On the track to immunize plants

Bachelor Thesis II

Bachelor Molecular Biotechnology

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1. Abstract

German

In der Tabakpflanze codiert das Resistenzgen *N* für ein Rezeptorprotein. Dieses erkennt den Tabakmosaikvirus als Angreifer, wobei anhand einer Signaltransudktion es zu einer hypersensitiven Immunantwort und somit zu Nekrose kommt. Als Startsignal dient die Dimerisation von 2 TIR Domänen, jedoch sind die molekularen Abläufe hinter der Dimerbildung noch nicht aufgeklärt. In dieser Arbeit wird untersucht, ob die Stabilisierung von Dimeren aus Varianten der TIR Domän zu einem sensibleren Immunrezeptor führt. Dafür wurde mit Hilfe von einer ortsspezifischen Mutagenese 12 spezifische Substitutionen in die DNA Sequenz des Dimerinterfaces eingebracht. Dann wurden die NTIR Mutanten als NusA-NTIR-GFP-„Sandwich“-Plasmid generiert und zur Analyse im Größenausschluss-Chromatographen weiter-gegeben. Wie erwartet haben alle Varianten ein Signal eher zum Monomeren des nativen NTIRs gezeigt, als zum Dimer vom *RPS4*. Das Protein des Resistenzgens von *Arabidopsis*, *RPS4*, weist eine hohe Tendenz zur Dimerisation auf, dass es auch ohne in Anwesenheit eines Liganden zur spontanen Dimerisierung kommt. In einem Bioassay diente *RPS4* als positive Kontrolle. Die nächsten Schritte werden sein die vielversprechendsten Substitutionen zu kombinieren. Das eigentliche Ziel ist die Herstellung eines verbesserten, pflanzlichen Immunrezeptors.

English

In the tobacco plant, the resistance gene *N* codes for a protein which recognizes the tobacco mosaic virus as attacker that leads to a hypersensitive response causing necrosis. The signaling starts by dimerizing of the TIR domains of N. The specifications of this dimer are undetermined yet, therefore the dimerization ability requires further examination. This project investigated if stabilizing dimeric TIR domain variants will lead to a more sensitive immune receptor. Hereby through a side-directed mutagenesis 12 specific substitutions were introduced into the DNA sequence of the dimer interface. Then the NTIR mutants were generated as NusA-NTIR-GFP-’sandwich’ plasmid and tested further by size exclusion chromatography. As expected the NTIR variants showed a signal more as monomers like the native NTIR, not as dimers like *RPS4*. The *Arabidopsis* resistance gene *RPS4* has a high tendency for dimerization, thus without the presence of a ligand TIR domains spontaneously dimerize. In a bioassay, *RPS4* has served as positive control. Future work will utilize the most promising substitutions in combination. The eventual goal is to engineer an improved plant immune receptor.

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2. Introduction

The Problem: agriculture challenges in the future

By 2050 the world's population will reach 9 billion people, hence agriculture is not only facing a higher demand for food, but also shortening water supplies and limiting farming land. Moreover, climate change may even aggravate the availability of natural resources for agricultural use. The resulting weather fluctuations have increased requirements for future crops [1]. Nowadays, approaches for these challenges are diverse. On the one hand, there are numerous seed bank initiatives working to preserve crop diversity. One effort led by Cary Fowler of the Global Plan of Action on Plant Genetic Resources established the world's most secure and largest gene bank, Svalbard Global Seed Vault, on a Norwegian Island for the UN Food and Agriculture Organization, and was adopted by 150 countries 1996 [2]. On the other hand, there have been a number of technical advances that aim to address this challenge. For example, since the 1970s, the biotech-company Monsanto [3] has dominated the global market with its glyphosate-based herbicide, called Round-Up, with the concomitant development of genetically engineered seeds, which are resistant to the weed-killer. This combination of advanced technologies leads to an increase of monoculture efficiency, resulting in stable and high yields [4]. However, as Aglika Edreva states (2004), "Presently disease control is largely based on the use of fungicides, bactericides and insecticides – chemical compounds toxic to plant invaders, causative agents or vectors of plant diseases. However, the hazardous effect of these chemicals or their degradation products on the environment and human health strongly necessitates the search for new, harmless means of disease control." [5] Thus, plant pathologists work on a different approach to avoid the use of hazardous chemicals.

The study of the molecular mechanisms of plant resistance disease aims to find strategies to improve plant immunity to promote protection against disease, rather than treating disease when it occurs, and can be thought of as a vaccination program for plants. "Increased understanding of the molecular basis of disease resistance will not only answer basic biological questions on the mode of action of resistance genes, but will facilitate efforts to engineer crops for resistance to disease ", says Pamela C. Ronald (1997) [6]. The plant immune system can be subdivided into an outer and inner defense line; both can recognize specific pathogens and activate an immune response. The first, outer line consists of extracellular surface pattern recognition receptors, which detect pathogen-associated molecular patterns receptors and hence leads to intracellular signaling, transcriptional reprogramming, and biosynthesis of small molecule metabolites

to limit the pathogen's spread. But pathogens have developed strategies to overcome the cell surface barrier, thus the second inner line steps in, relying on resistance genes, or so-called *R* genes, encoding for a polymorphic family of intracellular receptors [7]. The interactions of the products of plant *R* genes with pathogens are central to the gene-for-gene hypothesis proposed by Harold Henry Flor (1955): "For each resistance gene in the host there is a corresponding gene for avirulence in the pathogen conferring resistance and vice-versa" [8]. Generally, research in this field seeks to examine two open questions. One is how exactly the products of *R* genes interact with their specific avirulence proteins (AvrP). A second is how this interaction triggers an immune response in terms of a molecular mechanism. In this study, I focused on an archetype disease resistance gene from tobacco, known as the *N gene*, and its interaction with tobacco mosaic virus (TMV) as a model to help address the fundamental questions in the field. The foundation of this work is a more detailed understanding of the *N* gene's structure and function.

The *N* gene: function and structure

By interacting with the TMV, the product of the *N* gene recognizes it as a pathogen and induces an immune response. This plant-pathogen interaction is one of the earliest and most studied, with classical genetic experiments showing that *N* is a single dominant gene [9]. In addition, follow on research identified the helicase domain (HEL) of TMV's replicase protein as the main trigger for immune reactions [10]. Thus, the interaction of the products of the *N* gene with HEL provides a classic example of the gene-for-gene hypothesis, and leads to a form of programmed cell death, which in plants is called the hypersensitive response (HR). The HR response promoted by the N-HEL interaction shows interesting temperature sensitivity [11]. Upon the identification and cloning of the *Nicotiana glutinosa N* gene, one of the first molecularly identified *R* genes in plants, significant clues have been gleaned on its biological function, primarily based on the organization of its structural domains [12]. *N* gene codes for a receptor protein of the second defense line in the innate immune system and the protein is composed of three domains.

Fig.1: Schematic representation of the N protein: the N-terminal Toll-interleukin receptor-like (TIR) domain, the central nucleotide binding (NB) domain and the C-terminal leucine rich repeat (LRR) domain.

An N-terminal Toll-interleukin receptor-like (TIR) domain is believed to be a signaling component for the HR reaction. A central nucleotide binding (NB) domain coordinates ATP binding and hydrolysis, which leads to the structural changes that activate an immune response. A C-terminal leucine rich repeat (LRR) domain is thought to play a role in TMV recognition [13]. Interestingly, not only is the full-length N protein required for the HR response to TMV or HEL but an alternatively spliced variant of 652 residues is needed for full resistance [14]. Additionally, early mutagenesis studies have implicated important roles for several amino acid residues in TMV recognition and immune signaling. Recently, structural studies have been used to identify specific amino acids in related plant TIR domains that are involved in immune signaling, but a mechanism has not yet been elucidated [15].

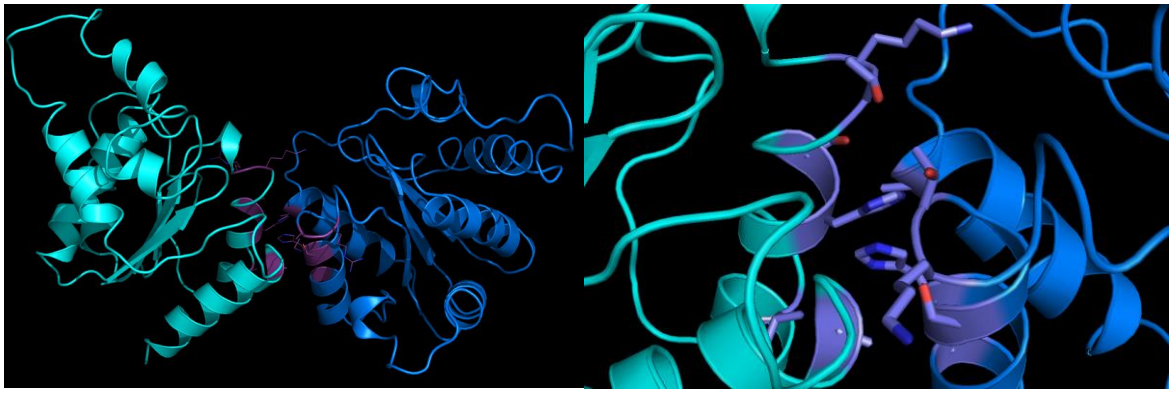

Fig.2: 3D model of two N-TIR domains generated through Rosetta. Left picture illustrates the positioning of two TIR monomers (cyan and blue) forming a dimer, while right picture zooms into the dimer interface (purple) and shows the amino acids aimed for modification.

This project's approach: TIR domain as effector module

The TIR domain is named based on its homology to the mammalian interleukin receptor and to the protein Toll found in *Drosophilia* [16]. Important clues for the role of the TIR domain in activating plant immunity come from structure studies. Maud Bernoux et al. (2011) clarify that: “Analysis of the structure combined with site-directed mutagenesis suggests that self-association is a requirement for immune signaling” and suggest distinct surface regions involved in self-association and signaling. In a broad spectrum of crops, such as potato, flax, tomato and also tobacco, different variants of TIR domains can be found [17]. Furthermore, in analyzing with the *Arabidopsis R* genes against *Pseudomonas syringae 4* (*RPS4*) and resistance *to Ralstonia solanacearum 1* (RRS1), Simon Williams et al. (2014) suggest that, “TIR domain hetero-dimerization is required to form a functional RRS1/*RPS4* effector recognition complex.” In this case both autoactive TIR domains of the *R* genes provide a model for analysis of the TIR domain of the *N* gene because of their “conserved TIR/TIR interaction interface” [18]. Because dimerization appears to be

an important attribute of TIR domain signaling, and plant TIR domains that self-associate can promote an HR reaction even in the absence of pathogen, the hypothesis for this research is that by stabilizing dimeric variants of the TIR domain of the N protein, it may be possible to engineer a more sensitive receptor to activate an immune reaction. The TIR domain of the N protein shows little if any tendency to self-associate, and unlike *RPS4*, which forms stable dimers, cannot itself elicit the hypersensitive response in plants. The rationale to generate stable NTIR dimers relied on using the laboratory's recently completed X-ray crystal structure (see Fig.2) of the domain to guide site-directed mutagenesis for replacing residues located at the putative dimerization interface. This interface is rather small for a protein-protein interaction (~1000-1400Å), quite polar, and not very hydrophobic. Therefore, the strategy involved inspecting the structure, focusing particularly on loop residues at interface regions 25-35 and 158-165, identifying non-conserved residues on the periphery and replacing them with bulkier, hydrophobic groups to increase association. The tendency of the variants to dimerize could then be evaluated *in vitro*, and promising candidates could be introduced *in planta* to examine a tendency to promote HR in an autoactive manner.

Fig.3: NTIR nucleotide sequence (cyan) with start codon in green, dimerization interface in purple and stop codon in red. The 181 amino acid long sequence is above the DNA.

To investigate the hypothesis, a side-directed mutagenesis system was developed to introduce twelve mutations into the NTIR at the region corresponding to signaling dimer interface. First these mutants were constructed in a pET-vector with an N-terminal NusA-tag that increases solubility which helps for later protein purification. Then the coding sequences were cloned into another plasmid with a C-terminal GFP (green fluorescence protein)-tag to enable screening of changes in quaternary structure, in this case monomers to dimer, in size exclusion chromatography (S.E. chromatography). Subsequently promising variants that showed display a tendency to self-associate would then be cloned into binary vectors to examine their ability to promote the hypersensitive response in plants.

3. Results

Mutagenesis System

Fig.4: Scheme representing the mutagenic method. Each fragment (vector backbone in blue, NusA-side in orange, GFP-side in green) is amplified with appropriate primers (arrows). The mutagenic primers do not bind 100% to the template because of the mutated sequence.

A collection of different point mutations were introduced at the dimerization site of the TIR domain to examine the mechanisms behind the dimerization and the consequent signaling. Specific amino acid substitutions were constructed using polymerase chain reaction (PCR) and Gibson Assembly (GA). This procedure involved the generation of two fragments for each mutant, each using one mutagenesis primer and one extension primer. In addition, the designed primers include those that introduce specific amino acid code changes and those that overlap sequences of a pET22bb-vector for the subsequent GA.

Determination of amino acids substitutions for TIR variants

The specific amino acid substitutions were following:

Substitutions	K25V	K25I	K25L	T26V	T26I	T26L	S29A	S29V	H30A	V34I	V34L	A163V

Tab.1: The substitutions were constructed at different positions indicated by the number while the letters precise the amino acid change using the one letter codes. For example: the first substitution is lysine (K) at position 25 of the 181 amino acid long TIR domain to valine (V).

Construction of TIR variants by PCR

The first step was to construct two mutant fragments by using appropriately designed primers in a PCR. A table with the primers and their melting temperatures (Tm) can be found in the Annex (Materials and Methods). Each reaction contained the same template, consisting of a plasmid with the wild-type TIR domain cloned as a TEV protease cleavable NusA fusion, two primers (one mutant primer, C1-D12, and one extension primer, either H7 or NusA-C), buffer, dNTPs, $MgSO_4$ and KOD polymerase. Occasionally, for

amplification reactions that yielded little or no product, up to 4% DMSO was included in the amplification reaction. The annealing temperature was set 5°C below the lowest primer Tm and the extension time was based on the size (bp) of the expected product. The second step was to purify the PCR products. The products of the amplification reactions as well a DNA ladder were loaded on a 1%-agarose gel in the presence of ethidium bromide to visualize their size under UV-light. Amplified fragments of the appropriate size were isolated from the agarose for subsequent purification using the Wizard® SV Gel and PCR Clean-Up System protocol. Eventually**,** the concentration of the purified DNA-fragment was quantified with a NanoDrop Spectrophotometer (a typical spectrum is enclosed in Annex, Appendix 1.).

Substi-tution	mutant primer	extension primer	template	concentra-tion (ng/µL)	fragment size	pmol/ µL
K25V-C	C1	H7	pET22b-NusA-Link-NTIR-D5C	88.8	507	0.273
K2V5-IC	C2	NusA-C	pET22b-NusA-Link-NTIR-D5C	60.4	746	0.133
K25I-C	C3	H7	pET22b-NusA-Link-NTIR-D5C	60.1	507	0.185
K25I-IC	C4	NusA-C	pET22b-NusA-Link-NTIR-D5C	41.3	746	0.091
K25L-C	C5	H7	pET22b-NusA-Link-NTIR-D5C	66.9	507	0.206
K25L-IC	C6	NusA-C	pET22b-NusA-Link-NTIR-D5C	37.2	746	0.082
T26V-C	C7	H7	pET22b-NusA-Link-NTIR-D5C	56.6	507	0.174
T26V-IC	C8	NusA-C	pET22b-NusA-Link-NTIR-D5C	69.4	746	0.153
T26I-C	C9	H7	pET22b-NusA-Link-NTIR-D5C	73.4	507	0.226
T26I-IC	C10	NusA-C	pET22b-NusA-Link-NTIR-D5C	61.4	746	0.135
T26L-C	C11	H7	pET22b-NusA-Link-NTIR-D5C	48.6	507	0.150
T26L-IC	C12	NusA-C	pET22b-NusA-Link-NTIR-D5C	57	746	0.125
S29A-C	D1	H7	pET22b-NusA-Link-NTIR-D5C	55.2	507	0.170
S29A-IC	D2	NusA-C	pET22b-NusA-Link-NTIR-D5C	28.6	746	0.063
S29V-C	D3	H7	pET22b-NusA-Link-NTIR-D5C	58.5	507	0.180
S29V-IC	D4	NusA-C	pET22b-NusA-Link-NTIR-D5C	25.1	746	0.055
H30A-C	D5	H7	pET22b-NusA-Link-NTIR-D5C	52.1	507	0.160
H30A-IC	D6	NusA-C	pET22b-NusA-Link-NTIR-D5C	34.2	746	0.075
V34I-C	D7	H7	pET22b-NusA-Link-NTIR-D5C	52.3	507	0.161
V34-IC	D8	NusA-C	pET22b-NusA-Link-NTIR-D5C	44.5	746	0.098
V34L-C	D9	H7	pET22b-NusA-Link-NTIR-D5C	42.9	507	0.131
V34-IC	D10	NusA-C	pET22b-NusA-Link-NTIR-D5C	44.2	746	0.097
A163V-C	D11	H7	pET22b-NusA-Link-NTIR-D5C	105	96	0.139
A163V-IC	D12	NusA-C	pET22b-NusA-Link-NTIR-D5C	45	1160	0.062

Tab.2: Overview of the PCRs and their purified yield. For each substitution two fragments were generated with a different primer pair to get a complement (i.e.: K25V-C) and an inverse complement fragment (i.e.: K25V-IC). The template used for all reactions was the pET22b-NusA-NTIR plasmid, and primers contained appropriate overlapping sequences to enable GA. The concentration of the PCR products in pmol per µL was calculated using the formula in Fig. 4. Additional data can be found in Annex, Appendix 1.

GA of plasmids containing mutationally altered TIR domains

GA was used to construct plasmids to express the modified TIR domains for screening. The plasmids were assembled from pairs of the newly-constructed fragments, which contained the same specific substitution, and the pET plasmid backbone. The amount of pET-vector in pmol/µL was calculated by having a concentration of 100ng/µL and a length of 6342bp with the following formula:

$$X\frac{ng}{\mu L} * (\frac{nmol}{Ybp})^{-1} * (\frac{650ng}{mol})^{-1} * \frac{10^3 mol * pmol}{nmol} = \frac{Zpmol}{\mu L}$$

Fig. 5: Formula to convert DNA concentrations X into Z pmol/µL knowing its length Y. 650 is the molecular weight of an average nucleotide.

Thus, the amount of vector used was 0.0243pmol/µL and a 3 to 5 fold excess of each fragment was needed for efficient GA. The fragments were mixed in appropriate amounts, GA Master Mix was added, and after incubation, the reaction mix was used for an electroporation transformation. After the electroporation of DNA into competent *E. coli* TOP 10 cells, 1mL SOC media was added and the culture was grown in a shaking incubator for 1h at 250rpm and 37°C. Thereafter the liquid bacteria culture was plated on LBamp agar media and placed in a 37°C incubator. 12 to 24h later the plates showed colonies indicating a successful plasmid assembly and transformation in contrast to a negative control reaction which contained only vector and GA mix, but no fragments.

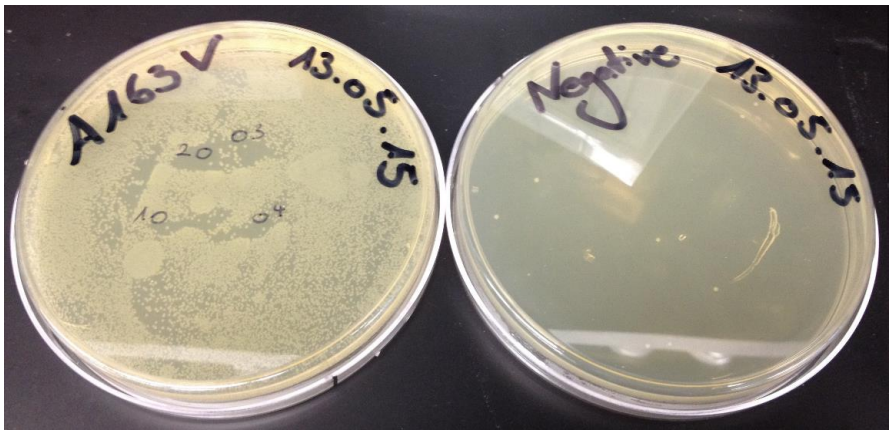

Fig. 6: Representative transformation results of a GA to construct modified TIR domains. The left plate with colonies is containing the substitution A163V representative for all created TIR mutants. The LBamp media plate is covered with numerous colonies after successful GA and transformation, because the generated plasmids carry a resistance gene for Ampicillin. On the right is a negative control showing that just the vector does not contain compatible ends for GA and therefore does not lead to colony growth.

Screening for positive colonies by Miniprep and restriction enzyme digestion (RE)

Four colonies from each plate were randomly selected and grown over night in 2mL LBamp. The next day, 1.5mL of each *E. coli* culture was used for minipreparation of plasmid DNA. The prepared plasmids in TE buffer are screened with an RE digest. The sequence of pET-NusA-Link-TEV-NTIR presents several RE sites to confirm proper assembly. As shown in Fig.6, I chose NdeI and HindIII as enzymes for the screening since they gave easily resolvable bands by agarose electrophoresis. Incubation should take over 1h at 37°C and the samples should be loaded on a 0.8%-agarose gel. The used pET sequence is added in the Annex (Appendix 2.)

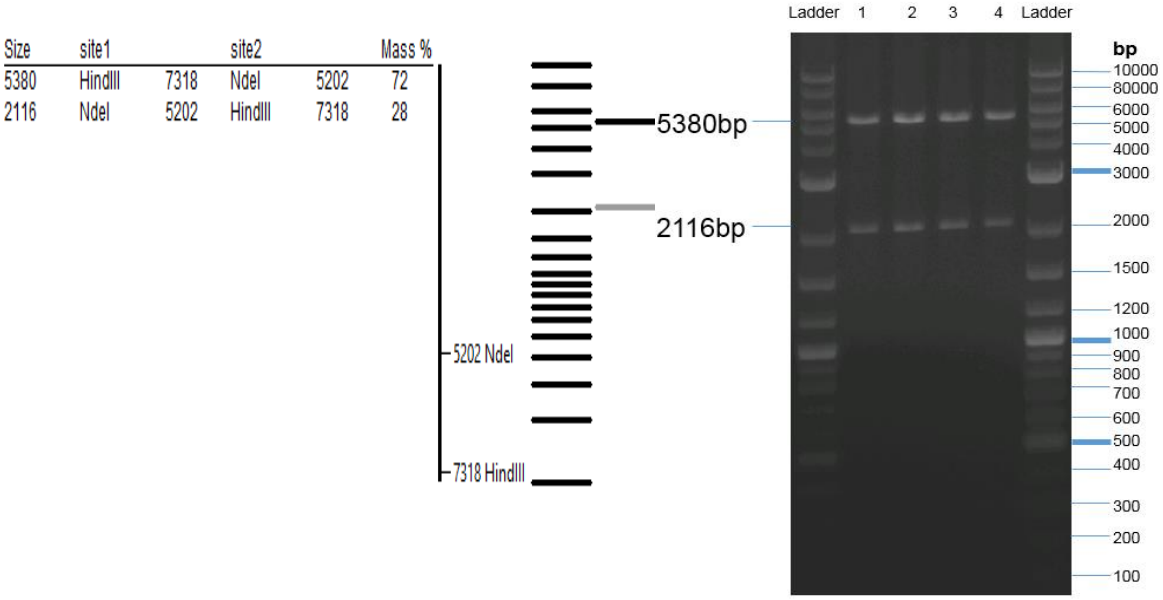

Fig. 7: On the left: expected restriction enzyme digest of a correctly assembled expression plasmid containing the tobacco NTIR gene. Two fragments of 5380 and 2116 bp would result after a restriction enzyme digestion of the assembled plasmid with NdeI and HindIII. On the right: The gel shows the expected bands.

Selection of positive colonies and isolation of plasmids

One of the positive clones was selected and the remaining 0.5mL of the original culture was used to inoculate 7.5mL LBamp. Plasmids were prepared from overnight cultures using the Wizard® *Plus* SV Minipreps DNA Purification System to generate a large quantity of high quality plasmid suitable for confirmation by DNA sequencing. Plasmids concentrations of over 120ng/µL were prepared to ensure a reliable sequencing reaction in order to validate of the appropriate substitution as well as to exclude any unwanted mutations in the TIR sequence resulting from PCR. In case the yield of the culture is too low, the samples are concentrated by centrifugation under a vacuum. A typical result from a sequencing reaction is enclosed in the Annex (Appendix 3.).

PCR Amplification of the NusA-NTIR variants for assembly into a GFP-vector

In order to screen the TIR variants for a tendency to dimerize, the next step was to clone the plasmids from the NusA vector to another vector to add a GFP-tag to the TIR domain. This would enable S.E. chromatography to capture monomer or dimer signals. The TIR mutants were cloned by amplifying the NusA-NTIR plasmids with a primer designed to anneal to the 5'-region of the NTIR gene but contained an extension sequence complementary to pET22b, and another primer to anneal the 3'-region of the NTIR gene and contained an extension sequence for the linker prior to GFP. This yielded a PCR product containing the mutant NTIR gene with overlapping sequences to the GFP-pET22b-vector. Thus, the isolated plasmids served as templates for another round of PCR using the F1 and TIR E4 extension primers. This reaction should result in the amplification of the NusA-NTIR sequence yielding a ~2000bp long fragment. Again a 0.8%-agarose gel helps to check and separate the reaction product.

Fig. 8: Picture of a 0.8%-agarose gel with 1% ethidium bromide under UV-light. The samples K25V and K25I showed the expected band at 2000bp and a band at 500bp, which is dispensable.

The amplified fragments are then assembled with GA and used to transform *E. coli*. These constructs, containing the TIR mutants in the GFP-vector, were given to Dr. Jim Parsons for an analysis of their expression and the molecular size of the resulting protein.

Producing a NusA-NTIR-GFP -'sandwich'

Surprisingly, neither wild-type nor the mutationally modified NTIR genes were able to be expressed in bacteria as C-terminal GFP fusions for size analysis. Therefore a different approach was needed to generate material for the structure screen. Since the laboratory uses a vector containing an N-terminal NusA-tag to express the TIR domain of the *N* gene

in a soluble manner, the ability of a 'sandwich' construct containing an N-terminal NusA-tag fused to the NTIR gene with a C-terminal GFP-tag was evaluated for expression. This new approach was successful with the wild-type NTIR gene, so we decided to use it for the NTIR mutants. Using the sequence-verified clones as templates, the primers F1 and TIRE4 were used to amplify the fragments corresponding to the NusA-NTIR region. The fragments were purified from agarose gels, and then used for another round GA into a vector containing GFP. The reactions were used to transform *E. coli*, and colonies screened with RE to verify the 'sandwich' construct. The same enzymes, NdeI and HindIII, were used for screening, resulting in two bands of 2881 and 5380bp. All positive clones were purified, concentrated and confirmed by DNA sequence analysis (see Annex, Appendix 4).

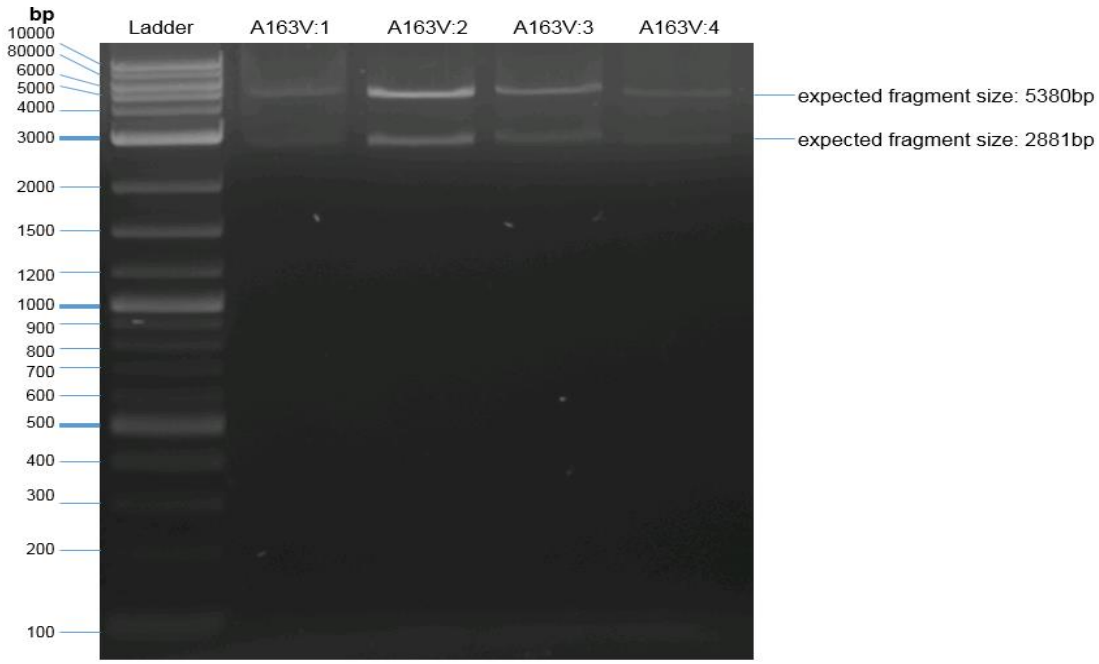

Fig.9: The agarose gel reveals bands under UV-light, whereby on the far left is the 2 log ladder used as a standard with annotated fragment size in base pair (bp). All samples, A163V:1, 2, 3 and 4, show expected bands at 5380bp and 2881 bp length.

Testing constructed TIR variants with S.E. chromatography

The confirmed constructed Nus A-NTIR-GFP 'sandwich'-plasmids were given to Dr. James Parson. The plasmids were transformed into *E. coli* BL21 (DE3) for efficient protein expression. The large scale cultures showed already a green color indicating that the GFP-tagged protein was highly expressed. Then the protein was purified and analyzed by S.E. chromatography. This chromatography was designed to test whether the NTIR-variants showed an increased tendency to form a dimer by following the GFP signal at 490nm. The native NTIR protein fused with a NusA-tag and GFP-tag serves as a

reference. An overview table of testing all TIR variants, as well as a graph with two *RPS4* mutants is added to Annex (Appendix 5.) A previous analysis of *RPS4* variants showed the left shift and thus the shortening of retention time when a dimeric variant is compared to a monomeric counterpart.

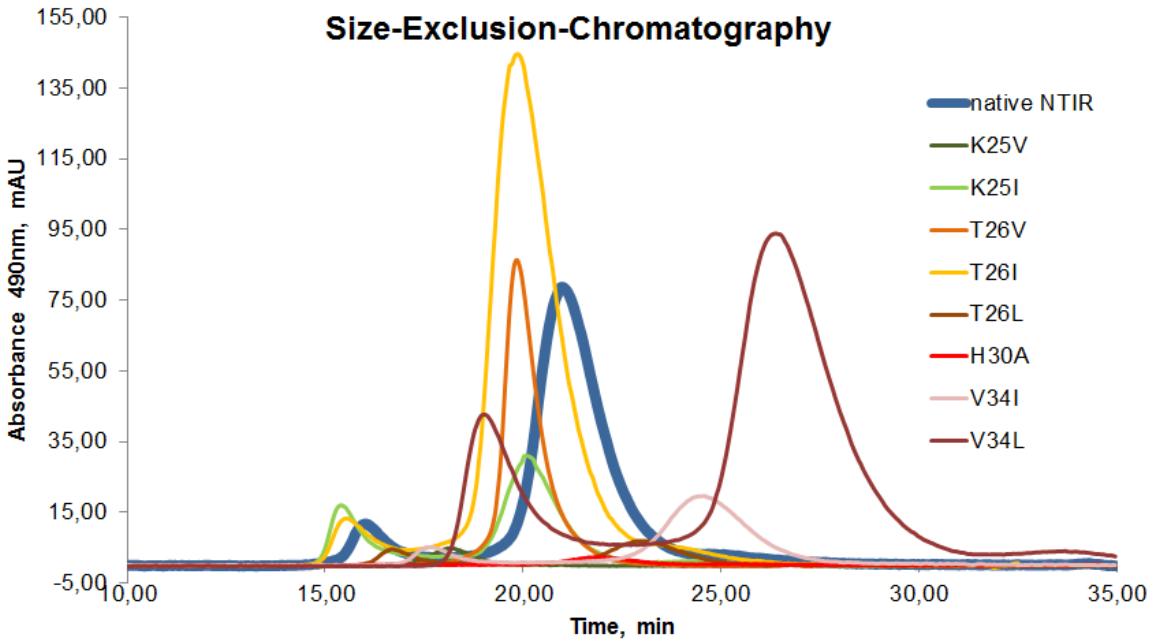

Fig.10: Graph representing the data collected from the chromatograph. The X axis is the time in min and the Y axis is the absorbance reporting the measured GFP signal at 490nm. The data of native NTIR (thick blue) serves as reference. The first peak at 16 min is aggregated material, the second peak at 21min is the eluent, which is the soluble monomer or dimer fraction and the last bump at 25min is residual small molecule absorption from the extract. In a S.E. chromatography the smallest particles leave the column first, therefore dimers and then monomers go through the filtration gel. While the TIR variants of K25 (greenish) and T26 (yellowish) show the right phenomenology (compare with Annex, Appendix 5.) of a risen tendency for dimerizing to native NTIR, the substitutions H30A, V34I and V34L have a disruptive effect and tend even more to monomers.

Bioassay in tobacco plants

An eventual goal is to test the effects of the TIR mutations *in vivo*. Therefore, transient expression of the TIR genes was investigated using *Agrobacterium tumefaciens*. Initial studies were conducted using the *RPS4* TIR domain since it strongly self-associates and promotes HR in the absence of pathogen. Thus, it serves as a useful comparison and positive control for future engineered dimeric TIR domains. *A. tumefaciens* cultures containing the *RPS4* TIR domain were grown in liquid YEP media at 28°C with shaking at 300rpm overnight. The cells were pelleted by centrifugation and resuspended in infiltration buffer containing MES, $MgCl_2$ and acetosyringone, the cell density was adjusted to an OD_{600} of 3.0 and the cells incubated for 3h. Afterwards, leaves of *N. benthamiana* are

infiltrated with a blunt end syringe. After four days, the zones of infiltration are examined to assess the level of necrosis. These initial studies established the feasibility of examining NTIR variants for their ability to promote HR by transient expression using *A. tumefaciens*.

Fig.11: On the right is a scheme giving the different mixtures out of four components indicating the four infiltration sides. On the left side, three days after infiltration leaves are showing necrosis (grey marks) on ingress sides of mixtures containing the HR activating *RPS4* agrobacteria culture. GFP containing cultures are added to detect component degradation in case of lack of necrosis. PZP is a suppressor which inhibits the plant cell's nucleases and it enhances the signaling through dimerization. "4" does not contain the self-reactive component RPS4 and as expected no signs of an immune response are shown.

4. Discussion

The goal

The goal of my project was to lay the foundation for evaluating the ability of the TIR domain of the tobacco *N* gene to dimerize and therefore promote HR in plants. This would be a significant step in engineering plants to respond to pathogens with an immune response signal. Firstly, twelve different TIR variants were constructed that introduced single amino acid substitutions at the dimerizing interface. Some of these changes should on one hand, have disruptive effects, but on the other hand, could also stabilize the dimer by providing increased hydrophobic surface to increase the interaction between monomers. Some of the NTIR variants are still in the process to be examined by the lab for their dimerization ability. This work established a system to test the hypothesis that stabilizing TIR dimers would increase the ease of immune signaling and thereby pave the way to engineer more sensitive immune receptors.

Future work

Although it was not expected that a single mutant would convert the monomeric NTIR domain into a stabilized dimer, like *RPS4*, the aim was to glean information on the substitutions that would contribute the most to dimerization. Follow on studies would involve combining the “best” substitutions, including mutations at other positions, to achieve the desired phenotype. The current rationale was to avoid interface residues that were highly conserved, since their substitution could destabilize the monomer. However, there could be merit in considering those targets, more extensive mutagenesis is required to generate stable dimers. During the course of my work, the PCR protocol to generate the fragments for each mutant as well as constructing the expression vectors were optimized, hence the parameters I developed, including annealing temperature, requirement for DMSO and template concentration, will have bearing on further work on the construction of dimeric NTIR domains.

The troubles

The initial strategy to express TIR-variants for structure screening using a GFP-tag in the pET-vector was unsuccessful. The preparation of large-scale cultures to yield the engineered proteins revealed that the GFP-fusion proteins did not express. This is an interesting outcome because for many proteins studied in the lab, the superfolded GFP-tag tends to increase protein solubility and therefore, benefits its expression [19]. To overcome the technical limitation, it was decided to generate a ‘sandwich’-plasmid with

the NusA and GFP flanking the TIR domain still in the pET-vector. When fused to a target protein, NusA increases protein stability and thus its expression, and this was seen for the NTIR-GFP constructs. The subsequent large-scale cultures of the various NTIR mutants showed a yellowish green color, which indicates high GFP-protein expression.

Indication for future infiltration experiments

In regard to the infiltration experiments, the observed results were unsatisfying. Over the project's span, only one of the numerous experiments showed necrosis symptoms in the expected time period of three to four days following infiltration. Despite the efforts to optimize all conditions following the agroinfiltration protocol [20], the symptoms were delayed over a week or never appeared at all. To assure that the products of the infiltrated genes were not degraded, controls were regularly performed in which the reaction components were mixed with a GFP-expressing construct and observed using a fluorescence microscope. In this way, the presence of the infiltrated components could be verified. There are several, probable causes for the lack of necrosis. Previous work has shown that the hypersensitive response is temperature dependent, and necrosis is inhibited at both high and low temperatures [21]. At the start of this project, the principal investigator moved the laboratory to a new space, which displayed wide swings in temperatures. Despite efforts to stabilize the temperature in the laboratory, including the installation of a new thermostat, the temperature fluctuations may have promoted unexpected changes in the physiology of the tobacco plants used for this analysis. Further investigations under controlled and consequently stable temperature conditions could lead to a more routine evaluation of pathogen-dependent and independent activation of the hypersensitive response and necrosis.

Summary

The multi-step procedure was a convenient approach to construct the TIR variants by applying different molecular biology methods/techniques as PCR, gel electrophoresis, DNA isolation and purification, GA, electroporation, and RE digest. Additionally the project provided an insight into the usage of different laboratory tools and equipment, as well as into sequence analyzing with different programs, chromatography, production of competent cells, and cell culture. My personal favorites were the conduct of infiltration experiments and the horticulture. All in all I learnt a variety of different laboratory skills and thanks to the repetitive procedure, I acquired a high routine and eventually I worked very efficient. My personal investigator guided me through the project with technical support and his expertise, especially with the first PCR problems and later on he proposed the

‘sandwich’-construct. However I could independently resolve challenges by trials with the aid of provided papers, manuals and protocols. Due to the insolubility of the initial NTIR-GFP construct, time was running out to eventually establish an infiltration experiment with the TIR-variants to test those *in vivo*. Nevertheless, the produced mutants will deliver essential clues for future efforts to engineer an enhanced TIR domain, and including the findings on the leucine rich repeats domain, this will lead to an advanced *N* gene and thus an improved immune response in plants.

5. Declaration of Originality

I, Janet Bakalarz, certify that the work presented in this bachelor thesis has been performed, written down and interpreted solely by myself, unless stated otherwise. No other person's effort has been used without acknowledgement in this thesis. All references and verbatim extracts were quoted and marked as such, as well as all sources of information including graphs and data sets are specified. I declare that this work is submitted in partial fulfillment for the degree of Bachelor in Science in Molecular Biotechnology and has not been handed in elsewhere in any other form for the acquisition of any other degree or qualification. Furthermore I confirm that the by myself-made copies are identical.

Date	Signature
31.08.2015	Janet Bakalarz

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7. Annex

Abbreviations

A. tumefaciens:	Agrobacterium tumefaciens
bp:	base pair
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid
E. coli:	*Escherichia coli*
EDTA:	ethylenediaminetetraacetic acid
GA:	Gibson Assembly
GFP:	green fluorescent protein
h:	hour(s)
HEL:	helicase domain of the replicase protein of the TMV
HR :	hypersensitive response
LBamp media:	Lucia Broth media with 100µg/mL of Ampicillin
MES:	2-(N-morpholino)ethanesulfonic acid
min:	minute(s)
N gene:	archetype member of the superfamily of plant resistance genes
NTIR:	TIR domain of the receptor protein coded by *N* gene
PCR:	polymerase chain reaction
RNA:	ribonucleic acid
RPS4:	*Arapidopsis* resistance gene against *Pseudomonas syringae 4*
s:	seconds
SOC media:	super optimal broth with catabolite repression media
TEV:	tobacco etch virus
TIR:	Toll interleukin-like receptor
Tm:	melting temperature
TMV:	tobacco mosaic virus
YEP media:	yeast extract peptone

Materials and Methods

Analyzing software programs

- APE a plasmid editor: http://biologylabs.utah.edu/jorgensen/wayned/ape/

Program used for handling DNA sequences

- Blast: https://blast.ncbi.nlm.nih.gov/

Online tool for sequences' alignments

- SnapGene: http://www.snapgene.com/

Software helped to visualize the final 'sandwich'-construct

- Rosetta: https://www.rosettacommons.org/

Sophisticated program to visualize and simulate 3D-structures of proteins and nucleotides

Primers

Name	Remark	Sequence	Tm (°C)	Length (bp)
C1	bN-K25V-C	GAAGATACCCGT**GTT**ACCTTTACCTCTCATCTG	61	33
C2	bN-K25V-IC	CAGATGAGAGGTAAAGGT**AAC**ACGGGTATCTTC	61	33
C3	bN-K25I-C	GAAGATACCCGT**ATT**ACCTTTACCTCTCATCTG	61	33
C4	bN-K25I-IC	CAGATGAGAGGTAAAGGTAATACGGGTATCTTC	61	33
C5	bN-K25L-C	GAAGATACCCGTCTGACCTTTACCTCTCATCTG	61	33
C6	bN-K25L-IC	CAGATGAGAGGTAAAGGTCAGACGGGTATCTTC	61	33
C7	bN-T26V-C	GAAGATACCCGTAAAGTTTTTACCTCTCATCTGTAC	60	36
C8	bN-T26V-IC	GTACAGATGAGAGGTAAAAACTTTACGGGTATCTTC	60	36
C9	bN-T26I-C	GAAGATACCCGTAAAATTTTTACCTCTCATCTGTAC	60	36
C10	bN-T26I-IC	GTACAGATGAGAGGTAAAAATTTTACGGGTATCTTC	60	36
C11	bN-T26L-C	GAAGATACCCGTAAACTGTTTACCTCTCATCTGTAC	60	36
C12	bN-T26L-IC	GTACAGATGAGAGGTAAACAGTTTACGGGTATCTTC	60	36
D1	bN-S29A-C	GATACCCGTAAAACCTTTACCGCTCATCTGTACGAAGTT	64	39
D2	bN-S29A-IC	AACTTCGTACAGATGAGCGGTAAAGGTTTTACGGGTATC	64	39
D3	bN-S29V-C	GATACCCGTAAAACCTTTACCGTTCATCTGTACGAAGTT	64	39
D4	bN-S29V-IC	AACTTCGTACAGATGAACGGTAAAGGTTTTACGGGTATC	64	39
D5	bN-H30A-C	CGTAAAACCTTTACCTCTGCTCTGTACGAAGTTCTG	62	36
D6	bN-H30A-IC	CAGAACTTCGTACAGAGCAGAGGTAAAGGTTTTACG	62	36
D7	bN-V34I-C	TCTCATCTGTACGAAATTCTGAACGATAAGGGT	62	33
D8	bN-V34I-IC	ACCCTTATCGTTCAGAATTTCGTACAGATGAGA	62	33
D9	bN-V34L-C	TCTCATCTGTACGAACTGCTGAACGATAAGGGT	62	33
D10	bN-V34L-IC	ACCCTTATCGTTCAGCAGTTCGTACAGATGAGA	62	33
D11	bN-A163V-WT-C	CGTGATAAAACCGATGTTGATTGTATTCGTCAGATTCGT	65	39
D12	bN-A163V-WT-IC	ACGAATCTGACGAATACAATCAACATCGGTTTTATCACG	65	39

NusA-C	Extension primer	TGGTGGATGAAGATAAACACACGATGGATATCGCCGTTGAAGC	70	43
H7	Extension primer	TGCTCGAGTGCGGCCGCAAGCTTT	70	24
F1	*RPS4*-pBI-M1-C	GCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG AAGGAGATATAC	65	57
TIRE4	TNP-D5C-S181-Link-IC	GCCAGAACCAGCAGCGGAGCCAGCAGAAATCTTAGACAGTTT AGAAGAAATCTGATCAAC	59	60

PCR

Thermocycler, PCR tubes, automatique micropipettes with sterile tips

Autoclaved H_2O	34	µL
*Buffer 10x	5	µL
*dNTPs	5	µL
*$MgSO_4$	3	µL
Primer 1 1000 nM	0.5	µL
Primer 2 1000 nM	0.5	µL
Template 50ng/µL	1	µL
*KOD Hot Start DNA polymerase	1	µL
Total	**50**	**µL**

(*components from Novagen 71086 | KOD Hot Start DNA Polymerase, EMD, USA)

The user protocol “TB341 Rev. D 0111JN” from http://www.emdmillipore.com was followed with varying components like adding DMSO or increasing the the total Mg-concentration and adjusting water. In addition the annealing temperature and extension time are adjusted depending on primers and expected fragment length.

Gelelectrophoresis

Gel electrophoresis apparatus and comb (70µLx6 dents or 30µLx 10 dents)

Agarose gel	37	mL
TBE buffer	300	mL
*2-Log DNA Ladder	5	µL
*Gel Loading Dye, purple (6x) with RNAse	3	µL
Sample	0.5	µL

(*components from NEB N3200L)

The agarose gels had 0.8, 1, or 1.2% (for <500bp, 500-700bp resp. <700bp long fragments) and were run under 80 V for 30 to 120 min. First the standard ladder was loaded, and then samples mixed with loading dye (15%). For later DNA purification the whole sample was loaded, for RE screenings just 10 µL.

Isolation and purification of DNA

After electrophoresis and under UV light, the right bands are cut out of the gel with a clean scalpel with collected in weighted microcentrifuge tubes for the next purification step. Hereby the user protocol Wizard® SV Gel and PCR Clean-Up System from https://promega.com was followed.

Quantification

Purified DNA can be quantified by using a NanoDrop Spectrometer. For blanking is sterile H_2O needed and 1-2µL of sample is loaded to get a good concentration measurement in ng/µL.

GA

Vector	1	µL
Fragment 1	~1	µL
Fragment 2	~1	µL
Total (sample)	~3	µL

Sample	~3	µL
*GA Master Mix	~3	µL

(*component from NEB)

- Measure the concentration of the vector with NanoDrop spectrometer
- Convert the concentration into amount (pmol/µL)
- Calculate the needed volume of fragment(s) (3 to 5 fold times excess to vector)
- Add vector and fragment(s) in a PCR tube, vortex briefly
- Add the same amount of Master Mix to the tube
- Put in Thermocycler and program for 15min and 50°C
- Let cool for at least 5 min for further transformation or store in -20°C freezer

Competent cells

E. coli TOP10	5	mL
E. coli BL21 (DE3)	5	mL
A. tumefaciens	5	mL

- Inoculate 500mL of L-broth with 1/100 volume of a fresh overnight *E. coli* culture.
- Grow the cells at 37 °C shaking at 300 rpm to an OD_{600} of approximately 0.5–0.7 (the best results are obtained with cells that are harvested at early- to mid-log phase; the appropriate cell density therefore depends on the strain and growth conditions).
- Chill cells on ice for ~20min. For all subsequent steps, keep the cells as close to 0°C as possible (in an ice/water bath) and chill all containers in ice before adding cells. To harvest, transfer the cells to a cold centrifuge bottle and spin at 4000 x g for 15min at 4°C.
- Carefully pour off and discard the supernatant. It is better to sacrifice the yield by pouring off a few cells than to leave any supernatant behind.
- Gently resuspend the pellet in 500mL of ice-cold 10% glycerol. Centrifuge at 4000 x g for 15min at 4°C; carefully pour off and discard the supernatant.
- Resuspend the pellet in 250mL of ice-cold 10% glycerol. Centrifuge at 4000 x g for 15 min at 4°C; carefully pour off and discard the supernatant.
- Resuspend the pellet in ~20mL of ice-cold 10% glycerol. Transfer to a 30mL sterile Oakridge tube.
- Centrifuge at 4000 x g for 15 min at 4°C; carefully pour off and discard the supernatant.
- Resuspend the cell pellet in a final volume of 1–2mL of ice-cold 10% glycerol. The cell concentration should be about 1–3 x 1010 cells/mL.
- This suspension may be frozen in aliquots on dry ice and stored at -80°C. The cells are stable for at least 6 months under these conditions.

Transformation: High Efficiency Electrotransformation of E. coli

Pulser, electroporation cuvette, culture tubes, plates, incubator, shaker 250rpm

Sample after GA	3.5+	µL
Competent cells (E.coli, A. tumefaciens)	50	mL
SOC media	1	mL
LBamp with agar for plates	30	mL

- Thaw the cells on ice. For each sample to be electroporated, place a 1.5 mL microfuge tube and either a 0.1 or 0.2cm electroporation cuvette on ice.
- In a cold, 1.5mL polypropylene microfuge tube, mix 40µl of the cell suspension with 1 to 2µL of DNA (DNA should be in a low ionic strength buffer such as TE).

Mix well and incubate on ice for ~1min. (Note: it is best to mix the plasmids and cells in a microfuge tube since the narrow gap of the cuvettes prevents uniform mixing.)

- Set the BioRad MicroPulser to “Ec1” when using the 0.1 cm cuvettes. Set it to "Ec2" or "Ec3" when using the 0.2 cm cuvettes.
- Transfer the mixture of cells and DNA to a cold electroporation cuvette and tap the suspension to the bottom. Place the cuvette in the chamber slide. Push the slide into the chamber until the cuvette is seated between the contacts in the base of the chamber. Pulse once.
- Remove the cuvette from the chamber and immediately add 1mL of SOC media to the cuvette. Quickly but gently resuspend the cells with a Pasteur pipette. (The period between applying the pulse and transferring the cells to outgrowth medium is crucial for recovering *E. coli* transformants. Delaying this transfer by even 1min causes a 3-fold drop in transformation. This decline continues to a 20-fold drop by 10min.
- Transfer the cell suspension to a 17 x 100mm polypropylene tube and incubate at 37°C for 1h, shaking at 225rpm.
- Plate on selective medium.

Rapid Plasmid Mini Prep

Culture	1.5	mL
STET	200	µL
Lysozym in TE buffer (10mg/mL)	20	µL
Isopropanol	200	µL
TE Buffer	50	mL

- Grow 2mL bacteria from 5 h to o/n at 37°C.
- Pour culture into 1.5mL microcentrifuge tube. (Save remaining culture for large-scale growth if needed.)
- Pellet bacteria 1min in microfuge. Decant supernatant, aspirate remaining.
- Suspend pellet in 200µL STET by vortexing. Add 20µL fresh 10mg/mL lysozyme.
- Place tubes in boiling water bath for 45s.
- Centrifuge immediately 5min in microcentrifuge (4°C or 25°C).
- Remove large clot with toothpick. Precipitate with 200µL Isopropanol at room temperature for 5-10min; spin 5 min in microcentrifuge. Should be able to see pellet. Decant supernatant; aspirate remainder; be careful not to lose pellet.

- Wash pellet by rinsing with 70% ethanol;
- Decant supernatant; invert tubes on rolled up paper towel to dry. Resuspend pellets in 50µL TE.

RE Digest

H20	34.5	µL
*BSA	0.5	µL
*Buffer 2	9	µL
*NdeI	3	µL
*HindIII	3	µL
Total (RE mix)	**50**	**µL**

RE mix	10	µL
Sample after Miniprep in TE buffer	10	µL

For a RE digest: use 10µL DNA in a 20µL reaction. Let the RE mix sit for over 1h at 37°C. Use Fluorchem to make pictures of the gel to evaluate the bands from the digestion.

Agroinfiltration experiment

- Grow agrobacterium cultures over night;
- Measure the OD_{600} of each culture and calculate the dilution with infiltration buffer to have the desired OD_{600} (between 1 to 3);
- Pellet for 1min cells in centrifuge;
- Discard media and resuspend in the required volume of buffer;
- Incubate for 2 to 3h, cover the tubes to prevent evaporation;
- Make desired mixtures of cultures containing different components;
- Use clean blunt-end syringes to inject on the basal side of leaves the agrobacterium solution;

Agarose gel (0.8/1/1.2%)

Agarose	0.8/1/1.2	g
Fill up TBE buffer	100	mL
Ethidium bromide	3.7	µL

Lucia-Broth (LB) media and plates

Bacto tryptone	10	g
Bacto yeast extract	5	g
NaCl	10	g
agar	15	g
Fill up deionized H_2O	1	L
1000x Ampicillin (100mg/mL)	1	mL

- Add 500mL of H_2O to a graduated cylinder;
- Add 10g tryptone, 5g yeast extract and 10g NaCl:
- Stir everything to bring into solution;
- For plates add 15g of agar;
- Add H_2O to total volume of 1L and divide 500mL into 1L flasks;
- Autoclave at liquid setting for 30 min in a basin making sure to loosen top;
- Make sure bench top has wiped down with ethanol;
- Remove sterile Petri dishes from plastic bag (save the bag for storage);
- Let LB cool to ~55°C;
- Add 1000x Ampicillin (100mg/ml) to LB Agar (optional);
- Pour a layer of LB Agar into each plate being careful to not lift the cover off excessively until half of the plate is filled;
- Let each plate cool over night;
- Store plates in plastic bags in fridge with: name, date and additives;

YEP media

Bacto tryptone	10	g
Bacto yeast extract	10	g
NaCl	5	g
Agar	15	g
Fill up deionized H_2O	1	L
Antibiotics in 1000x stocks	1	mL

Same instructions as for LB media.

SOC media

Bacto tryptone	2	%

Bacto yeast extract	0.5	%
NaCl	10	mM
KCl	2.5	mM
$MgCl_2$	10	mM
$MgS0_4$	10	mM
Glucose	20	mM
Fill up deionized H_2O	1	L

- Add 500mL of H_2O to a graduated cylinder;
- Add 10g tryptone, 5g yeast extract, 10g NaCl, 2.5g Kl, 10g $MgCl_2$ and 20g glucose;
- Stir everything to bring into solution;
- Add H_2O to total volume of 1L and divide 500mL into 1L flasks;
- Autoclave at liquid setting for 20min in a basin making sure to loosen top;
- Distribute after cooling off into 50mL Falcon tubes for easier handling.

Glycerol 10%

Glycerol	80	mL
Fill up deionized H_2O	800	mL

Autoclaved for 20 min.

STET

Sucrose 60%	6.6	mL
Triton X-100	2.5	mL
EDTA 0.5M	5	mL
Tris pH=8, 1M	2.5	mL
Deionized H_2O	33.3	mL
Total	**50**	**mL**

Autoclaved for 20 min.

TE buffer

Tris-HCL pH=8	10	mM
EDTA	1	mM

Autoclaved for 20 min.

TBE buffer

Tris Base	108	g
Boric Acid	55	g
EDTA pH=8, 0.5M	40	mL
Fill up deionized H_2O	10	L

Infiltration buffer

MES	34.5	µL
MgCl2	0.5	µL
Acetosyringone	9	µL
Fill up deionized H_2O	3	µL

Appendix

1. Construction of TIR variants by PCR

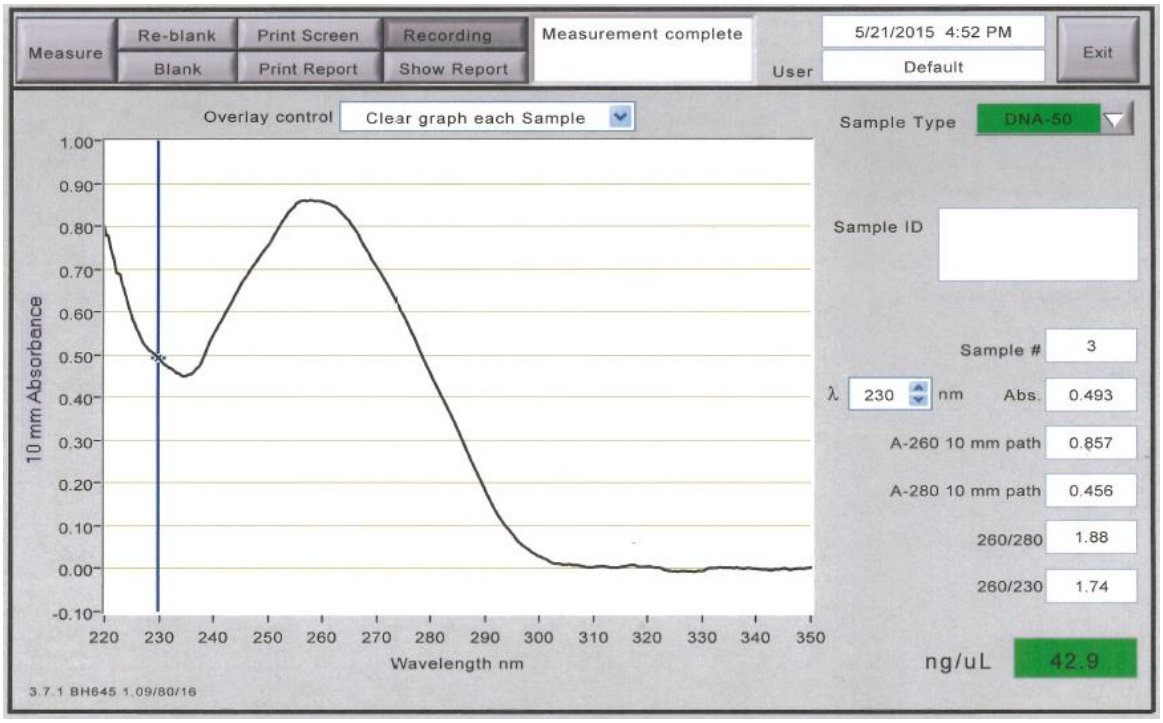

Fig.I: NanoDrop output of measuring DNA concentration of amplified V34L-C. Nucleotides have their highest absorbance at 260nm, therefore the spectrum's peak should be around that wavelength. A deviated peak and irregularities in the curve indicate impurities in the DNA sample.

Subsitution	annealing temperature (°C)	extension time (s)	changes to protocol	volume taken for GA (µL)
K25V-C	56	10	added DMSO	0.44
K2V5-IC	56	15	added DMSO	0.90
K25I-C	56	10	reduced Mg	0.65
K25I-IC	56	15	reduced Mg	1.32
K25L-C	56	10	reduced Mg	0.58
K25L-IC	56	15	reduced Mg	1.47
T26V-C	55	10		0.69
T26V-IC	55	15		0.79
T26I-C	55	10		0.53
T26I-IC	55	15		0.89
T26L-C	55	10		0.80
T26L-IC	55	15		0.96
S29A-C	59	10		0.71
S29A-IC	59	15		1.91
S29V-C	59	10		0.67
S29V-IC	59	15		2.18
H30A-C	57	10	added DMSO	0.75

H30A-IC	57	15	added DMSO	1.60
V34I-C	57	10		0.75
V34-IC	57	15		1.23
V34L-C	57	10		0.91
V34-IC	57	15		1.24
A163V-C	60	20	added DMSO	0.86
A163V-IC	60	20	added DMSO	1.94

Tab.I: Additional information about the individual PCRs to the overview table Tab.2. In addition the needed volume for a successful GA is given too.

2. Screening for positive colonies by Miniprep and RE digestion

agatatacatATGGGCAGCCATCATCATCATCATCACAGCGCCAACAAAGAAATTTTGGCTGTAGTTGAAGCCGTATCCAATGAAAAGGCGCTACCTC
GCGAGAAGATTTTCGAAGCATTGGAAAGCGCGCTGGCGACAGCAACAAAGAAAAAATATGAACAAGAGATCGACGTCCGCGTACAGATCGATCGCAAA
AGCGGTGATTTTGACACTTTCCGTCGCTGGTTAGTTGTTGATGAAGTCACCCAGCCGACCAAGGAAATCACCCTTGAAGCCGCACGTTATGAAGATGA
AAGCCTGAACCTGGGCGATTACGTTGAAGATCAGATTGAGTCTGTTACCTTTGACCGTATCACTACCCAGACGGCAAAACAGGTTATCGTGCAGAAAG
TGCGTGAAGCCGAACGTGCGATGGTGGTTGATCAGTTCCGTGAACACGAAGGTGAAATCATCACCGGCGTGGTGAAAAAAGTAAACCGCGACAACATC
TCTCTGGATCTGGGCAACAACGCTGAAGCCGTGATCCTGCGCGAAGATAGCTGCCGCGTGAAAACTTTCGCCCTGGCGACCGCGTTCGTGGCGTGCTC
TATTCCGTTCGCCCGGAACGGTGGCGCGCAACTGTTCGTCACTCGTTCCAAGCCGGAAATGCTGATCGAACTGTTCCGTATTGAAGTGCCAGAAATCG
GCGAAGAAGTGATTGAAATTAAAGCAGCGGCTCGCGATCCGGGTTCTCGTGCGAAAATCGCGGTGAAAACCAACGATAAACGTATCGATCCGGTAGGT
GCTTGCGTAGGTATGCGTGGCGCGCGTGTTCAGGCGGTGTCTACTGAACTGGGTGGCGAGCGTATCGATATCGTCCTGTGGGATGATAACCCGGCGCA
GTTCGTGATTAACGCAATGGCACCGGCAGACGTTGCTTCTATCGTGGTGGATGAAGATAAACACACGATGGATATCGCCGTTGAAGCCGGTAACTTGG
CGCAGGCGATTGGCCGTAACGGTCAGAACGTGCGTCTGGCTTCGCAGCTGAGCGGTTGGGAACTCAACGTGATGACCGTTGACGACCTGCAGGCTAAG
CATCAGGCGGAAGCGCACGCAGCGATCGACACCTTCACCAAATATCTCGACATCGACGAAGACTTCGCGACTGTTCTGGTAGAAGAAGGCTTCTCGAC
GCTGGAAGAATTGGCCTATGTGCCGATGAAAGAGCTGTTGGAAATCGAAGGCCTTGATGAGCCGACCGTTGAAGCACTGCGCGAGCGTGCTAAAAATG
CACTGGCCACCATTGCACAGGCCCAGGAAGAAAGCCTCGGTGATAACAAACCGGCTGACGATCTGCTGAACCTTGAAGGGGTAGATCGTGATTTGGCA
TTCAAACTGGCCGCCCGTGGCGTTTGTACGCTGGAAGATCTCGCCGAACAGGGCATTGATGATCTGGCTGATATCGAAGGGTTGACCGACGAAAAAGC
CGGAGCACTGATTATGGCTGCCCGTAATATTTGCTGGTTCGGTGACGAAGCGGGCGCCAACAATAACTCGAATAACAACGCGAGCGAGAATCTTTATT
TTCAGGGCGCCATGgCTTCTTCTTCTTCTTCTTCTCGTTGGTCTTATGATGTTTTTCTGTCTTTTCGTGGTgaagATACCCGTAAAACCTTTACCTCT
CATctgTACGAAGTTCTGAACGATAAGGGTATCAAGACCTTCCAGGATGATAAACGTCTGGAATATGGTGCTACCATTCCAGGTGAACTGTGTAAAGC
TATTGAAGAATCTCAGTTCGCTATCGTTGTGTTTTCTGAAAACTACGCTACCTCTCGTTGGTGTCTGAACGAACTGGTTAAGATCATGGAGTGTAAGA
CCCGTTTCAAGCAGACCGTTATCCCAATTTTCTATGATGTTGATCCATCTCATGTTCGTAACCAGAAAGAATCTTTTGCTAAAGCCTTTGAAGAACAT
GAAACCAAGTACAAGGATGATGTTGAAGGTATTCAGCGTTGGCGTATTGCTCTGAACGAAGCTGCTAACCTGAAAGGTTCTTGTGATAACCGTGATAA
AACCGATGCTGATTGTATTCGTCAGATTGTcGAcCAGATTTCTTCTAAACTGTGTAAGATTTCTtaagcttgcggccgcactCGAGCAccaccaccac
caccactgagatccggctgctaacaaagcccgaaaggaagctgagttggctgctgccaccgctgagcaataactagcataacccctttggggcctctaa
acgggtcttgaggggttttttgctgaaaggaggaactatatccggatA

Fig.II: NusA-NTIR sequence: NdeI(brown), Linker (yellow), NusA (orange), NusA-C primer (grey), TEV-Link (yellow), NTIR (cyan), signaling interface residues (purple), HindIII (brown).

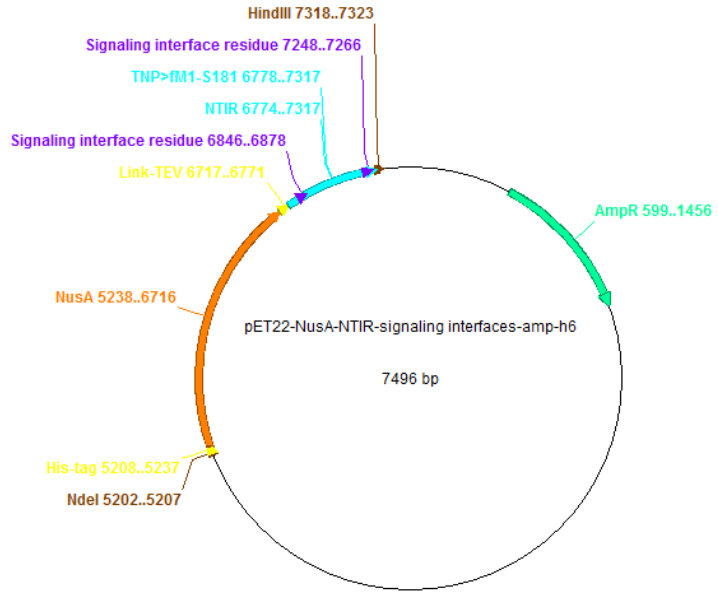

Fig.III: plasmid map of pET22b-NusA-NTIR.

3. Selection of positive colonies and isolation of plasmids

```
>150423-25_M02_C7_T7terminator.ab1      1405
NNNNNNNNNNCTCNTTCGGGCTTTGTTAGCAGCCGGATCTCAGTGGTGGT
GGTGGTGGTGCTCGAGTGCGGCCGCAAGCTTAAGAAATCTTAGACAGTTT
AGAAGAAATCTGATCAACAATCTGACGAATAACATCAGCATCGGTTTTAT
CACGGTTATCAACAGAACCTTTCAGGTTAGCAGCTTCGTTCAGAGCAATA
CGCCAACGCTGAATACCTTCAACATCATCCTTGTACTTGGTTTCATGTTC
TTCAAAGGCTTTAGCAAAAGATTCTTTCTGGTTACGAACATGAGATGGAT
CAACATCATAGAAAATTGGGATAACGGTCTGCTTGAAACGGGTCTTAGCC
TCCATGATCTTAACCAGTTCGTTCAGACACCAACGAGAGGTAGCGTAGTT
TTCAGAAAACACAACGATAGCGAACTGAGATTCTTCAATAGCTTTAGACA
GTTCACCTGGAATGGTAGCACCATATTCCAGACGTTTATCATCCTGGAAG
GTCTTGATACCCTTATCGTTCAGAACTTCGTACAGATGAGAGGTAAAAAC
TTTACGGGTATCTTCACCACGAAAAGACAGAAAAACATCATAAGACCAAC
GAGAAGAAGAAGAAGAAGCCATGGCGCCCTGAAAATAAAGATTCTCG
CTCGCGTTGTTATTCGAGTTATTGTTGGCGCCCGCTTCGTCACCGAACCA
GCAAATATTACGGGCAGCCATAATCAGTGCTCCGGCTTTTTCGTCGGTCA
ACCCTTCGATATCAGCCAGATCATCAATGCCCTGTTCGGCGAGATCTTCC
AGCGTACAAACGCCACGGGCGGCCAGTTTGAATGCCAAATCACGATCTAC
CCCTTCAAGGTTCAGCAGATCGTCAGCCGGTTTGTTATCACCGAGGCTTT
CTTCCTGGGCCTGTGCAATGGTGGCCAGTGCATTTTTAGCACGCTCGCGC
AGTGCTTCAACGGTCGGCTCATCAAGGCCTTCGATTTCCAACAGCTCTTT
CATCGGCACATAGGCCAATTCTTCCAGCGTCGAGAAGCCTTCTTCTACCA
GAACAGTCGCGAAGTCTTCGTCGATGTCGAGATATTTGGTGAAGGTGTCG
ATCGCTGCGTGCGCTTCCGCCTGATGCTTAGCTTGCAGGTCGTCAACGGT
CATCACGTTGAGTTCCCAACCGCTCAGTTGCAAAGCCANNCCNACGTTCT
GACCGTTNCGGCAATCGCCTGGNCCAANTACCGGCTTCAAGGGNANTNCC
ATCGGGGNTTANCTTCNCCNCNNNNNNNAANCAACGTTGGCGGGNCCTTG
NNTTAACNNAANTGGCCNGGNTNNNNNCCNNNGNAANNNNNNNANCCNNNC
CCCCNNNNTTANGAANCCNGCTGAANCCGGNCCCNNANTNNNNNGAGGCC
TCCGG
```

Fig.IV: Sequence result of sample T26V provided by Macrogene. Sequencing started from t7terminator and a total of 1405 bp were read. “N” mark an undetermined base due to ambiguous or weak signals.

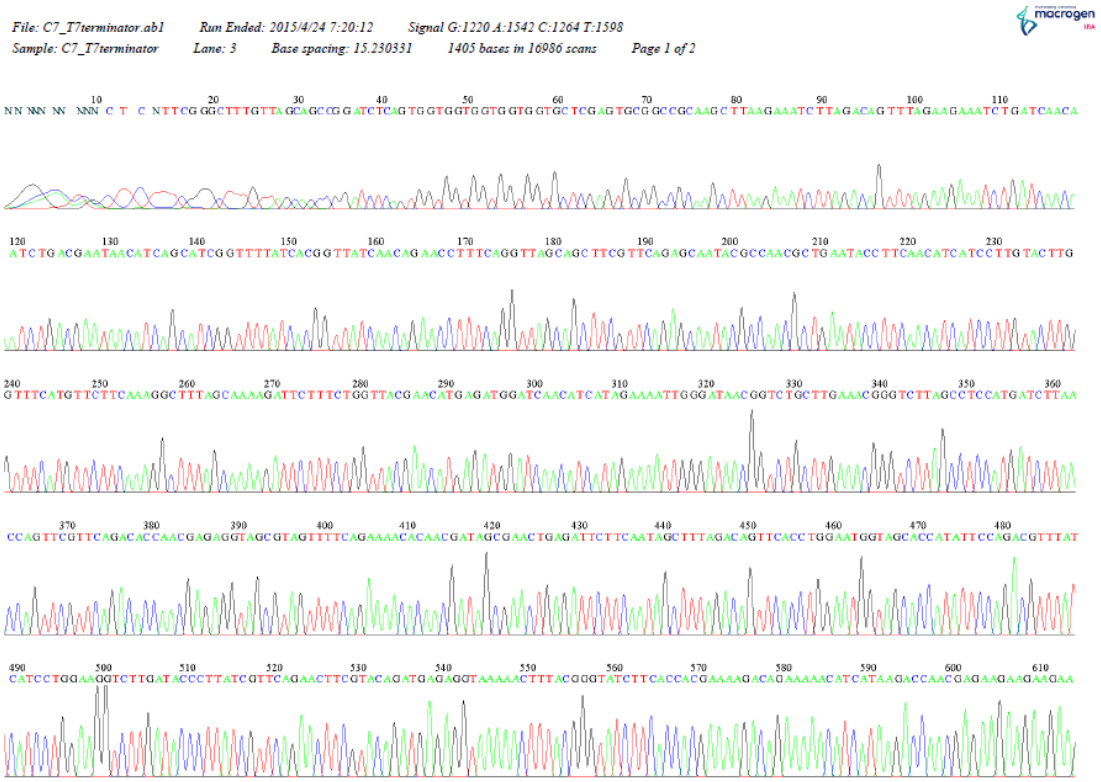

Fig.V: Signal result of sample T26V provided by Macrogen. The different colors indicate the different bases and the higher the peak, the stronger the signal. Usually at the start, the signals are weak and therefore the sequencing is “blurry”.

```
                                                                  START
Query  1    GGCGCCAACAATAACTCGAATAACAACGCGAGCGAGAATCTTTATTTTCAGGGCGCCATG  60

Sbjct  668  GGCGCCAACAATAACTCGAATAACAACGCGAGCGAGAATCTTTATTTTCAGGGCGCCATG  609

Query  61   GcttcttcttcttcttcttctCGTTGGTCTTATGATGTTTTTCTGTCTTTTCGTGGTGAA  120

Sbjct  608  GCTTCTTCTTCTTCTTCTTCTCGTTGGTCTTATGATGTTTTTCTGTCTTTTCGTGGTGAA  549
                         T
Query  121  GATACCCGTAAAACCTTTACCTCTCATCTGTACGAAGTTCTGAACGATAAGGGTATCAAG  180

Sbjct  548  GATACCCGTAAAGTTTTTACCTCTCATCTGTACGAAGTTCTGAACGATAAGGGTATCAAG  489
                         V (substitution T26V)
Query  181  ACCTTCCAGGATGATAAACGTCTGGAATATGGTGCTACCATTCCAGGTGAACTGTGTAAA  240

Sbjct  488  ACCTTCCAGGATGATAAACGTCTGGAATATGGTGCTACCATTCCAGGTGAACTGTCTAAA  429

Query  241  GCTATTGAAGAATCTCAGTTCGCTATCGTTGTGTTTTCTGAAAACTACGCTACCTCTCGT  300

Sbjct  428  GCTATTGAAGAATCTCAGTTCGCTATCGTTGTGTTTTCTGAAAACTACGCTACCTCTCGT  369

Query  301  TGGTGTCTGAACGAACTGGTTAAGATCATGGAGTGTAAGACCCGTTTCAAGCAGACCGTT  360

Sbjct  368  TGGTGTCTGAACGAACTGGTTAAGATCATGGAGGCTAAGACCCGTTTCAAGCAGACCGTT  309

Query  361  ATCCCAATTTTCTATGATGTTGATCCATCTCATGTTCGTAACCAGAAAGAATCTTTTGCT  420

Sbjct  308  ATCCCAATTTTCTATGATGTTGATCCATCTCATGTTCGTAACCAGAAAGAATCTTTTGCT  249

Query  421  AAAGCCTTTGAAGAACATGAAACCAAGTACAAGGATGATGTTGAAGGTATTCAGCGTTGG  480

Sbjct  248  AAAGCCTTTGAAGAACATGAAACCAAGTACAAGGATGATGTTGAAGGTATTCAGCGTTGG  189

Query  481  CGTATTGCTCTGAACGAAGCTGCTAACCTGAAAGGTTCTTGTGATAACCGTGATAAAACC  540

Sbjct  188  CGTATTGCTCTGAACGAAGCTGCTAACCTGAAAGGTTCTGTTGATAACCGTGATAAAACC  129

Query  541  GATGCTGATTGTATTCGTCAGATTGTCGACCAGATTTCTTCTAAACTGTGTAAGATTTCT  600

Sbjct  128  GATGCTGATGTTATTCGTCAGATTGTTGATCAGATTTCTTCTAAACTGTCTAAGATTTCT  69
          STOP
Query  601  TAAGCTT  607

Sbjct  68   TAAGCTT  62
```

Fig.VI: Sequence alignment of native NTIR (Query) and NTIR (Sbjct) with T26V substitution using Blast. In green is the starting codon of NTIR, in red is the stop codon and yellow marks the expected mutation.

4. Producing a NusA-NTIR-GFP -'sandwich'

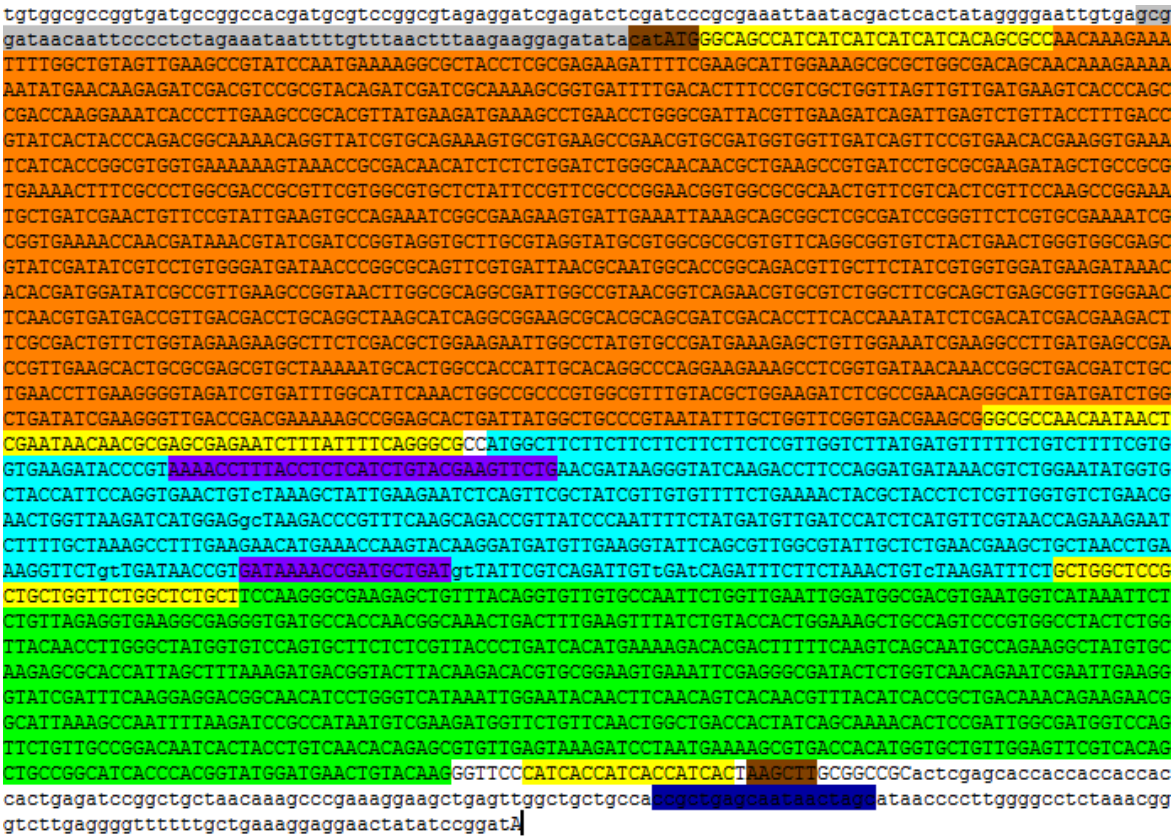

Fig.VII: NusA-NTIR-GFP-'sandwich': F1 primer (grey) NdeI(brown), Linker (yellow), NusA (orange), TEV-Link (yellow), NTIR (cyan), signaling interface residues (purple), HindIII (brown), T7 terminator (blue).

Substitution	1. concentration (ng/µL)	2. concentration (ng/µL)
K25V	71.5	124.8
K25I	63	226.6
K25L	90	192.3
T26V	38.8	118
T26I	52.7	235.7
T26L	49.4	131
S29A	41.6	134.4
S29V	40.9	135.5
H30A	50.1	140.5
V34I	51.5	134
V34L	44	218
A163V	97.4	126.3

Tab.II: List of the yielded concentrations in ng/µL of the 'sandwich'-construct containing the different substitutions. The 1.concentration was measured directly after the purification and the yield was not high enough for sequencing, which requires at least 120 ng/µL DNA for a reliable result. The 2.concentration is measured after each sample got concentrated down with a vacuum spin. After this last step the different constructs are stored in a -20°C freezer.

```
>150710-20_M23_D5_T7terminator.ab1      1342
NNNNNNNNNCNTCNTTCGGNCTTTGTTANCAGCCGGATCTCAGTGGTGGT
GGTGGTGGTGCTCGAGTGCGGCCGCAAGCTTAGTGATGGTGATGGTGATG
GGAACCCTTGTACAGTTCATCCATACCGTGGGTGATGCCGGCAGCTGTGA
CGAACTCCAACAGCACCATGTGGTCACGCTTTTCATTAGGATCTTTACTC
AACACGCTCTGTGTTGACAGGTAGTGATTGTCCGGCAACAGAACTGGACC
ATCGCCAATCGGAGTGTTTTGCTGATAGTGGTCAGCCAGTTGAACAGAAC
CATCTTCGACATTATGGCGGATCTTAAAATTGGCTTTAATGCCGTTCTTC
TGTTTGTCAGCGGTGATGTAAACGTTGTGACTGTTGAAGTTGTATTCCAA
TTTATGACCCAGGATGTTGCCGTCCTCCTTGAAATCGATACCCTTCAATT
CGATTCTGTTGACCAGAGTATCGCCCTCGAATTTCACTTCCGCACGTGTC
TTGTAAGTACCGTCATCTTTAAAGCTAATGGTGCGCTCTTGCACATAGCC
TTCTGGCATTGCTGACTTGAAAAAGTCGTGTCTTTTCATGTGATCAGGGT
AACGAGAGAAGCACTGGACACCATAGCCCAAGGTTGTAACCAGAGTAGGC
CACGGGACTGGCAGCTTTCCAGTGGTACAGATAAACTTCAAAGTCAGTTT
GCCGTTGGTGGCATCACCCTCGCCTTCACCTCTAACAGAGAATTTATGAC
CATTCACGTCGCCATCCAATTCAACCAGAATTGGCACAACACCTGTAAAC
AGCTCTTCGCCCTTGGAAGCAGAGCCAGAACCAGCAGCGGAGCCAGCAGA
AATCTTAGACAGTTTAGAAGAAATCTGATCAACAATCTGACGAATACAAT
CAGCATCGGTTTTATCACGGTTATCACAAGAACCTTTCAGGTTAGCAGCT
TCGTTCAGAGCAATACGCCAACGCTGAATACCTTCAACATCATCCTTGTA
CTNTGGTTTCATGTTCTTCAAAGGCTTTAGCAAAAGATTCTTTCTGGTTA
CGAACATTGAGATGGATCAACATCCTAGAAAATTNGGGATAACGGNCTGC
TTTNAANCGGGTNTTACACTCCTGATGTTNAACCAGTTCGTTCANCACCA
CNGAGAGAGGAGGGGGGNTTTTNCNNAAAANNNNNNNANNGGNAACGGGN
ATTTTNNNNAANTTTTNCCAGTTCCNNCCGCGAGGGNAGANCCNNAATTC
CNAANGTGTTTTNNTNNGGGNAGAGGGGNNNNNACCNTTCNNTTTTTNAG
ATCTNNNNNCGCCANANNAANGAANGATTNGNNNNNNNTTNG
```

Fig.VIII: Sequence result of sample H30A provided by Macrogene. Sequencing started from t7terminator and a total of 1342bp were read. “N” mark an undetermined base due to ambiguous or weak signals.

```
Query  391   AGTACAAGGATGATGTTGAAGGTATTCAGCGTTGGCGTATTGCTCTGAACGAAGCTGCTA  450
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     AGTACAAGGATGATGTTGAAGGTATTCAGCGTTGGCGTATTGCTCTGAACGAAGCTGCTA  60

Query  451   ACCTGAAAGGTTCTGTTGATAACCGTGATAAAACCGATGCTGATGTTATTCGTCAGATTG  510
             ||||||||||||||  ||||||||||||||||||||||||||  ||||||||||||||||
Sbjct  61    ACCTGAAAGGTTCTTGTGATAACCGTGATAAAACCGATGCTGATTGTATTCGTCAGATTG  120

Query  511   TTGATCAGATTTCTTCTAAACTGTCTAAGATTTCTGCTGGCTCCGCTGCTGGTTCTGGCT  570
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   TTGATCAGATTTCTTCTAAACTGTCTAAGATTTCTGCTGGCTCCGCTGCTGGTTCTGGCT  180

Query  571   CTGCTTCCAAGGGCGAAGAGCTGTTTACAGGTGTTGTGCCAATTCTGGTTGAATTGGATG  630
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   CTGCTTCCAAGGGCGAAGAGCTGTTTACAGGTGTTGTGCCAATTCTGGTTGAATTGGATG  240

Query  631   GCGACGTGAATGGTCATAAATTCTCTGTTAGAGGTGAAGGCGAGGGTGATGCCACCAACG  690
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   GCGACGTGAATGGTCATAAATTCTCTGTTAGAGGTGAAGGCGAGGGTGATGCCACCAACG  300

Query  691   GCAAACTGACTTTGAAGTTTATCTGTACCACTGGAAAGCTGCCAGTCCCGTGGCCTACTC  750
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   GCAAACTGACTTTGAAGTTTATCTGTACCACTGGAAAGCTGCCAGTCCCGTGGCCTACTC  360

Query  751   TGGTTACAACCTTGGGCTATGGTGTCCAGTGCTTCTCTCGTTACCCTGATCACATGAAAA  810
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   TGGTTACAACCTTGGGCTATGGTGTCCAGTGCTTCTCTCGTTACCCTGATCACATGAAAA  420

Query  811   GACACGACTTTTTCAAGTCAGCAATGCCAGAAGGCTATGTGCAAGAGCGCACCATTAGCT  870
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   GACACGACTTTTTCAAGTCAGCAATGCCAGAAGGCTATGTGCAAGAGCGCACCATTAGCT  480

Query  871   TTAAAGATGACGGTACTTACAAGACACGTGCGGAAGTGAAATTCGAGGGCGATACTCTGG  930
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   TTAAAGATGACGGTACTTACAAGACACGTGCGGAAGTGAAATTCGAGGGCGATACTCTGG  540

Query  931   TCAACAGAATCGAATTGAAGGGTATCGATTTCAAGGAGGACGGCAACATCCTGGGTCATA  990
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
Sbjct  541   TCAACAGAATCGAATTGAAGGGTATCGATTTCAAGGAGGACGGCAACATCCTGGGTCATA  600

Query  991   AATTGGAATACAACTTCAACAGTCACAACGTTTACATCACCGCTGACAAACAGAAGAACG  1050
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   AATTGGAATACAACTTCAACAGTCACAACGTTTACATCACCGCTGACAAACAGAAGAACG  660

Query  1051  GCATTAAAGCCAATTTTAAGATCCGCCATAATGTCGAAGATGGTTCTGTTCAACTGGCTG  1110
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   GCATTAAAGCCAATTTTAAGATCCGCCATAATGTCGAAGATGGTTCTGTTCAACTGGCTG  720

Query  1111  ACCACTATCAGCAAAACACTCCGATTGGCGATGGTCCAGTTCTGTTGCCGGACAATCACT  1170
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   ACCACTATCAGCAAAACACTCCGATTGGCGATGGTCCAGTTCTGTTGCCGGACAATCACT  780

Query  1171  ACCTGTCAACACAGAGCGTGTTGAGTAAAGATCCTAATGAAAAGCGTGACCACATGGTGC  1230
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   ACCTGTCAACACAGAGCGTGTTGAGTAAAGATCCTAATGAAAAGCGTGACCACATGGTGC  840

Query  1231  TGTTGGAGTTCGTCACAGCTGCCGGCATCACCCACGGTATGGATGAACTGTACAAG  1286
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   TGTTGGAGTTCGTCACAGCTGCCGGCATCACCCACGGTATGGATGAACTGTACAAG  896
```

Fig.IX: Sequence alignment of native NTIR-GFP (Query) and NTIR-GFP (Sbjct) with H30A substitution using Blast. Sequencing reactions are usually not longer than 1500bp, therefore it is not possible to check if the whole NusA-NTIR-GFP fragment is correct. However, the NusA-NTIR was verified beforehand, thus the linking sequence between NTIR and GFP (yellow: Link TEV) is used to check the correct 'sandwich'-construct.

5. Testing constructed TIR variants with S.E. chromatography

Fig.X: Plasmid map of pET22b-NusA-NTIR-GFP with essential restriction enzyme sides.

NTIR variant	Stages of testing				Result
	expression	solubility	protein gel	S.E. chromatography	
K25V	yes	yes	yes	yes	↑
K25I	yes	yes	yes	yes	↑
K25L	no	-	-	-	↓
T26V	yes	yes	yes	yes	↑
T26I	yes	yes	yes	yes	↑
T26L	yes	yes	yes	yes	↑
S29A	yes	yes	yes	not purified yet	-
S29V	not started				-
H30A	yes	yes	yes	yes	↓
V34I	yes	yes	yes	yes	↓
V34L	yes	yes	yes	yes	↓
A163V	yes	yes	yes	not purified yet	-

Tab.III: Overview of testing the TIR variants for dimerization changes. First the NusA-NTIR-GFP-construct is cloned into an expression vector; S29V testing has not been started at the time of submission. All variants expresses expect of K25L, without expression no protein can be tested further and it indicates the instability and disruptive character of the mutation. The GFP-proteins were also examined for solubility and run on a protein gel. Eventually a large scale culture is set to purify a high enough amount of protein for S.E. chromatography. Also S29A and A163V have not been purified yet. The chromatography results in retention times. Latter are compared with the native NTIR outcome, the retention shows the positive (↑) or negative (↓) change in dimerizing tendency of NTIR due to the substitution. While K25V, K25I, T26V, T26I and T26L increase dimerizing; K25L, H30A, V34I and V34L reduce dimerization rate respectively result in a disrupted mutant.

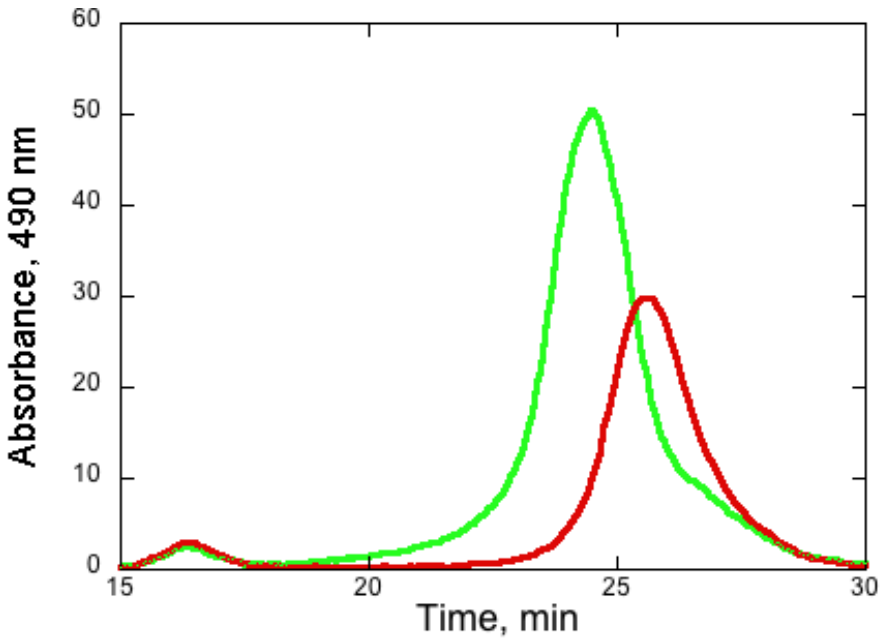

Fig.XI: This graph represents the data collected over a S.E. chromatography analysis of *RPS4* mutant R30S(green) and of *RPS4* mutant S33A H34A(red). This representation is used to illustrate that the dimeric mutant, *RPS4* R30S, shows up between the aggregate peak at 16 min and the monomer peak of *RPS4* S33A H34A at 26min. This phenomenology serves as control for the dimer testing of the TIR variants with native NTIR.

Tables and figures register

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First at all, I want to acknowledge Dr. Edward Eisenstein, who gave me to possibility to work in his research group at IBBR, a collaboration of University of Maryland and other institutions in the USA. Dr. Eisenstein did not just provide me with guidance during and around my internship, but he also supported the deployment of my project and my laboratory skills. Next, my gratitude goes to Dr. Jim Parson who helped me and gave me advice whenever I needed it. In addition, his findings flowed into this thesis. Many special thanks go to Christine Kim and Ryan Cooper, who assisted me through my first steps and introduced me to horticulture. Moreover, I am thankful for the companionship of Sean Barker, who also contributed to this work, Paula Kleyman and Torrance Wang during lab days. Besides the whole friendly and handful staff from IBBR, I want to thank the Xiao-Lab and especially Harley King.

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