Riedl et al., 2005; Bao et al., 2007; Qi et al., 2010). 3D modeling of the NB-ARC domain of R proteins, using Apaf-1 as a template, revealed that three of the four sub-domains are present, but that the ARC3 domain is absent (Albrecht and Takken, 2006; Takken et al., 2006).

The importance of the NB-ARC domain for R protein function is underscored by the observation that many mutations in the NB-ARC domain result in either inactive or autoactivating proteins that trigger defence signalling in the absence of a pathogen (Albrecht et al., 2003; Takken et al., 2006). Previously, we showed that the CC-NB-ARC domains of the two tomato R proteins I-2 and Mi-1.2 bind and hydrolyse ATP (Tameling et al., 2002). Biochemical analysis of two constitutively active I-2 mutants showed that these proteins were affected in ATP-hydrolysis (S233F and D238E), but not in nucleotide binding. When these mutations were combined with a mutation in the P-loop that disturbs ATP-binding (K207R) the autoactivation phenotype was abolished (Tameling et al., 2006). These results show that nucleotide binding is required for I-2-mediated defence signalling (Tameling et al., 2002). Yeast two-hybrid (Y2H) experiments revealed differences in the interaction patterns of two I-2 interacting protein with wild-type I-2 and ATPase and ATP-binding mutants of the protein, suggesting different conformations of the R protein depending on its nucleotide binding state (chapter 3). Our biochemical data support the concept of a conformational change as the I-2N•ATP complex was found to be less stable than the I-2N•ADP complex (Tameling et al., 2006). Together these data formed the basis of a model in which an R-protein acts as a molecular switch whose activation and conformation is controlled by its ability to bind and/or hydrolyse ATP. The model proposes that in the absence of a pathogen the NB-ARC-LRR protein is in the OFF-state (resting state). The presence of a pathogen encoded Avr protein is detected by the LRR domain that upon recognition induces a conformational change in the
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NB-ARC domain. This change allows the release of bound ADP and subsequent binding of ATP, which triggers a second conformational change. This second change releases the signalling potential of the R protein, possibly by the liberation of the N-terminal effector domain. The intrinsic ATPase activity of the R protein attenuates the signalling response and returns the protein to its resting state (Takken et al., 2006).

Although the nucleotide binding state and ATPase activity has now been analyzed for a number of STAND proteins, the role nucleotide binding has in signalling remains poorly understood and is controversial (Kim et al., 2005; Riedl et al., 2005; Ueda et al., 2006; Bao et al., 2007; Duncan et al., 2007; Marquenet and Richet, 2007; Qi et al., 2010; Williams et al., 2011). To address this issue we set out to investigate the nucleotide binding properties of three different R proteins. Besides I-2 we used the potato Rx protein that confers resistance to potato virus X (Bendahmane et al., 1999). For Rx much data is available on its intramolecular interactions in relation to its activation state. Another advantage of Rx is the availability of a large collection of mutants, a cloned elicitor (Coat protein of PVX) and the possibility to detect tagged-Rx \textit{in planta}. We included in our analysis also a non-\textit{Solanaceous} R protein, the barley MLA27 protein. This CC-NB-ARC-LRR R protein confers resistance to barley powdery mildew (Seeholzer et al., 2010). Heterologous production and isolation of the three R proteins was optimized and the purified proteins were analyzed for their ability to bind and hydrolyze nucleotides. The data obtained support the molecular switch model as proposed for I-2 and provides new leads for future studies addressing the molecular basis underlying R protein functioning.

RESULTS

\textbf{Heterologously produced Rx and I-2 do not exhibit ATPase activity \textit{in vitro}}

To analyse the ATPase activity of Rx we set out to heterologously produce the protein in \textit{E. coli}. Because we expected that production of full-length protein would be technically demanding, we also choose to produce a truncated form lacking the LRR domain, and thus only encompassing the CC and NB-ARC domains (RxΔLRR). Besides wild-type Rx, a K176R mutant was created for both full-length and the RxΔLRR variant. This P-loop mutation is predicted to abolish nucleotide binding and is intended to serve as a negative
control in the ATPase assays. All four constructs were cloned into pGEXT4-1 vectors for expression in E. coli.

![Image](image.png)

**Fig. 1. Production of full-length Rx and the truncated RxΔLRR variant in E. coli BL21 (DE3) cells**

Coomassie stained SDS-PAGE showing total protein content of E. coli BL21 cells expressing Rx full-length or RxΔLRR wild-type proteins before (1) and after induction with IPTG (2). IPTG-induced cells were lysed and after centrifugation the soluble fraction (3) and the “inclusion body” pellet (4) was loaded on gel. M = protein marker lane.

All Rx variants were successfully produced in E. coli, as exemplified in Fig. 1 for the wild-type Rx and RxΔLRR proteins. Addition of IPTG induced protein expression and resulted in the appearance of bands whose sizes corresponded to those predicted for the full-length (134kDa) and the truncated versions of Rx (81kDa) (Fig. 1, lanes 2). After lysis of the E. coli cells the majority of Rx was found in aggregates (inclusion bodies) (Fig. 1, lanes 3 and 4). Since the truncated protein (RxΔLRR) was produced in higher amounts than the full-length Rx protein (Fig. 1) we continued with production of the shorter variants. Both RxΔLRR wild-type and K176R mutant were isolated and refolded from solubilised inclusion bodies. Refolding was essentially done using the procedure described by Tameling and co-workers (Tameling et al., 2002) (Table 1; Fig. S1). As an internal control also wild-type I-2ΔLRR protein was refolded from inclusion bodies. Of the refolded proteins ATPase activity was assessed by measuring their ability to convert α32P-ATP into α32P-ADP. The radioactive reaction products were subsequently separated by thin layer chromatography (TLC) and detected using phosphoimaging (Storm, Molecular dynamics) (Tameling et al., 2002). As a positive control for the ATPase assay wild-type I-2ΔLRR protein that previously had been
purified from inclusion bodies (described in (Tameling et al., 2002; Tameling et al., 2006)) was included. Unlike the original batch of I-2ΔLRR protein neither the refolded I-2ΔLRR nor the RxΔLRR proteins showed ATPase activity exceeding that of the negative control (Table 1; Fig. S2).

The lack of ATPase activity could be due to improper refolding of the proteins isolated from the inclusion bodies. The refolding procedure is based on step-wise dilution of the protein-denaturing urea using dialysis. Other refolding methods (Patra et al., 2000; Tameling et al., 2002), including the commercially available protein refolding kit (Protein Refolding Kit (Pierce)) were applied, but none of these procedures yielded RxΔLRR proteins exhibiting ATPase activity (Table 1; Fig. S1).

Next, to avoid solubilisation and refolding, we focused on the soluble protein fraction present in the *E. coli* cell lysates. Although I-2ΔLRR and RxΔLRR could be GST-purified in small amounts from the soluble fraction, no specific ATPase activity exceeding background activity was detected for either of them (Table 1; Fig. S1 and S2). To increase the yield of soluble Rx proteins derivatives *E. coli* strain BL21 were used (Lucigen Corporation) that are optimized for the expression of toxic proteins. As can be seen in the Fig. 2, both RxΔLRR wild-type and the K176R mutant were successfully expressed in all four BL21 lines and could be GST-affinity purified from the soluble fractions. Derivative C41pLysS gave the highest expression of the RxΔLRR fragment (Fig. 2; line 3), and was therefore selected to produce the wild-type and P-loop mutants of RxΔLRR and I-2ΔLRR. All four Rx and I-2 derivatives were affinity-purified using their GST-tag and analysed for their ability to hydrolyse ATP. No specific ATPase activity was detected that exceeded that of the GST control. This low level activity is likely attributable to a contaminating ATPase that binds to the beads and co-purifies with the R protein fragments (Table 1; Fig. S2).
Fig. 2. RxΔLRR\textsuperscript{wt} and RxΔLRR\textsuperscript{K176R} GST-affinity purified from soluble protein lysates of four different \textit{E. coli} BL21 strains

Coomassie stained SDS-PAGE showing RxΔLRR wild-type and the K176R mutant produced in four different \textit{E. coli} BL21 (Lucigen) strains (1-C41; 2-C43; 3-C41pLysS; 4-C43pLysS). To estimate the amount of purified Rx protein, a known amount (0.5 µg) of BSA- bovine serum albumin protein was loaded as a reference.

The lack of specific ATPase activity could imply that the RxΔLRR proteins are not correctly folded when produced in \textit{E. coli}, regardless whether they are isolated from inclusion bodies or from the soluble fraction. Possibly bacteria do not possess the capacity for proper R protein folding. Therefore, expression of these proteins in the yeast \textit{Pichia pastoris} was explored. Although we could reproduce production of the flax NB-LRR protein M in this system, we were unable to produce Rx in \textit{P. pastoris} (data not shown; Schmidt et al., 2007). Production of the positive control shows that this system is able to express NB-LRR proteins, but apparently Rx has specific requirements that are not met by this yeast (Table 1).

To summarize, from the various expression and purification methods explored the highest yield of soluble RxΔLRR protein was obtained using \textit{E. coli} C41pLysS. This strain was also used to produce I-2ΔLRR. However, none of the purified proteins had detectable \textit{in vitro} ATPase activity as they were unable to hydrolyse radioactive α32P-ATP.

**Extraction and detection of nucleotides in \textit{E. coli} produced NB-LRR R proteins**

Besides mis-folding, an alternative explanation for the low intrinsic ATPase activity of the \textit{E. coli}-produced soluble I-2ΔLRR and RxΔLRR proteins is that they are locked in a specific nucleotide-bound state. For the structurally related NB-ARC proteins CED-4 and Apaf-1 it has been shown that a nucleotide remains bound during the entire purification procedure...
(Riedl et al., 2005; Yan et al., 2005). A tightly bound nucleotide in a purified R protein could block ADP-ATP turnover and thereby block ATPase activity.

Table 1. Expression and ATPase activity of Rx variants obtained using different methods for protein production and purification.

<table>
<thead>
<tr>
<th>Production of Rx and RxΔLRR</th>
<th>Expression</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Production in E. coli (BL21; DE3); plasmid: pGexT4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSTRxhis6 &amp; GSTRxΔLRR</td>
<td>Yes (Fig. S1A)</td>
<td>No (Fig. S2A)</td>
</tr>
<tr>
<td>Purification from soluble fraction (GST pull down)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solubilization: 8 M Urea  Refolding: Step-wise dialysis (Tameling et al., 2002)</td>
<td>Yes (Fig. S1B)</td>
<td>No (Fig. S2A)</td>
</tr>
<tr>
<td>Solubilization: 2M Urea, 12,5 pH  Refolding: dilution (Patra et al., 2000)</td>
<td>Yes (Fig. S1C)</td>
<td>No</td>
</tr>
<tr>
<td>Solubilization: 6M Guanidine  Refolding: Kit Pierce</td>
<td>Yes (Fig. S1D)</td>
<td>No</td>
</tr>
<tr>
<td><strong>Production in E. coli (BL21; Lucigen); plasmid: pGexT4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSTRxhis6 &amp; GSTRxhis6dLRR</td>
<td>Yes (Fig. 2)</td>
<td>Non Specific (Fig. S2B)</td>
</tr>
<tr>
<td><strong>Production in P. pastoris (GS115); plasmid: pPIC9</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rxhis6 &amp; RxdLRRhis6</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Production in P. pastoris (X33); plasmid: pPICZ</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>His9Rx &amp; His9RxdLRR (Schmidt et al., 2007)</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>GSTRx-c-mychis6 &amp; GSTRxdLRRc-mychis6</td>
<td>No</td>
<td>ND</td>
</tr>
</tbody>
</table>

To test this hypothesis the CC-NB-ARC domains of E. coli C41pLysS produced RxΔLRR and I-2ΔLRR wild-type, and their P-loop mutants were affinity-purified using their GST-tags. Nucleotides were extracted from proteins bound to the glutathione agarose-beads by denaturing the proteins with 60% methanol. Subsequently, a luciferase-based ATP Determination Kit (Molecular probes; A22066) was used to detect and quantify any ATP
released from the proteins. As a positive control for the ATP-detection assay known amounts of ATP were spiked into the reaction mixture after sample measurement.

Nucleotides were extracted from 150 pmol RxΔLRR wild-type protein or from the same amount of the corresponding P-loop mutant. In both extracts only trace amounts of ATP could be detected, which were estimated to be around 0.07 pmol. From 150 pmol of the wild-type I-2ΔLRR protein around 0.3 pmol of ATP could be extracted, while the extract from the P-loop mutant contained less than 0.03 pmol of bound ATP. Although the wild-type protein carried 10x more ATP than the P-loop mutant in both cases the amount of bound nucleotide was very low as compared to the amount of input protein and much lower than expected from a 1:1 protein:nucleotide ratio. These results indicate that the bulk of the purified R protein fragments do not co-purify with bound ATP.

To test whether the samples contained ADP rather than ATP, an enzymatic pyruvate kinase assay was used to convert ADP into ATP. The hence formed ATP could be measured using the optimized ATP determination assay. Although the positive control (spiked ADP) revealed that the assay functioned and is highly sensitive (detection level ∼1 pmol), all extracts were found to contain < 1 pmol of ADP per 150 pmol protein.

To summarize, neither ADP nor ATP was found to co-purify with *E. coli* C41pLysS-produced RxΔLRR or I-2ΔLRR proteins. These negative data imply that the inability to hydrolyse ATP by these purified proteins is not due to the proteins being locked into an ADP or ATP-bound state preventing nucleotide exchange, but rather suggest mis-folding of the proteins.

**Full-length MLA27 does co-purify with ADP**

The successful production of full-length, wild-type NB-LRR protein MLA27 from insect cell is described in (Maekawa et al., 2011). This group provided us with ~2.7 mg of the purified protein. To assess correct folding we set out to determine whether full-length MLA27 protein does co-purify with a bound nucleotide (Maekawa et al., 2011). For this the luciferase-based ATP/ADP identification and quantification method that is described before was employed. Instead of a methanol extraction we used a boiling step to release potentially bound nucleotides from the protein. The advantage of the latter method is that no additional components are added to the sample that might affect the enzymatic assay.
Fig. 3. Elution profiles of MLA and nucleotides from NAP5 gel filtration columns

Purified MLA27 was separated on NAP5 gel filtration columns and in each fraction the amount of ADP and protein was determined. ADP (blue diamonds) co-elutes with the MLA protein (red dots), but is also found in later fractions. ADP does not co-elute with the negative control, BSA, a non-ADP binding protein (green triangles). BSA eluted in the same fraction as MLA27. Protein concentrations were determined using SDS-PAGE and confirmed using a Nanodrop. ADP concentrations were identified using a luciferase-based ATP determination kit (see material and methods).

Despite the high sensitivity of the ATPase assay (detection level <<1 pmol of ATP), no ATP was detected in the supernatant obtained after boiling and pelleting 70 pmol (7.5 µg) of MLA27 protein. Direct assaying for the presence of protein-bound ADP was not possible, because MLA27 was stored in a buffer that contains 5 µM ADP. To remove unbound nucleotides, MLA27 was subjected to gel-filtration (NAP-5; GE Healthcare) and the fractions obtained were analyzed for the presence of ADP and protein. Bovine serum albumin (BSA) dissolved in the same ADP-containing buffer served as a negative control. Both BSA and MLA27 eluted in fractions ranging from 200-600 µl and peaked in the fraction eluting at approximately 400 µl. For ADP detection, the ATP assay was adjusted by adding pyruvate kinase and its substrate to convert ADP into ATP. No ADP was detected in the fractions eluting from the BSA-loaded column, but in the MLA-containing fractions ADP was present and this elution profile correlated with that of MLA (Fig. 3). We calculated the total amount of co-eluting ADP (202±26 pmol) to be approximately 43% of the amount of MLA27.
(471±42 pmol) in these fractions. Assuming that NB-ARC proteins such as MLA27 have one nucleotide-binding site, this indicates occupancy of the protein with ADP of around 43%.

Notably, the ADP concentration remained elevated in the later fractions that did not contain MLA27. We assumed that this is free ADP that dissociated from the protein while migrating through the column. Free ADP migrates more slowly and will therefore elute in later fractions. This hypothesis is consistent with the observation that ADP was not detected in the corresponding BSA reference fractions until elution of the column void-volume (±1 ml). Comparing the ADP concentrations in the fractions eluting between 200-900 µl with that of the BSA control revealed an MLA27-specific amount of 289±42 pmol ADP in the total eluate. This value implies that a third of the nucleotides dissociated from MLA27 while migrating through the column. As the protein first elutes around 5 minutes, this value indicates a relatively stable MLA27-ADP complex with a half-life exceeding 5 minutes. Taken together, we conclude that at least 61% of the purified MLA27 protein is ADP-bound when loaded on the column. This high occupancy indicates that the NB domain of the bulk of the purified full-length NB-LRR protein is properly folded and capable of ADP binding.

No conformational changes in MLA27 are recorded using tryptophan florescence spectroscopy

Based on our findings described in Chapter 3 we predict that the conformation of R proteins differs depending on its nucleotide binding state. Here we wanted to investigate whether such conformational changes in NB-LRR R proteins during dissociation and/or exchange of bound nucleotides can be detected. Access to correctly folded, full-length ADP-bound MLA27 created an opportunity to test various methods to measure conformational changes. The low concentration and limited amount of protein available requires a highly sensitive method. Since MLA27 protein contains 9 tryptophans (isoform MLA27-1 (Seeholzer et al., 2010)) the suitability of tryptophan fluorescence spectroscopy was explored. After excitation tryptophan emits its fluorescence in a spectral range from 300 to 350 nm depending on the polarity of the local environment. Hence, changes in fluorescence of this aromatic residue can be used as a proxy to study changes in overall protein conformation. To test if this method is applicable for MLA27 we examined whether changes could be measured in the fluorescence spectrum while unfolding/denaturing the protein. For this purpose MLA27 was incubated at
increasing temperatures and the fluorescence spectrum was measured at 15 °C intervals starting at 20 °C. As a reference MLA27 kept at 20 °C was used. The first change in the emission spectra between the reference and heat-treated sample was observed after incubation at 35 °C and the difference increased at higher temperatures (Fig. 4A). Besides temperature-induced denaturation also a detergent was used to induce unfolding. MLA27 was exposed to increasing concentrations of Sodium-dodecyl sulphate (0-500 µM SDS). After each SDS addition the tryptophan emission spectra were recorded for the treated and reference sample. Treatment with 75µM SDS caused the first detectable change in the spectrum indicative for a conformational change (Fig. 4B). These two experiments indicate that conformational changes of MLA27, likely caused by (partial) unfolding triggered by either increased temperatures or by SDS, can be monitored using tryptophan fluorescence spectroscopy.

Next, we examined whether tryptophan fluorescence spectroscopy might also be applicable to detect conformational changes of MLA27 during dissociation of ADP and/or re-binding of nucleotides. To mimic the empty state of MLA27, the protein was incubated with an access of EDTA to chelate ions of magnesium thereby removing the co-factor necessary for ADP binding. As a reference MLA27 was stored in a buffer with an access of ADP and lacking EDTA. Any difference in the spectra between these two treatments would indicate a difference in the conformation of these proteins that would be attributable to a difference in their nucleotide binding state: ADP bound versus empty. As can be seen in Figure 5, no difference was observed between the spectra, which would imply that there is either no rebinding or the predicted change is too small to be detected by this method.
Chapter 5

A

Fig. 4. Tryptophan fluorescence spectra of MLA27 upon temperature- or Sodium Dodecyl Sulfate (SDS)-induced denaturation

After excitation at 295 nm the emitted tryptophan fluorescence [counts/s] of MLA27 was recorded between 310-390 nm using a fluoro spectrophotometer. The ratio between the highest (355 nm) and the lowest count (315 nm) were plotted (Fluorescent Intensity Ratio F355/F315) to reveal differences in tryptophan fluorescence indicative for a conformational change of the protein.

A) The F355/F315 ratio of MLA27, incubated for 3 min. at increasing temperatures: 20°C, 35°C, 50°C, 65°C, 80°C, 95°C, was measured after each incubation step (MLA-T red dots). MLA27 protein not exposed to temperature changes was used as a reference (MLA-R blue triangles)

B) The F355/F315 ratio of MLA27 at increasing concentrations of Sodium Dodecyl Sulphate (SDS): 0, 25, 50, 75, 100, 150, 200, 250, 500 µM was measured (MLA-T red dots). As control MLA protein not exposed to SDS was used (MLA-R blue triangles)

In a second experiment we used MLA27 that had been subjected to gel-filtration to remove unbound ADP. Incubating the protein in a buffer without ADP should result in dissociation of the bound ADP eventually resulting in the “empty state”. Subsequent addition of an access of ADP could result in ADP re-binding and a possible conformational change. However, incubating the purified proteins in a buffer with or without ADP resulted in identical spectra. Assuming that ADP can be re-bound, this indicates that either there is no conformational change, or alternatively that the change is not detectable using this method (Fig. 5).

To conclude, dissociation of ADP did not result in a measurable change of the tryptophan fluorescence spectra nor did incubation of the “empty” protein with ADP. Since major conformational changes of MLA27 during physical or chemical denaturation were readily detected using this method, these results imply that, if conformational chances occur after ADP dissociation these are too subtle to be detected using tryptophan fluorescence spectroscopy.
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Fig. 5. Tryptophan fluorescence spectra of MLA27 incubated with or without EDTA and in presence or absence of ADP
MLA27 was incubated for 120 min. either with EDTA (1mM) (MLA+EDTA: green diamonds) or without EDTA (MLA-EDTA: yellow square).
Unbound ADP was removed from the protein storage buffer by running MLA27 on a desalting column (NAP-5).
The purified protein was subsequently incubated in a buffer without ADP (MLA-ADP: blue triangles) or with 5 µM ADP (MLA+ADP: red dots). Tryptophan fluorescence was to record after excitation at 295nm. The emission spectrum between 310-390 nm was recorded using a fluorospectrophotometer [counts/s]. Changes in the spectrum where depicted by plotting the fluorescence intensity ratios F355/F315.

DISCUSSION

Models for R protein activation
Although the first plant R gene has been cloned almost 20 years ago the molecular mechanism underlying R protein regulation and activation remain elusive. Using a biochemical approach we here show, for the first time, that an intact, full-length R protein co-purifies with a nucleotide. The finding that barley MLA27, a member of the plant NB-LRR proteins, is specifically bound to ADP is in line with previous studies that implied that nucleotide binding is important for the functioning of STAND proteins (Tameling et al., 2002, 2006; Kim et al., 2005; Riedl et al., 2005; Yan et al., 2005; Bao et al., 2007; Duncan et al., 2007; Marquenet and Richet, 2007; Qi et al., 2010; Williams et al., 2011). For MLA27 we measured a nucleotide occupancy of ~61% that was reduced to ~43% after gel filtration. These high
percentages indicate that the protein forms a relatively stable complex with ADP. The observation that wild-type MLA27 is ADP-bound provides direct experimental support for the ‘switch-model’ that proposes that the resting state of an NB-LRR R protein is ADP-bound (Takken et al., 2006; Tameling et al., 2006).

Our data are in agreement with those from a recent analysis of a near full-length flax M protein produced in the yeast *Pichia pastoris*. The wild-type NB-LRR M protein was also found to co-purify exclusively with ADP. Interestingly, an autoactive variant (M-D555V) co-purified both with ADP and ATP (Williams et al., 2011). These data provide direct support for the proposition that the ATP-state of the NB-LRR protein represents the activated state. Unfortunately, we could not perform similar experiments for MLA27 as production and purification of autoactive or loss-of-function mutants failed (data not shown). Nevertheless, the collective biochemical data obtained independently for I-2, M and MLA27 are all consistent with the switch model, making it a well-supported model. Besides the switch model for the activation of R proteins a competing model has been proposed that is based on the analysis of a single R protein fragment. The NB-ARC-LRR fragment of the tobacco N protein was produced in *E. coli* and found to bind ATP rather than ADP. *In vitro* incubation of the ATP-bound N protein fragment with the corresponding viral Avr protein induced ATP hydrolysis. Based on these data the authors proposed that the ADP state rather than the ATP state is the activated state for this protein and that ATP hydrolysis is required for R protein activation (Ueda et al., 2006). Besides these two models, a third model has recently been proposed for RPS2 and RPM1, two Arabidopsis proteins. This model lacks biochemical support and is solely based on the observed homology of these two NB-LRR proteins to CED-4. This third model proposes that NB-LRR R proteins are constitutively ATP-bound and activation occurs by releasing a constitutive negative regulation without the need of nucleotide exchange/hydrolysis (Qi et al., 2010).

Although the “switch” model gained most experimental support up till now, the other two models cannot be excluded at this stage and different models might apply to different classes of NB-LRR R proteins. A serious limitation of all models is that they are largely based on *in vitro* data generated using a very limited number of R proteins. Future studies should therefore include additional R proteins and should also analyze their nucleotide binding state *in planta* before and after pathogen perception. These experiments could reveal whether all R proteins
function the same mechanistically or employ different mechanisms as implied for other STAND members.

**Conformational changes in NB-LR R proteins**

A unifying theme for all STAND proteins is the key-role of nucleotide binding and the ability to switch from an auto-inhibited to an activated state. Regardless whether nucleotide exchange or hydrolysis transduces activation into signalling, in all cases a conformational change is proposed that switches the NB-LRR protein from an inactive to a signalling-competent state. In an attempt to visualize these conformational changes we explored tryptophan fluorescent spectroscopy. Using this technique we were able to detect major conformational changes that were induced by either chemical or physical denaturation of MLA27. The same method, however, failed to capture conformational changes upon dissociation and/or possible re-binding of ADP (Fig. 4 and 5). This negative result could either indicate that: a) there is no rebinding, b) there are no major conformational changes upon nucleotide (re)binding or c) that the method is not sensitive enough to detect such changes. To discriminate between these options it is essential to include nucleotide binding- and ATPase-mutants, which are unfortunately currently unavailable as the mutants were unstable hampering their production (data not shown).

As an alternative method to detect conformational changes in proteins the use of circular dichroism was examined. Pilot experiments showed that the amounts of MLA27 available to us were below the detection threshold of the equipment (data not shown). Since it is difficult to upscale protein production, alternative methods will be required to monitor dynamic differences in MLA27 conformation. It would be worthwhile to investigate whether, similar as reported for I-2 and its derivatives (Chapter 3), the yeast two-hybrid interactions of MLA27 with its N-terminal interactor WRKY (Shen et al., 2007) could serve as a proxy to monitor MLA27 nucleotide-binding depended conformational changes. Alternatively, to more directly measure conformational changes one could try to measure in planta difference in FRET (fluorescence resonance energy transfer) or FLIM (fluorescence lifetime imaging) of MLA27 variants fused to two different fluorescent pairs. If there are major conformational changes upon exposure to the Avr protein this might result in a shift in the fluorescence spectrum (Pietraszewska-Bogiel and Gadella, 2010).
Heterologous production of R proteins

The very limited amounts of MLA27 restricted a detailed biochemical characterization of this protein, such as measuring its ATPase activity or the Km for nucleotide binding. Difficulties in obtaining sufficient amounts of soluble NB-LRR proteins is a generic bottleneck as also exemplified in this chapter and by Maekawa and coworkers (2011). Notably, out of the 20 MLA alleles expressed in a variety of expression systems, only MLA27 could be successfully produced and purified. MLA27 is the first - and so far only - example of a truly full-length NB-LRR R protein heterologously produced in amounts that allow (limited) biochemical analyses (Maekawa et al., 2011). The M and L6 flax resistance proteins could only be produced in *P. pastoris* when respectively the first 21 or 29 amino acids of their N-terminal domains were removed (Schmidt et al., 2007; Williams et al., 2011). We could reproduce the production of the nearly full-length M protein using the *P. pastoris* expression system (data not shown), but our attempts to express Rx or truncated variants using the same methodology failed (Table 1). This negative result implies that the yeast system does not provide a generic expression system for the production of R proteins or R protein domains.

Expression and purification of RxΔLRR protein from *E. coli*, either from soluble fraction or after refolding from inclusion bodies, resulted in microgram amounts of protein (Fig. 1 and 2; Table 1). Unfortunately, none of the *E. coli* produced RxΔLRR proteins showed ATPase activity that exceeded background levels. This result could indicate either a mal-folded protein or lack of ATPase activity in Rx (Table 1). However, as also I-2, produced using the same procedures did not exert ATPase activity, this result indicates that the employed method is not suitable to produce enzymatically active R proteins. Furthermore, the soluble Rx proteins purified from *E. coli* BL21 C41Slys (Lucigen) did not co-purify with nucleotides providing additional support for the hypothesis that the *E. coli*-produced proteins are not correctly folded.

Until now the only (near) full length R proteins that have been produced relied on eukaryotic expression systems: MLA27 in insect cells and M and L6 in yeast. It is therefore plausible that a eukaryotic protein folding machinery seems essential for the production of correctly folded R proteins. Besides expression, a second bottleneck is the purification of these inherently unstable proteins. Nevertheless, the production of full length MLA27, its purification and subsequent analysis of its nucleotide binding state described in this chapter shows that it is
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feasible to overcome these hurdles. The generated data therefore not only provides support for the switch-model, but more importantly they describe a production- and purification method that can be employed for these multi-domain proteins.

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MATERIAL AND METHODS

Production and purification of Rx from E. coli BL21 (DE3).
The I-2ΔLRR wild-type and a K207R mutant cloned in a pGEXT4-1 vector was used as a positive control for production of NB-LRR proteins from E. coli BL21 and the ATPase assays (Tameling et al., 2002). The Rx containing plasmids encode either wild-type or the K176R mutant of full-length Rx or a truncated version that lacks the LRR domain (RxΔLRR). The full-length variants are fused N-terminally to gluthatione S-transferase (GST) and C-terminally to 6 residues of histidine (6xHIS), while the shorter variants (RxΔLRR), encompassing amino acids from 1 to 426, are fused N-terminally to a GST tag. Construction of Rx and RxΔLRR wild-type in pGEXT4-1 vector is described in chapter 3. The K176R mutants of Rx and RxΔLRR were cloned following the same cloning strategy as used for the wild-type variants. As donor plasmid pBAD: Rx and RxΔLRR constructs containing the indicated mutations were used. The latter constructs where a gift from H, Keller (Wageningen University, NL) (chapter 3).

Rx and RxΔLRR wild-type and K176R mutant were expressed as GST-fusion proteins from the pGEXT4-1 derived constructs using E. coli BL21 (DE3). Expression was induced by the addition of 1mM IPTG followed by a 4h incubation while decreasing the temperature from 37°C to 25°C. Cells were harvested by low speed centrifugation and lysed with lysozyme (1mg/ml), Triton-X (1%) followed by sonification (Patra et al., 2000). After centrifugation (12000xg for 15 min.) GST-tagged proteins were affinity purified from the soluble fraction using Glutathione Sepharose beads (Healthcare, Sweden). Expression, purification and renaturation of proteins from inclusion bodies was performed as described before (Patra et al., 2000; Tameling et al., 2002), or according to the instructions provided with the Protein Refolding Kit (Pierce). Protein concentrations were estimated either using Coomassie blue stained SDS-PAGE by comparing to 0.25, 0.5 or 1µg BSA as a standard or determined using a Bradford assay using BSA as a standard (Bradford, 1976).
Chapter 5

Construct preparation, production and purification of Rx from *P. pastoris*

A *BamHI/NotI* fragment coding Rx was isolated from pGEXT4-1-Rx vector (chapter 3) and ligated into pPIC9alpha vector (Invitrogen) digested with *BamHI* and *NotI*, which removes the plasmid-encoded signal peptide for extracellular secretion. The resulting plasmid codes for full-length Rx fused C-terminally to 6xHIS. To obtain a C-terminally 6xHIS tagged RxΔLRR construct a pBAD vector containing RxΔLRR was digested with *NcoI* and *AgeI*. The RxΔLRR encoding fragment was used to replace the full length Rx in pGEXT4-1-Rx previously treated with *NcoI* and *AgeI*. This cloning strategy resulted in a pGEXT4-1 plasmid encoding RxΔLRR fused C-terminally to 6xHIS.

In addition, constructs encoding different variants of Rx protein fused to various tags were made in the pPICZa vector (Invitrogen). To equip RxΔLRR N-terminally with 9 residues of Histidine, a PCR product was generated using primers FP1707 (TTT CGA ACA TGG GAC ATC ATC ATC ACC ATC ATC ACC ATC ATA TGG CTT ATG CTG CTG TTA C) and FP1708 (GGG GTA CCC TAA TTC ATG TTT CGA GCT TCC CTC) and pGEXT4-1:RxΔLRR as a template. The obtained product was digested and the *BstBI/KpnI* fragment was ligated to pPICZa vector digested with the same enzymes. The resulting plasmid encodes RxΔLRR fused C-terminally to 6xHIS.

Amplification with primers: FP1711 (CCC AAG CTT GGG CCC AGA ATG GGG TCC CCT ATA GGT TAT TGG) and FP1712 (GGG GTA CCT TCA TGT TTC GAG CTT CCC TC) on plasmid pGEXT4-1:RxΔLRR allowed equipment of RxΔLRR N-terminally with a GST tag. Two, a *HindIII/NcoI* and a *NcoI/KpnI*, fragments of this PCR product were ligated to pPICZa vector previously digested with *HindIII* and *KpnI*. The resulting plasmid encodes RxΔLRR protein N-terminally fused to GST and C-terminally fused to c-myc and 6xHIS originated from pPICZa vector. This plasmid, digested with *NheI/KpnI*, was used to ligate a PCR product amplified on pGEXT4-1:Rx with primers FP1709 (TGA TCA GCT AGC GGA CCG A) and FP1710 (GGG GTA CCT AGG TAC GCG TAG AAT CGA GAC).

Rx and RxΔLRR wild-type and K176R proteins encoded by pPIC9 vector were expressed in *P. pastoris* strain GS115, while pPICZa-based expression of different Rx variants was done in *P. pastoris* strain X-33. Yeast transformation and protein expression and purification was performed according to the instruction of Invitrogen. Detection of the protein in a total protein extract was done by western blots probed with a pentaHIS antibody (Qiagen). As a positive control for production of protein from *P. pastoris* and detection on western blot we included a construct expressing HA-tagged M protein from flax (Schmidt et al., 2007).
Production and purification of RxΔLRR and I-2ΔLRR from *E. coli* BL21 (Lucigen).

*E. coli* BL21 (Lucigen) was transformed with pGEXT4-1 plasmids encoding either RxΔLRR (both wild-type or the K176R mutant) or I-2ΔLRR (both wild-type and the K207R mutant) (Tameling et al., 2002). The RxΔLRR construct has an N-terminal GST and a C-terminal 6xHIS tag. Expression of RxΔLRR or I-2ΔLRR, wild-type and mutants was induced by incubating the cells with 0.5mM IPTG for 6h at 18°C. Cells were lysed by sonification on ice for 5 min. After centrifugation (12000xg for 15 min.) proteins were purified from the soluble fraction using Glutathione Sepharose beads (Healthcare, Sweden). The concentration of the purified proteins was estimated on SDS-PAGE gel stained with Coomassie blue by comparison to 0.25, 0.5 or 1µg BSA.

**Nucleotide extraction and ADP-ATP determination**

- **Methanol-based extraction of nucleotides from RxΔLRR and I-2ΔLRR variants**

  GST-tagged RxΔLRR and I-2ΔLRR wild-type and mutants were affinity purified using Glutathione Sepharose beads. Based on SDS-PAGE we estimated that of each protein around 750pmol was bound to the beads (total volume 1200 µl of 50% beads slurry). To extract protein-bound nucleotides, the beads were pelleted and semi-dry beads were overnight incubated with 60% of MeOH at -20°C. Subsequently the supernatant was isolated and freeze-dried and the obtained pellet was resuspended in 50 µl HPLC grade water (equivalent to 14.8pmol/µl of protein). A volume of 10 µl was used to detect and quantify nucleotides using an ATP/ADP determination luciferase-based kit (Molecular probes, A22066). Experiment was repeated three times.

- **Buffer exchange and extraction of nucleotides of MLA27**

  To determine whether MLA27 is nucleotide bound a buffer exchange step was required to clear ADP from the storage buffer (10µM MgCl₂, 10mM Tris pH 8, 100 mM NaCl, 5µM ADP). Gel filtration columns (NAP5; GE Healthcare) were pre-equilibrated with the buffer mentioned above but lacking ADP. Subsequently, 150 µl of MLA27 (105µg) protein solution was loaded and 350 µl of buffer was added. The protein was eluted using 1 ml of buffer and collected in fractions of ± 150µl. Bovine serum albumin (BSA) (0.7 mg/ml) dissolved in the same buffer was used as a reference. Protein concentrations in the fractions were quantified by comparison to a BSA standard on a Coomassie stained SDS-PAGE gel followed by measuring the intensity of the bands using adobe photoshop CS4. Protein concentrations were verified using a NanoDrop Spectrophotometer (ND-1000).

  The presence of ATP was independently determined tree times using the original MLA27 protein solution (1.4µg and 7µg), while ADP measurements were performed on the fractions obtained after gel filtration. Nucleotides were extracted from MLA27 by boiling the protein for 5 min. followed by a 2 min. centrifugation (14000xg) step. All gel filtration experiments were repeated twice and nucleotide determination of each fraction was done in duplicate.

- **ATP/ADP luciferase-based determination kit**

  Released ATP was quantified using an ATP determination kit (Molecular probes, A22066) according to the instructions given by the manufacturer. In brief, 2-20 µl of the sample was added to 200µl reaction solution and luminescence was recorded in time using a LKB-Wallac luminometer (1250). When luminescence stabilized, 5
or 10 pMol ATP was spiked in and luminescence was recorded until stable. The nucleotide concentration in each sample was calculated by comparison to this internal standard.

For ADP determination, 3µl pyruvate kinase (Sigma-Aldrich) and its substrate Phospho(enol)pyruvic acid monosodium salt hydrate (2.5 mM; Sigma-Aldrich) was added to convert ADP into ATP, the latter being detected using the above described method. As internal standard 5 pMol ADP was spiked in to monitor both the enzymatic conversion of ADP into ATP as well as the bioluminescence generated after consumption of the released ATP.

**Tryptophan fluorescent spectroscopy**

To detect denaturation-associated conformational changes, 20µl of MLA27 protein (0.75µg/µl) was exposed either to increasing temperatures (20°C - 95°C) or to increasing concentrations of Sodium-dodecyl sulphate (0-500 µM SDS). The volume of each sample was adjusted up to 200µl with storage buffer (10µM MgCl₂, 10mM Tris pH 8, 100 mM NaCl, 5µM ADP). The fluorescence spectrum emitted by the tryptophans in MLA27 was measured using a fluorescence spectrometer (PTI: Photon Technology International with Felix32 software and MD-5020 UV lamp) using quartz cuvettes (QS 10.00 mm Hellma) after 3 minutes incubation at 15°C intervals.

For SDS induced denaturation MLA27 was incubated for 1 minute at step-wise (25µM or 50µM) increased SDS concentration and the fluorescence spectrum was recorded. To detect conformational changes due to alterations in the protein-nucleotide binding state 1mM EDTA was supplemented to 20µl of MLA27 protein in its standard buffer (0.75µg/µl) and the emitted spectra were compared (assayed at 0, 5, 10, 15, 30, 60, 90, 120 min. counted starting from the moment of adding EDTA) with non-EDTA treated protein. Alternatively, ADP was removed from the protein by running it on a desalting column and 40µl samples of purified MLA27 (0.37µg/µl) were incubated simultaneously in storage buffer: one with and the other without 5µM ADP. Spectra were measured and compared every 30 min for 2h. Each experiment was done in duplo.
**SUPPLEMENTARY FIGURES**

**Fig. S1. Production of Rx variants in *E. coli* BL21 (DE3)**

A) Coomassie stained SDS-PAGE showing RxΔLRR wild-type (RxΔLRR<sup>wt</sup>) and the K176R derivative (RxΔLRR<sup>K176R</sup>). Proteins were GST-affinity purified from the soluble protein fraction obtained after lyses of *E. coli* BL21 (DE3). To estimate the protein concentration 2, 5 and 10 µl from total 300 µl were loaded on SDS-PAGE and compared to known amounts (0.25, 0.5, 1 µg) of BSA-bovine serum albumin protein.

B) Coomassie stained SDS-PAGE showing RxΔLRR wild-type (RxΔLRR<sup>wt</sup>) and the K176R mutant (RxΔLRR<sup>K176R</sup>). Proteins were purified from the inclusion bodies obtained after IPTG-induction and lyses of *E. coli* BL21 (DE3). The inclusion bodies were solubilised with 8M Urea and refolded using step-wise dialysis (Tameling et al., 2002). To estimate the protein concentrations 0.5, 1 and 5 µl from total around 5 ml were loaded on the SDS-PAGE and compared to known amounts (0.25, 0.5, 1 µg) of BSA-bovine serum albumin protein.

C) Coomassie stained SDS-PAGE showing RxΔLRR wild-type (RxΔLRR<sup>wt</sup>) protein purified from inclusion bodies obtained after IPTG-induction and lyses of *E. coli* BL21 (DE3). Inclusion bodies were solubilised with 2M Urea at pH 12.5 and refolded by dilution (Patra et al., 2000). To estimate the protein concentrations 2, 5 and 10 µl from total 17.5 ml were loaded on the SDS-PAGE and compared to known amounts (0.25, 0.5, 1 µg) of BSA-bovine serum albumin protein.

D) Coomassie stained SDS-PAGE showing RxΔLRR wild-type (RxΔLRR<sup>wt</sup>) protein purified from inclusion bodies obtained after IPTG-induction and lyses of *E. coli* BL21 (DE3). Inclusion bodies were solubilised with 6M Guanidine and refolded using eight different refolding solutions (1-8) (Pierce Protein Refolding Kit). To estimate protein concentrations 5 µl from total 1 ml were loaded on the SDS-PAGE and compared to known amounts (0.25, 0.5, 1 µg) of BSA-bovine serum albumin protein.
Fig. S2. ATPase assays of Rx and I-2 proteins variants produced in *E. coli* BL21 (DE3) or *E. coli* BL21 (Lucigen; C41pLysS).

A) RxΔLRR and I2ΔLRR wild-type proteins isolated from *E. coli* BL21 (DE3) lysates and either purified from inclusion bodies (IB) or from the soluble fractions using GST-affinity purification (GST). The obtained proteins were incubated for 0, 30 or 60 min with 5µM [α32P]ATP, with or without Mg2+. Reactions were subjected to Thin Layer Chromatography (TLC) and autoradiographed to identify the production of [α32P]ADP.

B) RxΔLRR and I2ΔLRR: wild-type (wt) and mutants proteins (K176R and K207R respectively) GST-affinity purified from soluble fractions of *E. coli* BL21 (Lucigen; C41pLysS) lysates were incubated for 0, 30 or 60 min with 5µM [α32P]ATP, with or without Mg2+. Reactions were subjected to Thin Layer Chromatography (TLC) and autoradiographed to identify the production of [α32P]ADP. Produced and purified in the same way proteins encoded by empty vector (pGexT4) served as a negative control for ATPase assay.

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