Master Thesis Report
Phytopathology Department
Master Programme Plant Biotechnology

Yeast two-hybrid screening for Rx binding proteins and random mutagenesis study of the RanGAP2-WPP domain

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Abstract

Rx is a potato resistance protein of the Nucleotide-Binding Leucine-Rich Repeats (NB-LRR) immune receptors family, which confers extreme resistance to Potato Virus X (PVX). Rx is a Coiled-Coiled (CC) type of NB-LRR and large part of the mechanism for downstream signalling triggered by the receptor is still unknown. Previous research has defined particular roles for each of the domains. Whilst the LRR domain is associated to a main recognition role, the CC-NB domains were considered for downstream signalling and activation, respectively. Later publications suggested that the NB domain is also involved in signalling; however, the way in which signalling is triggered, remains unknown. Moreover, previous results showed that interaction with the RanGTPase Activation Protein 2 (RanGAP2) is required for resistance. RanGAP2 is localized to the outer side of the Nuclear Envelope (NE). The interaction with RanGAP2 is given through the N-terminal domain (WPP) which binds to the CC domain of Rx. Expression of both peptides *N. tabacum* and *N. bethamiana* is sufficient to trigger HR.

Recent publications showed that RanGAP2 plays a role as a retention factor in the NE, influencing the partitioning of Rx in the cytoplasm and the nucleus (despite the lack of a NLS in Rx) and this balance is required for the resistance. Many questions are still unanswered in terms of the regulation of the Rx activation complex. Also around the role that RanGAP2 may have in the activation complex of Rx, as well as in the characteristics of the interactions Rx-RanGAP2. To explore what other proteins may be involved in downstream signalling, a yeast-two-hybrid screen was conducted having as baits the Rx-NB and Rx-CC-NB domain and screened against a cDNA library derived from dying seedlings of transgenic Cf4/Avr4 tomato undergoing HR. In parallel a mutant library of the RanGAP2 WPP domain was developed and screened for loss of HR; aiming at identifying amino acid residues involved in binding to the CC domain of Rx; using the information in an *ab initio* modelling of the WPP domain.

Results from the Y2H were discouraging since all selected candidates were found to be autoactive colonies. On the other hand, the mutant screen yielded a group of candidates showing lower levels of HR. A few of these candidates were analyzed and sequenced. The mutants showed either low levels of proteins or production of truncated proteins. Consequently, further research is needed to reach the second objective of the project.

Acknowledgments

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Introduction

In agriculture, crops are threatened by basically two kinds of stresses: non-biotic (environmental conditions) and biotic. Pathogens and pests are one of the oldest problems suffered by producers in all regions of the planet. Worldwide - since approximately 1950-1960's- biotic stress has been dealt mostly with chemical inputs, which together with breeding strategies and genetic improvement led to what has been known as the green revolution (Johnson, 1972).

However, even though when in 2009 alone 3 billion kg of pesticides were sprayed in crops fields in the world (Peshin and Dhawan, 2009), biotic stress still accounted for approximately 40% losses in food and fibre production (Kole et al., 2010). Moreover, side effects of chemical inputs in agriculture has taken a sever toll in the environment and scientist and policy makers are constantly stressing that current production technology will not be enough to cover the food, feed, fibre and energy demands for the years and generations to come (Kern, 2002, United Nations. Dept. of Economic and Social Affairs. Population Division., 2007). According to the United Nation's population projections, there will be a world population between 9.5 and 10 billion people in 2050, which will have to be supported by every time more limited resources like arable land and water(United Nations. Dept. of Economic and Social Affairs. Population Division., 2007). Hence, there is a continuous need for improving food production capacity in a constantly changing and increasingly growing world.

The development of the plant biotechnology field and the use of modern molecular biology tools in phytopathology, allows scientist to unveil the complex interactions between plants and pathogens. By understanding the way in which plants defend themselves from pathogens, researchers and producers alike are expected to be closer to more efficient and sustainable means of production applied to agriculture.

1. The plant immune system

Plants, like any other living organism, have evolved different strategies to cope with environmental challenges. Unlike animals, plants cannot escape from a direct threat by mobilizing, which immediately place them at disposal of herbivores,

pathogens, parasites as one of the basic links of the food chain in general (Gurevitch et al., 2006). Despite the apparent state of fragility, plants are resistant to most pathogens (Dangl and Jones, 2001), suggesting the existence of an effective defence mechanism at physical, physiological and molecular level (Gurevitch et al., 2006). Defence strategies evolved in plants are as wide as there are threats in nature (Gurevitch et al., 2006).

In the line of plant defences, the basics are physical barriers preventing insect attacks. These barriers are mostly structural components of the plant, namely lignin, wax layers and trichomes (Gurevitch et al., 2006). There are also known metabolites that give an evolutionary advantage to the plant survival, such as terpines, phenolic compounds, alkaloids or even volatiles intended to enhance indirect defence mechanisms from the ecosystem (Bonsi, 1999, Dicke and Baldwin, 2010). Other mechanisms have also been identified such as RNA interference as response to virus infection (Lin et al., 2007) and systemic acquired resistance (SAR) in which upon infection in a localized point, plants may rely on hormone signalling through the salicylic acid (SA), jasmonic acid (JA) or ethylene pathways to activate defence genes systemically (Durrant and Dong, 2004, Carr et al., 2010).

Interactions between hosts and pathogens are the result of a coevolution process in which both groups are under constant pressure to develop mechanisms of survival, either by infecting a host or by preventing infection (Thompson and Burdon, 1992). In plants, this evolutionary arms race was first introduced by Flor in 1942, whose work associated the concept of “R” proteins as a protein related to resistance, able to recognize virulence factors, therefore making the pathogen avirulent. These factors are named “Avr” proteins. Flor also began to study the inheritance of such traits (Thompson and Burdon, 1992, Flor, 1942, Flor, 1954). Further research would differentiate defence mechanisms in non-specific responses and specific recognitions of pathogens in a gene-for-gene manner (Dangl and Jones, 2001), as described in section 1.1.

Cellular mobility in plants is heavily constricted by cell walls, hence immune receptors are localized in all cells allowing a localized response in case of infection (Jones and Dangl, 2006) . Multiple receptor have been described (Dangl and Jones, 2001) however, upon activation they seem to activate similar responses which ultimately may ends in localized Programmed Cell Death (PCD), typically described

as necrotic spots on the infected tissue. This response is described as the Hypersensitive Response (HR)(Jones and Dangl, 2006).

1.1 PTI and ETI and resistance evolution

Evolution seem to have favoured an expanded innate immune system in plants based on immune receptor proteins, over the adaptive immune system based on specialized defence cells and recombination of immune receptors developed in higher mammals (Ausubel, 2005) (Chisholm et al., 2006). However, plants and mammals alike have shown presence of a largely conserved molecular mechanism of response referred to as Pathogen/Pattern Recognition Receptors (PPRs). These receptors recognize hostile organisms based on common Microbe-Associated Molecular Patterns (MAMPs) also known as Pathogen-Associated Molecular Patterns (PAMPs) (Ausubel, 2005, Jones and Dangl, 2006). Mammalian innate immunity is given by with Toll-Like Receptors (TLRs) and Nod-like receptors (NLRs). Nod like receptors resemble plant NB-LRRs, as detailed below (Ausubel, 2005, Dangl and Jones, 2001).

The first line of plant defences are mediated by molecular recognition of Pathogen-Associated Molecular Patterns (PAMPs), which in principle are molecules found across microbial species (Chisholm et al., 2006). Such molecules are often structural like chitin in fungi; flagelin, peptidoglycans and lipopolysaccharides in bacteria; or glucans in oomyces (Thomma et al., 2011, e. g. Ayers et al., 1976, Felix et al., 1993, Dow et al., 2000, Gust et al., 2007, Erbs et al., 2008). These molecules are elicitors of defence responses in plants, activating a set of defences generally referred to as PAMP Triggered Immunity (PTI)(Jones and Dangl, 2006).

PTI response has been reported to activate a complex network of signalling cascades which not necessarily activate HR mechanisms (Nurnberger and Kemmerling, 2009). Upon activation of PPRs, Mitogen Activated Protein Kinase (MAPKs) are considered to be responsible for triggering a signalling cascade that would up regulate defence genes to either produce callose depositions in the cell wall; phytoalexins or to produce oxygen reactive species (ROS), and –in some cases- trigger HR (Pandey and Somssich, 2009). However, among the different defence strategies, up regulation of genes activated by PPRs seems to trigger swift transcriptome changes carefully regulated to not always led to cell death (Pandey

and Somssich, 2009). In part is performed by a network of WRKY transcription factors (TF) which is thought to control activation or suppression of defence signalling pathways (Eulgem and Somssich, 2007).

Until recently, induction of a SAR state was thought to be triggered by ETI alone (Durrant and Dong, 2004), however Mishina et al (2007) has proven that PTI response also induces a SAR state in Arabidopsis (Mishina and Zeier, 2007). It has been described that SAR can be activated through the SA pathway upon infection of biotrophic pathogens, whilst JA pathway seem to be triggered by necrotrophic pathogens and chewing insects (Mur et al., 2006). Despite their normally antagonistic function, it has been reported that plant cells are able to rapidly adjust signalling even by regulating JA and SA pathways synergistically (Pandey and Somssich, 2009, Mur et al., 2006). In this regard, regulation of a partially interconnected pathway signalling network has also been shown to be related to WRKY TFs (Eulgem and Somssich, 2007). Likewise, negative or positive regulation of each pathway may have an effect on what set of defence genes are transcribed, shaping therefore defence response at cellular level for example, from callose deposition to activation of PCD. This careful regulation becomes of vital importance for survival, keeping in mind that HR always implies a loss of productive tissue for the plant. (Eulgem and Somssich, 2007, Nurnberger and Kemmerling, 2009, Nurnberger and Scheel, 2001, Pandey and Somssich, 2009)

Notwithstanding the broad effective basal defence system, some pathogens are still able to infect plants. Successful infections by virulent pathogens are required to have either a high tolerance to the defence system of the host plant, or on the other hand, suppression of defence responses (Nurnberger and Kemmerling, 2009). Is this regard, evolution has pressured pathogens to develop mechanisms for interrupting, inhibit, or, on the other hand, manipulate defence mechanisms in plants for their own benefit (Nurnberger and Kemmerling, 2009, Chisholm et al., 2006).

Pathogens that become virulent have evolved effectors to avoid recognition, just to expose some examples; the LysM effector Ecp6 from Cladosporium fulvum which sequesters oligosaccharide fragments of chitin to avoid chitin-triggered PTI (de Jonge et al., 2010). It has been also reported that Pseudomonas syringae effectors AvrPto, AvrRpt2 and AvrRpm1 inhibit PTI in susceptible plants (Chisholm et al.,

2006). Effectors are so specifically adapted to hosts that it has been reported that Pseudomonas AvrRpt2 is released inside the cell (via type III secretion system) as an inactive protease which would be later on activated by a native plant cyclophilin (such as Arabidopsis ROC1), and eventually inactivating the plant's defence response (Coaker et al., 2005). It has been also reported that fungal pathogens are able to influence the JA and SA pathways to favour their infection by influencing the response controlled by phytohormones (Chisholm et al., 2006).

However, evolution forced plants to recognize and develop specific immune proteins to recognize these specialized effectors and trigger a defence response, being HR the most frequent result. This kind of resistance is known as Effector Triggered Immunity (ETI) (Jones and Dangl, 2006) and responds to a gene-for-gene interaction (Flor, 1971). In this case, plants that have a specific R gene able to recognize the presence of an effector protein from a pathogen become resistant to their infection, whilst the pathogen becomes "avirulant". However if any of the alleles from the pathogen or the plant are absent or mutated, infection may still occur (Dangl and Jones, 2001, Jones and Dangl, 2006).

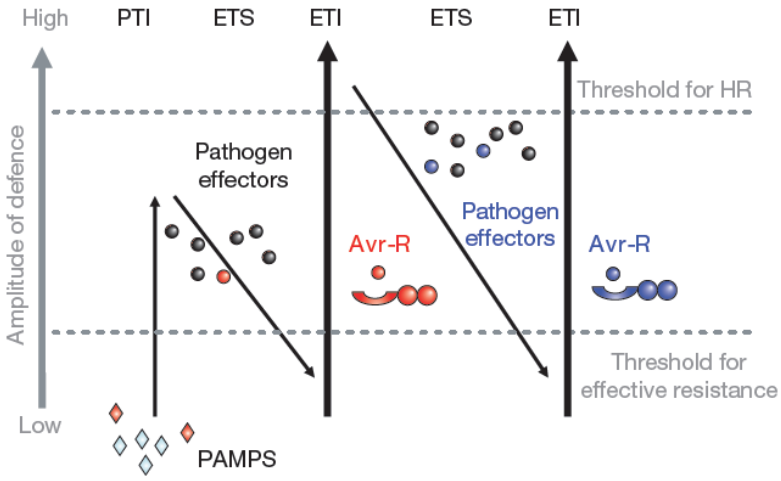

Figure 1. The zig-zag model introduced by Jones and Dangl to explain co evolution of pathogens and plants. When PAMPs cannot be recognized by the plant, resistance is low. However when PAMPs are recognized, the plant has a high resistance level given by a PAMP-Triggered Immunity response (PTI). Nonetheless, pathogens evolve effectors to circumvent PTI, causing Effector-Triggered Susceptibility (ETS), until the plant evolves an R gene to recognize an effector presence and re-establishes immunity by an Effector Triggered Immunity (ETI) response. If the pathogen is able to establish ETS again, the cycle will be a continuous process of co-evolution, (Jones and Dangl, 2006)

Just like some pathogens overcame PTI, there are also effectors found that are able to overcome ETI either (Chisholm et al., 2006). Nevertheless, plants have also

evolved different R genes that recognize a cognate Avr gene directly or indirectly (Jones and Dangl, 2006). In plant-pathogen interactions, mutations in effectors can occur rather frequently, therefore, forcing plants to have different mechanisms for developing R genes. One of these strategies is hypothesized as the "guard model" in which an R gene does not recognize an effector protein directly but it rather triggers defence by perceiving changes in a target protein compromised by the effector (Dangl and Jones, 2001, Van der Biezen and Jones, 1998). The coevolution of plant-pathogen interactions is hypothesized that, once breached the PTI, ranges from ETI to effector triggered susceptibility until the plant evolves a receptor which will trigger ETI again. This process has been described as the zig-zag model of Jones and Dangl (Jones and Dangl, 2006) shown in Figure 1.

1.2R proteins: models of structure and function

Resistance protein are classified according to their domain structure, describing two large groups of proteins: the Nucleotide-Binding Leucine-Rich Repeat (NB-LRR) and the extracellular LRR (eLRR) proteins (Chisholm et al., 2006)(Figure 2.). However, despite the large number of R genes reported, among the two groups, there has been described in total only 5 subclasses of proteins which collectively, determine the vast majority of resistance proteins to bacterial, fungi, oomyces, nematodes and viral pathogens (Chisholm et al., 2006, Dangl and Jones, 2001).However several R genes like Hm1 in maize or Rpg1 in barley –among others- do not fit any of the five commonly described structures (Martin et al., 2003).

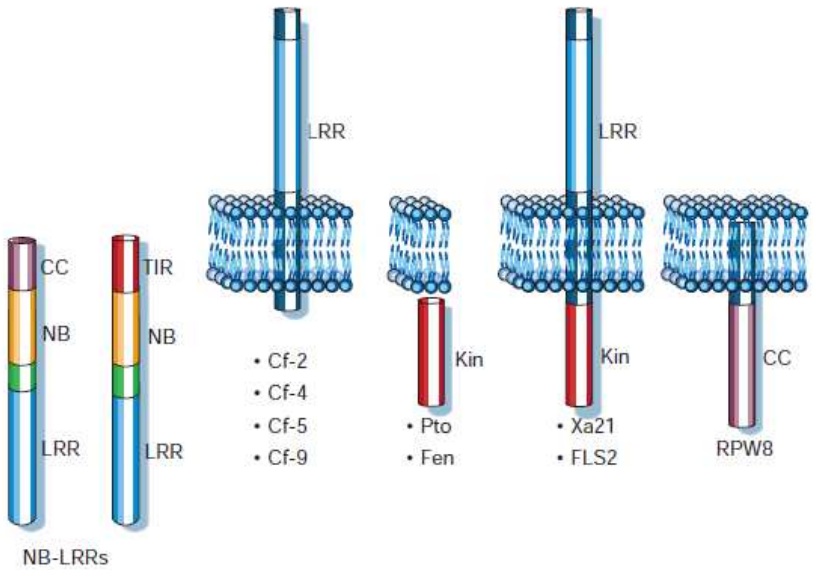

Figure 2. Domains representation mayor groups of R proteins identified to date. (Dangl and Jones, 2001)

Extracellular LRR proteins have been described mostly as transmembrane proteins classified by their domains architecture (Figure 2.) in: receptor like proteins (RLPs) and receptor like kinases (RLK) (both have a transmembrane domain and an extracellular LRR); Pto like receptors; and the putative Signal Anchor (SA):Coiled Coil (CC) receptor like protein(Jones, 2001, Chisholm et al., 2006) (Dangl and Jones, 2001).

From the RLPs, one of the most studied families of genes are the tomato Cf R proteins, conferring resistance to *Cladosposrium fulvum* effector proteins (De wit et al., 1993; Joosten et al., 1994; Tör et al, 2009). These RLPs have an extracellular LRR, a transmembrane domain (TM) and a short cytoplasmic region without any know function (Jones et al., 1994, Fritz-Laylin et al., 2005). Although a signalling domain has not been documented yet in Cf proteins, it is hypothesised that signalling occurs through interaction with other proteins (Chisholm et al., 2006). Receptor like kinases (RLK) such as Xa21 from rice confers resistance to *Xanthomonas oryzae* and has an extracellular LRR, a TM domain, and a cytoplasmic kinase as a signalling domain (Martin et al., 2003, Jones, 2001). Ultimately, a far less common type of receptor proteins are; Tomato Pto like proteins, which confers resistance to *Pseudomonas syringae* and has a Serine/Threonine kinase domain with a myristylation motif at its N terminus, (Martin et al., 1993, Jones, 2001) (Martin et al., 2003). And finally the SA:CC proteins RPW8.1, RPW8.2 which have been cloned from Arabidopsis and confers

resistance to a broad range of powdery mildew pathogens, however, only 5 homologous genes are known in Arabidopsis (Jones, 2001, Martin et al., 2003, Xiao et al., 2001).

It has been frequently reported that the largest group of R proteins found until now belong to the NB-LRRs proteins (Martin et al., 2003, Takken and Tameling, 2009, Chisholm et al., 2006, Jones, 2001, Jones and Dangl, 2006, Dangl and Jones, 2001). This family of proteins, despite some documented exemptions (Wirthmueller et al., 2007), generally do not contain a nuclear localization signal, suggesting that they are mostly cytoplasmicly located (Takken et al., 2006). Nonetheless, as further explained in 1.2, potato Rx has been shown to be carefully partitioned in the cell (Tameling et al., 2010). Different authors highlight details in organization and structure of R proteins; describing a largely shared C terminal LRR domain, a central nucleotide binding domain generally involved in self-regulation and activation, and an N terminal domain that is thought to be associated to signalling (Jones and Dangl, 2006, Jones, 2001, Dangl and Jones, 2001).

There is a class of NB-LRR proteins found in animal cells that has striking similarities to the innate immune receptors described across plants (Ausubel, 2005, Chisholm et al., 2006, Dangl and Jones, 2001). NB-LRR proteins from animal cells have also shown to play a role in regulating apoptosis and innate immunity (Ausubel, 2005, Dangl and Jones, 2001). Their surprising structural analogy suggests that this large family of proteins may have been developed early in the speciation process (Fritz-Laylin et al., 2005, McHale et al., 2006). However, this can also be the results of convergent evolution (Ausubel, 2005). In general plant NB-LRRs can be divided in two protein sub families according to their N terminal domain, namely the Coiled Coil (CC) receptor protein (CC-NB-LRR) and the Toll and Interleukin 1 receptor (TIR) protein (TIR-NB-LRR) (Dangl and Jones, 2001).

By analysing the functioning of each domain of NB-LRRs, a carefully regulation behind each R protein becomes evident. It is hypothesized that plant R proteins, although may initiate signalling through different mechanisms (not all of which are know), seem to trigger similar defence pathways that involves the transcription of conserved defence genes (Coll et al., 2011, van Doorn et al., 2011)

The LRR motif is a common feature found in thousands of protein ranging from viruses up to animal cells (McHale et al., 2006). The LRR domain is the longest domain in RLPs; the LRR repeat is a structural repetitive motif of 20-30 amino acids (Tameling and Takken, 2007); although in R proteins shorter sequences of 14 residues have been identified, with a characteristic sequence pattern rich in leucines (LxxLxxLxLxxxNxLxGxIPxxLGx, (Kajava, 1998)). This particular domain is suited to mediate protein-protein interactions and ligand binding (Kobe and Deisenhofer, 1994). Moreover, LRR domains are thought to have a dual function having a negative regulatory role in the NB-LRR (via protein intra-molecular interactions with the NB domain (Tameling and Takken, 2007)), and also a positive regulatory role, by translating the detection of a specific effector into activation of the protein (McHale et al., 2006, Takken and Tameling, 2009). Little is known about the mechanism by which the LRRs detect a pathogen. However it is known that some R proteins can bind directly to an effector, others bind indirectly through interaction with a mediating protein (following the guard model described in 1.1) (Takken and Tameling, 2009, Tameling and Takken, 2007, McHale et al., 2006). Notwithstanding the mechanisms, LRR domains are responsible for specific recognition of effectors, and the highly polymorphic nature of their non-conserved residues (represented by an "x" in the motif), which are predicted to be solvent exposed, suggests that those have an important role in specificity (McHale et al., 2006).

Linked to the LRR, is the NB domain (Figure 2). The core nucleotide binding domain in plant R proteins is fused to two other sub domains named ARC1 and ARC2 together forming the NB-ARC domain (Takken et al., 2006). This domain is also detected in two proteins involved in apoptosis in metazoans; Apaf-1 in mammals and Ced-4 in nematodes. The NB-ARC domain harbours motifs that are conserved within the signal transduction ATPases with numerous domains (STAND) proteins, of which the mammalian Nod-like receptors, playing a role in innate immunity, and Apaf-1 playing a role in apoptosis are also members (Dangl and Jones, 2001)(Leipe et al., 2004). However, these proteins contain a different N-terminal domain such as the caspase-activating recruitment domain (CARDs). It has been reported that STAND proteins usually function as molecular switches in signalling pathways (McHale et al., 2006)(Leipe et al., 2004). It has been proposed that conformational changes induced by a nucleotide binding site regulates activation of R proteins, introduced in the activation model presented by Takken

& Tameling (Figure 3)(Tameling et al., 2006, Takken and Tameling, 2009). Furthermore, in light of recent evidence, plant R proteins could have a unique characteristics with respect to this particular family of proteins, since it has been found that the NB domain in the potato protein Rx might play a role in initiating downstream signalling, as transient expression of the NB domain of Rx alone in tobacco has proven to trigger an HR (Rairdan et al., 2008) (Takken and Tameling, 2009). Nonetheless, there are still many open questions and debates regarding downstream signalling of R proteins.

The N-terminal side of most plant NB-LRRs have a TIR or a CC domain. The TIR domain has been linked to downstream signalling in Toll-like receptors from animal cells (Feys and Parker, 2000). In plants the CC domain is hypothesised to interact with either, signalling proteins triggering downstream signalling or to interact in protein complexes including chaperones and co-chaperones to activate a specific R protein, or else, with proteins being targeted by effectors according to the guard model (guardees) (McHale et al., 2006, Takken et al., 2006, Collier and Moffett, 2009, Lukasik and Takken, 2009). Given the predicted protein-protein interaction characteristics of this domain (McHale et al., 2006), it is still unknown how recognition and signalling are differentiated in many plant R proteins. There are, however, documented cases like for Rx in which the CC domain has proven to interact with a RanGAP protein that is important for balancing the nucleocytoplasmic distribution and stabilization of Rx, which is essential for Rx-mediated resistance to PVX (see section 1.3) (Tameling and Takken, 2007, Tameling et al., 2010, Slootweg et al., 2010).

Nonetheless, there are still many questions surrounding the mechanism by which Rx is able to trigger downstream signalling. This thesis report includes experimentation and results oriented to search for these answers.

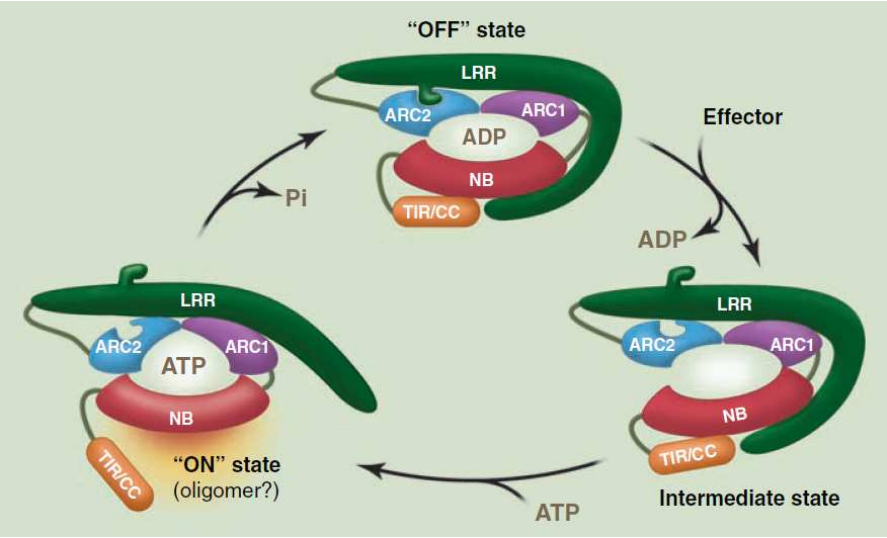

Figure 3. Model introduced by Takken and Tameling to explain intra-molecular autoregulation of NB-LRR proteins to avoid autoactivation (Takken and Tameling, 2009). In this model the NB-ARC domain is proposed to act as a molecular switch regulating the signalling activity of the NB-LRR.

1.3 Rx protein localization and partitioning

The Rx protein encoded on chromosome V is a CC-NB-LRR protein responsible for providing extreme resistance to Potato Virus X (PVX) found in potato cultivar *Cara*. There was another locus reported (on chromosome XII that also confers extreme resistance to the virus (Bendahmane et al., 1995). This turned out to be mediated by the Rx2 gene (Bendahmane et al., 2000). The resistance conferred suppresses PVX proliferation in plant tissue, and moreover, this response does not trigger an HR and is therefore called extreme resistance. (Bendahmane et al., 1999, Bendahmane et al., 1995). The Rx protein is most closely related to the potato proteins Rx2, encoded on chromosome XII, Gpa2, at the same locus as Rx, and the pepper protein Bs2, which confer resistance to PVX, the nematode *Globodera pallida*, and the bacterium *Xanthomonas campestris*, respectively (Rairdan and Moffett, 2006).

Activation of the Rx complex induces disruption of the intra-molecular interactions between the different domains (Moffett et al., 2002). The coat protein (CP) of PVX has been identified as the elicitor that activates Rx, which subsequently induces the extreme resistance response (Bendahmane et al., 1999, Bendahmane et al., 1995).

Research on this immune receptor has shown interaction of Rx with Ran GTPase–Activating Protein 2 (RanGAP2) (See section 2.), which is mediated by the N-terminal domains of both proteins. In Rx this is the CC domain and in RanGAP2 this is the Tryptophan-Tryptophan-Proline (WPP) domain (Tameling and Baulcombe, 2007)(Sacco et al., 2007). Furthermore, virus-induced gene silencing of *RanGAP2* in transgenic *N. benthamiana* plants carrying Rx has been reported to impair Rx-mediated resistance to PVX, which suggested that the nucleocytoplasmic trafficking that is regulated by RanGAP2, might be important for Rx function (Tameling and Baulcombe, 2007) Recent data, however, sheds light on the localization of Rx in the cell, which has been reported to situate in both the cytoplasm and the nucleus, despite its lack of a NLS (Tameling et al., 2010, Slootweg et al., 2010). Remarkably, when Rx is transiently coexpressed with RanGAP2 or the RanGAP2-WPP domain in *N. benthamiana*, its localization in the cell changes completely as the nucleocytoplasmic distribution strongly shifts towards the cytoplasm (Tameling et al., 2010). Furthermore, virus-induced gene silencing of *RanGAP2* resulted in an increased nuclear import of Rx. Together these data indicate that RanGAP2 serves as a cytoplasmic retention factor balancing the nucleocytoplasmic distribution of Rx. A balanced distribution is important for Rx function, because a shift in the equilibrium either towards the nucleus or to the cytoplasm impairs the function of the protein (Slootweg et al., 2010, Tameling et al., 2010).

2. Nucleocytoplasmic trafficking

Different researchers have found that some R proteins need to be located at the nucleus for functioning, and yet, very few R proteins have shown to contain a discernible NLS motif (Wirthmueller et al., 2007, Slootweg et al., 2010) (Shen and Schulze-Lefert, 2007). This paradox suggests the existence of a non-explored nuclear signalling mechanism allowing proteins without and NLS to be carried into the nucleus (Tameling et al., 2010). To understand the role that nuclear trafficking proteins may have in regulation of downstream signalling from Rx, it is necessary to study further the mechanism by which the subcellular distribution of Rx is kept in a careful balance.

RanGAPs are proteins involved in the regulation of the nucleocytoplasmic trafficking system occurring at the nuclear envelope (NE) (Meier, 2005). In plants,

trafficking of proteins heavier than 50 KDa across the NE through the nuclear pore complex (NPC) requires an active and meticulous import system involving importin proteins α & β, RanGTPases, (RanGDP/GTP), RanGAPs and other proteins involved in conformational changes and stability of the system (Meier, 2005, Liu and Coaker, 2008, Meier, 2007, Stewart, 2003).

Nuclear import of proteins initiates with the protein binding via its NLS to an importin α. The importin α subsequently binds to importin β after which the whole complex is imported into the nucleus through the nuclear pores (Meier, 2005). In the nucleus the complex dissociates when the importin β binds to Ran-GTP of which the concentration is much higher in the nucleus than in the cytoplasm. The importin β protein in complex with Ran-GTP is exported from the nucleus into the cytoplasm. At the outside of the NE RanGAP is localized which stimulates the GTPase activity of Ran-GTP when it comes out of the NPC, by which it rapidly converts to its Ran-GDP form. This hydrolysis leads to a conformational change that induces the release of Importin β in the cytoplasm ready for another round of import. Also the importin α protein is recycled and exported back to the cytoplasm. The whole nuclear trafficking system is driven by a sharp gradient of Ran-GDP to Ran-GTP over the NE, which is maintained by RanGAP in the cytoplasm and by additional factors in the cytoplasm and nucleus (Meier, 2005, Liu and Coaker, 2008, Meier, 2007, Stewart, 2003) (Xu et al., 2007).

2.1 RanGAP2 involvement in Rx-mediated defence response

Nuclear localization of several R proteins -besides Rx- has driven scientists to hypothesize that a complex and vast regulation process in plant cells based on the controlled trafficking system at the NE is important for R protein function (Xu et al., 2007, Meier, 2005, Liu and Coaker, 2008).

The plant nuclear trafficking system has been less studied than in the animal or yeast systems. However, much of its details have also been explored in plants. For example RanGAP proteins have been found anchored to the NE by a different mechanism that is unique to plants (Meier, 2005, Liu and Coaker, 2008, Meier, 2007, Xu et al., 2007). Plant RanGAPs possess a plant-specific N-terminal WPP domain with a characteristic WPP motif that is essential for targeting RanGAP to the NE through association with WPP domain interacting proteins (WIPs) and WPP-domain-interacting tail-anchored proteins (WITs)(Meier et al., 2010).

However, why is there a nucleocytoplasmic trafficking protein interacting with an R protein and is playing such an important role for R protein functioning? It has been reported that the WPP domain of RanGAP2 requires an extended CC domain to be targeted to the NE that is present in the nuclear anchor WIP and WIT proteins. Although not an extended CC domain, Rx does contain a CC domain which could bind to the WPP domain in a similar fashion as the WIPs and WITs (Liu and Coaker, 2008). Nonetheless, potato RanGAP1 shares nearly 68% of identity with potato RanGAP2 and does not interact with Rx as strongly as RanGAP2, giving an indication of the specificity of the interaction (Sacco et al., 2007, Tameling and Baulcombe, 2007, Tameling et al., 2010).

This information raises important questions towards the regulation of the Rx activation complex. Is it possible that the lack of an NLS in Rx is compensated by associating an NLS-containing protein? Does RanGAP2 have additional roles in the signalling complex of Rx besides serving as a cytoplasmic retention factor? What surface in the RanGAP2 WPP domain is involved in the binding to Rx and to the nuclear anchor proteins? Are there more proteins involved in the Rx complex? Is it RanGAP2 determining activation of the signalling complex by influencing in the partitioning of Rx proteins in the cell? Could this mechanism also apply to other R protein models?

To answer the question whether more proteins are part of the Rx complex a yeast-two-hybrid (Y2H) screen was started previously. As baits the Rx-NB and Rx-CC-NB domains were chosen and screened against a cDNA library derived from dying seedlings of transgenic Cf4/Avr4 tomato undergoing HR. Per bait more than hundred colonies from the initial screening plates were picked and stored as glycerol stocks. Only a few colonies were further analyzed so far. The aim of this MSc thesis was to further test the growth behaviour of the remaining colonies on the various selective plates in order to identify true Rx interactors. Secondly, a parallel objective was to identify key residues of RanGAP2 involved in the interaction between the WPP domain of RanGAP2 and the CC domain of Rx. This should reveal which amino acids from the Rx-binding surface, which will be used as constraints for the *ab initio* modelling of the WPP domain. To this end, a mutant library of the RanGAP2 WPP domain was developed and these mutants were screened for loss of HR in the transient coexpression with Rx-CC-NB in *Nicotiana tabacum*. For the Y2H screening results have proven to be discouraging, since among the 50 picked candidates no potential interactors were found. The mutant

screen was more successful, as several mutants have shown loss or lower levels of HR. From only a few candidates the sequence could be analyzed. The identified mutations lead to lower protein levels or to truncated proteins which likely caused a lower response. Therefore, further research is still needed to pinpoint key residues of RanGAP2 involved in Rx binding.

3. Results

3.1 Yeast-two-hybrid library screen

Results from previous publications have shown that the NB domain of Rx was sufficient to trigger a defence response *in planta* (Rairdan et al., 2008), likewise the CC domain of Rx has been identified to interact with proteins inside the cytoplasm (Tameling and Baulcombe, 2007). Based on this information and encouraged by previous publications of interactors identified by Y2H system, we decided to perform a library screen searching for potential interactors of the NB and CC-NB domains using a Matchmaker™ GAL4 Two-Hybrid system as detailed in methodology (Section 5.3).

Two bait constructs were created previously by ligating the Rx-NB domain and Rx-CC-NB fragments in the GAL4-bait plasmid pGBKT7 by which a fusion with the DNA binding domain (DNA-BD) was created. The prey library was derived from mRNA samples isolated from dying seedlings (Cf4/Avr4 tomato plants) and their parental lines (de Jong et al., 2002). These seedlings are the offspring of a cross between the transgenic Moneymaker (MM) Cf-4 line and the transgenic MM line carrying the cognate elicitor AVR4. Because of the Cf-4/Avr4 production the seedlings will undergo HR, however they can be rescued at 33°C. Shifting the seedlings to 22°C initiates the execution of HR. For the cDNA library seedlings were harvested for mRNA isolation at four specific time points after the shift to 22°C (0h, 3h, 5h and 8h). In a similar way seedlings from the parental lines were harvested at the same time points. mRNA of all samples were mixed together in identical quantities and used for cDNA synthesis in the library construction. The cDNAs were cotransformed with the linear pGADT7-Rec vector by which the cDNAs recombined in the prey vector (Nora Ludwig, personal communication). Aliquots of these prey containing yeast cells were used for the Y2H screening. In the pGADT7 vector the preys are fused to the activation domain (AD). Maps of the vectors can be

seen as appendix 1 and 2. Four different marker genes for selection were used according the specifications of the provider: tryptophan, leucine, adenine, histidine and *lacZ*. However, from the four reporter genes tryptophan and leucine are markers selecting for transformation only, since the bait plasmid has a gene encoding for tryptophan synthesis and the prey plasmid has a gene encoding for leucine synthesis (Clontech Yeast Protocols Handbook, Madison, USA).

3.1.1 Making of the library and screening strategy

The development of the prey library and the transformation to the yeast strain AH109 was performed previously by Nora Ludwig and Daniela Sueldo. They also transformed the two baits to strain Y187. The subsequent mating was performed by Nora Ludwig according to the protocol publish by Bickle et al. (Bickle et al., 2006). In the screening with the Rx-NB and the Rx-CC-NB baits, 6 x 10^7 and 1.2 x 10^8 colonies were screened respectively. This was based on plating a fraction of the mating reaction on transformation selective –WL media only selecting for the presence of both plasmids but not for bait and prey interaction. For the Rx-NB screening 192 colonies were picked that appeared on the selective -AHWLplates. For the Rx-CC-NB screening 192 colonies were picked from the –HWL+3AT (2.5 mM) selective media. The 3-Amino-1,2,4-triazole (3-AT) inhibitor was used to prevent the leakiness associated to the histidine marker (Clontech Yeast Protocols Handbook, Madison, USA). So in total 384 colonies has to be analyzed further. Only a few of these colonies were previously characterised (Nora Ludwig, personal communication). All these colonies had been inoculated in liquid cultures and stored as glycerol stocks previously. This was the status of the Y2H screenings at the start of my thesis.

The bait plasmids were coded as follows: Rx-NB in pGBKT7 hereafter referred to as 1005 (after its code SOL1005) and Rx-CC-NB in pGBKT7 hereafter referred to as 1001 (after its code SOL1001) (Table 1).The negative control of the screen was a combination of empty pGBKT7 plus empty pGAD7. The positive control was a combination of pGBK7-p53 (murine) plus pGADT7-SV40, a large T antigen. P53 interacts with SV40 in yeast, according to the manual (Clontech Yeast Protocols Handbook, Madison, USA).

Table 1. Labelling of constructs and combinations used in the Y2H screen

Construct	Label
pGBKT7	Empty bait
pGBKT7+Rx-NB	1005
pGBKT7+Rx-CC-NB	1001
pGADT7	Empty prey
pGADT7+cDNA	cDNA Clone
pGBKT7+p53	Bait pos. Control
pGADT7+sv40	Prey pos. Control
Combinations	
Sample	1001/1005 + cDNA Clone
Negative Ctrl.	Empty bait + Empty prey
Positive Ctrl.	Bait control + Prey control
Empty bait control	Empty bait + cDNA clone
Retransformation	1001/1005 + cDNA clone

Further characterization of the 384 colonies was performed by taking two approaches from which the potential candidates could be evaluated: growth performance in selective media and DNA profiling.

3.1.2 Growth in selective media

The first approach was to test the potential interaction strength *in vivo* by growing the yeast on selective plates lacking leucine and tryptophan (-WL) as controls, and histidine and adenine (-AHWL and -HWL) for interaction. Due to the reported leakiness of the histidine marker, three different concentrations of the 3-AT inhibitor were used to obtain more stringent selection plates: 2,5 mM, 10 mM and 15mM. Growth was evaluated at 3 and 7 dpi (Table 2).

Finally, activation of the *LacZ* gene was tested by the β-galactosidase overlay assay. For this assay the colonies were grown on -WL plates. The occurrence of blue colonies was evaluated at 30, 60 and 90 minutes (Figure 5).

Table 2. Evaluation sample of the results of the growth on selective media. Candidates in bold fitted the selection criteria and were retransformed.

Colony	-WL	-AHWL	-HWL 2.5mM 3 AT	-HWL 10mM 3 AT	-HWL 15mM 3 AT	α X-GaL
01 B2-A8	+	+	+	/	/	+
05 B2-H6	+	+	+	+	+	/
01 B1-F1	**+**	**+**	**+**	**+**	**+**	**+**
01 B1-C4	+	/	+	+	+	/
05 B1-C8	+	/	+	+	+	-
01 B1-B9	+	+	+	+	+	-
05 B2-A7	**+**	**+**	**+**	**+**	**+**	**+**
01 B2-F2	+	/	+	/	-	/
05 B1-A12	+	+	+	+	/	+
05 B1-B4	+	+	+	+	+	-
01 B2-B5	**+**	**+**	**+**	**+**	**+**	**+**

(+)Positive growth. (/)Compromised performance (-) No growth

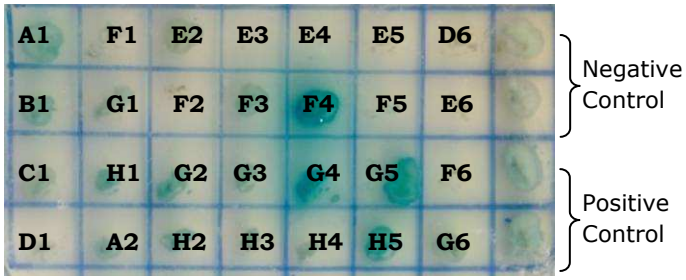

Figure 5. Example of galactosidade assay at 60 min. Response shown corresponds to plate 1 of the 1005 screening grown on -WL plates, overlaid with low-melting agarose containing α X-GAL and incubated at 30°C. Samples F4, G4, G5 and H5 showed better response than the positive control.

3.1.3 DNA profiling

In parallel, DNA profiling of the constructs were performed by colony PCR using primers JV001 and J002 designed by Jack Vossen (Table 7). The DNA fragment length coding for the preys ranged between ~300 and ~2100 bp. (Figure 6). Subsequently, digestion with the frequent cutter AluI restriction enzyme resulted in multiple patterns ranging between 0-4 bands and between ~2100 bp and ~200 bp (Figure 7).

Pattern studies were performed and groups of clones showing similar lengths and digestion patterns were clustered to avoid repetition in the selection. A disadvantageous characteristic the yeast-two-hybrid system is that a

cotransformant colony may activate transcription of the reporter genes without having a real interaction with the prey. The DNA contained in either the prey or the bait can trigger transcription on its own. This situation is referred to as auto-activation (Legrain et al., 2001). Colony PCR was also performed to search for the autoactivator 7B-8, previously found in a screening with the same library by Henriek Beenen (unpublished data). Only 5 colonies were positive from both screenings. This low incidence led to omit this procedure in the search of other known autoactivators.

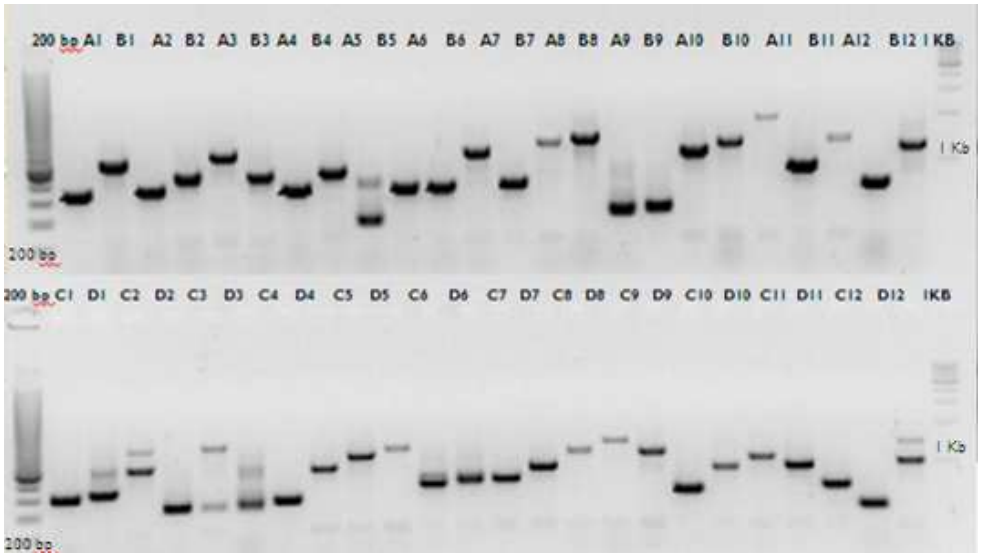

Figure 6. Colony PCR. Results shown from A1-D1 of plate 2 screening 1005 ran on a 1% agarose gel.

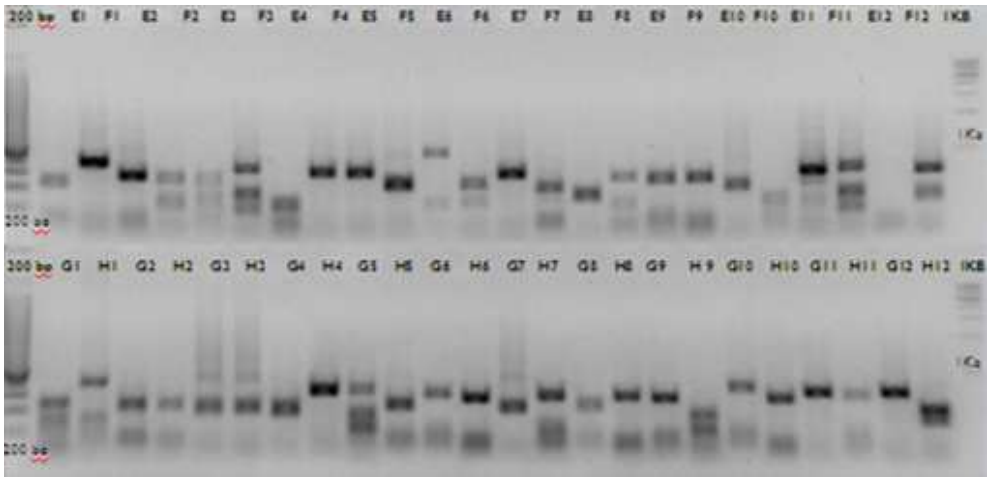

Figure 7. Digestion of colony PCR products with Alu1. Results shown from E1-H12 of plate 2 screening 1005 ran on a 1% agarose gel.

DNA profiling consisted in sorting colonies according to the insert size amplified by PCR (Figure 6). To this arrangement of data, information coming from the digested products was included. Colonies were arranged by insert size as first criteria, and

secondly, by digestion pattern. Clusters of colonies showing the same insert size and digestion pattern were suspected to have similar prey plasmids. Concomitantly, all candidates were selected based on growth performance in selective media, apparent healthiness of the colony and efficiency in conversion of α X-GAL. Detailed information on screen evaluation results for the 396 colonies can be found in Appendix 3 30 potential candidates for 1001 screen and 20 potential candidates for the 1005 screen (Table 3)

Table 3. Final list of colonies selected from screenings 1001 and 1005 to be retransformed. Code represents bait (1001/1005), number of plate (B1/B2) and location of the colony from the original microtiter plate.

Bait 1001			Bait 1005	
01B1-A1	01B1-F1	01B2-D10	05B1-A12	05B1-H5
01B1-A6	01B1-H11	01B2-D12	05B1-B7	05B2-A3
01B1-A7	01B2-A2	01B2-E11	05B1-C10	05B2-A7
01B1-B2	01B2-A3	01B2-E7	05B1-C7	05B2-C8
01B1-B5	01B2-A6	01B2-E8	05B1-D10	05B2-D8
01B1-C1	01B2-A7	01B2-F6	05B1-F10	05B2-E1
01B1-C10	01B2-B5	01B2-G2	05B1-F4	05B2-E10
01B1-C12	01B2-C12	01B2-G8	05B1-G2	05B2-E3
01B1-C2	01B2-C7	01B2-H1	05B1-G4	05B2-E4
01B1-C7	01B2-C9	01B2-H7	05B1-H2	05B2-G8

The final step in screening potential interactors was to re-test candidates in a new co-transformation to identify false positive results by autoactivation. Prey plasmids coming from the 50 selected clones were isolated and cotransformed in the yeast strain *PJ694a* following a LiAc protocol (Clontech Yeast Protocols Handbook, Madison, USA). Each prey clone was tested in two ways, repeating the original cotransformation with the bait insert (1001/1005) plus a parallel test with an empty bait. The objective of this final step was to prove that only the interaction between the prey clone and the bait insert was activating transcription of the reporter genes. In absence of the bait insert, activation should not occur.

Retransformants were initially inoculated in –WL and stamped for evaluation of growth on selective media on the following plates: -AHWL, -HWL + 10mM 3AT, -HWL + 15mM 3AT and –WL.

There were two kinds of results, a) none of the retransformants showed any difference compared to the empty bait control at 3 or 7 dpi; and b) positive growth of the empty bait control versus negative growth of the retransformants. Data suggests that none of the selected candidates identified were potential interactors, but rather autoactive colonies (Figure 8).

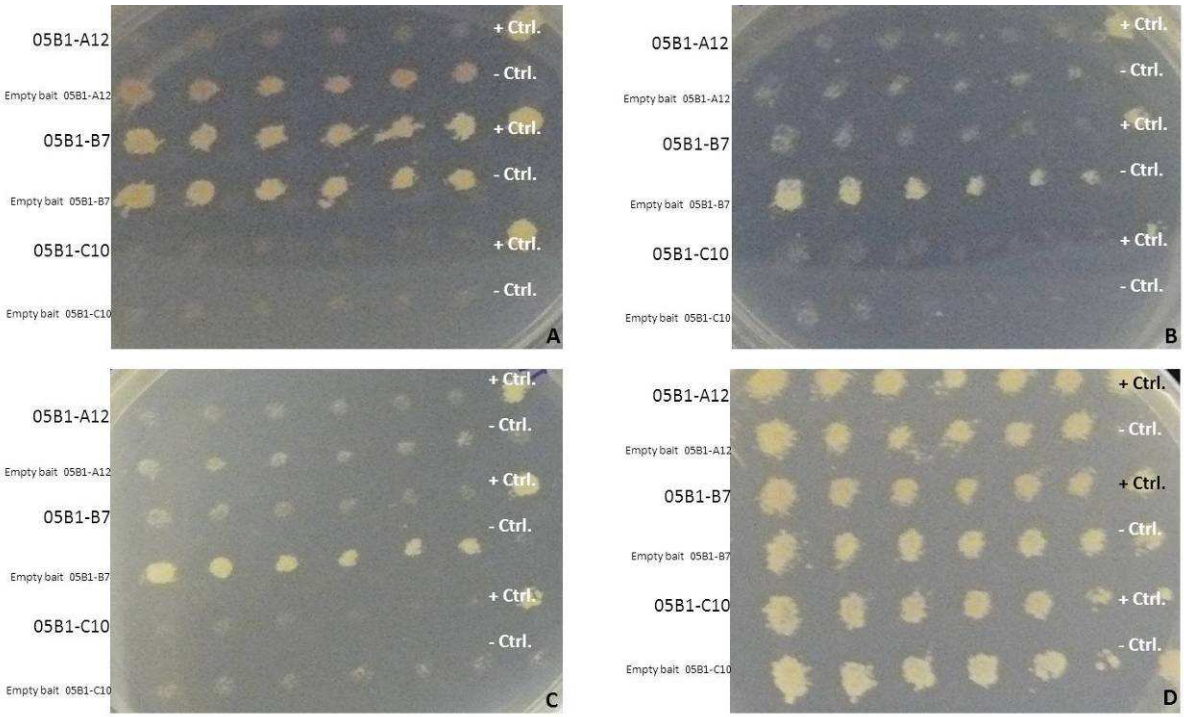

Figure 8. Retransformation results from three colonies of 1005 screening at 7 dpi. Results represents the two kind of responses found among the total amount of colonies: no difference between retransformant and empty bait control (05B1-B7 & 05B1-C10), and negative growth of retransformant versus positive growth of empty bait control (05B1-A12). Selective media: a) –AHWL; b) –HWL + 15mm 3AT; c) –HWL + 10mm; d) –WL.

3.2 WPP domain random mutagenesis process

As it has been addressed already in 2.1; the WPP domain of RanGAP2 is essential for the interaction with the Rx CC domain (Tameling and Baulcombe, 2007)(Sacco et al., 2007). The WPP domain of RanGAP1 and RanGAP2 share nearly 53% identity. Nevertheless, RanGAP1 association with Rx could not be detected in co-IPs(Tameling 2007). Later, yeast-two-hybrid studies showed that RanGAP1 is able to interact with Rx in yeast. In agreement similar to RanGAP2, RanGAP1 was also able to sequester Rx in the cytoplasm, albeit to a lower extent. Together with the co-IP data this indicates that RanGAP1 binds with lower affinity to Rx (Tameling et

al., 2010). Based on this information and on previous mutagenesis studies oriented to study gain or loss of a specific target (Tang et al., 2011, Tameling et al., 2010, Tameling et al., 2006, Bendahmane et al., 2002), we decided to generate a library of mutants for RanGAP1 and RanGAP2, to screen for gain and for loss of Rx interaction mutants.

3.2.1 Cloning of the WPP domains of RanGAP1 & RanGAP2 from *Nicotiana benthamiana*

RanGAP1-ΔC-Δstop and RanGAP2-ΔC-Δstop were amplified from plasmids Sol162 and SLDB3151. The PCR products were purified and ligated into the pENTR D-Topo plasmid. Sequencing and digestion with Pst I, EcoRI and BamHI confirmed successful cloning in this Gateway® compatible vector. This resulted in two entry clones that that were used for the subsequent LR reaction, recombining the *RanGAP* sequences into the destination vector pBIN-KS-GFP (SOL2095). The resulting plasmids were pBIN-KS-Rg2-ΔC-GFP (SOL3100) and pBIN-KS-Rg2-ΔC-GFP (SOL3102) (see Methods). Successful cloning was verified by sequencing and digestion with HindIII and KPNI (Figure 9).

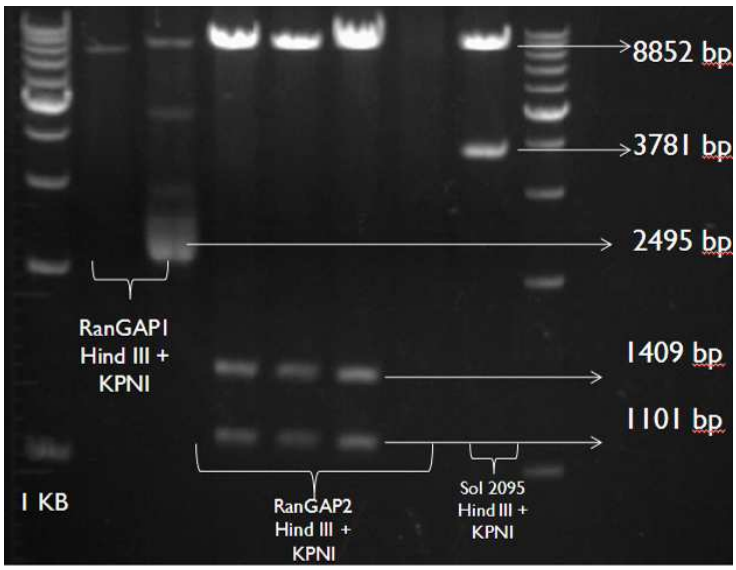

Figure 9. Digestion pattern of plasmids RanGAP1-ΔC-GFP, RanGAP2-ΔC-GFP and destination vector digested with HindIII+PKNI. Lanes 2-5 +7 showed the expected band pattern corresponding to the digestion of the right construct matching the *in silico* analysis done in Infomax Vector NTI®. Lane 1 failed and 6 was a negative control.

3.2.2 Coexpression response of RanGAP1-ΔC-GFP and RanGAP2-ΔC-GFP with Rx-CC-NB *in planta*.

The main objective of the WPP mutagenesis library was to analyze loss of Rx interaction based on mutations observed. As described in the methodology, the evaluation system was *Nicotiana tabacum*. Screening for loss of Rx interaction in co-IPs would not be feasible. Therefore we made use of the HR phenotype that was observed previously. Namely, when RanGAP2 or only the RanGAP2 WPP domain was co-expressed with Rx-CC-NB-HA a strong HR is triggered (Sacco et al., 2007; Tameling et al., 2010). This is not the case when RanGAP1 is co-expressed with Rx-CC-NB-HA (Tameling et a., 2010). The induced HR is a consequence of the increased Rx-CC-NB-HA levels that is the result of the Rx stabilisation mediated by interaction with RanGAP2. So, the induction of HR is an indicator of the Rx-RanGAP2 interaction. In order to test stability of both RanGAP-GFP fusion constructs, a set of coinfiltration in *N. tabacum* were tested with optical densities (OD) ranging from 0.1 until 1. The response from these coinfiltrations also set a minimum background for further evaluation, establishing therefore the minimum OD in which WT RanGAP2 was still able to trigger HR when coexpressed with RX-CC-NB.

Coinfiltrations in *N. tabacum* were performed with *Agrobacterium tumefaciens* strain C58C1 with the pCH32 helper plasmid under controlled conditions described in methodology. The leaves were evaluated at 7 days post infiltration (dpi).

Results shown in Figure 10 indicated similar levels of HR response triggered from ODs ranging 0.1 to 1.0 for RanGAP2-ΔC-GFP when co-expressed with Rx-CC-NB-HA, which indicated the flexibility of the OD range which is important for the large screening. As recently reported (Tameling et al., 2010), RanGAP1 appeared to have a weak interaction with Rx. Co-expression of RanGAP1 with Rx-CC-NB-HA does not induce an HR (Tameling et al., 2010), however we found that co-expression with RanGAP1-ΔC-GFP did induce a weak HR (Figure 10 b). Since the full length RanGAP1 does not induce an HR this result was not expected. However, this is likely the cause of higher expression levels of RanGAP1-ΔC compared to the full RanGAP1. This was indeed found for RanGAP2 and RanGAP2-ΔC (Tameling et al., 2007). The aim was to screen the RanGAP1-ΔC-GFP mutant library in combination with Rx-C-NB-HA to search for mutants that induced an HR. However, Figure 10 shows that the mild response triggered by the WT RanGAP1-ΔC would be hardly

distinguishable from a potential gain of function mutant. Because of this high background level, we decided not to proceed with the RanGAP1-ΔC screening of the experiment; since any effort evaluating what level of gain of function would have been acquired by the mutations becomes futile.

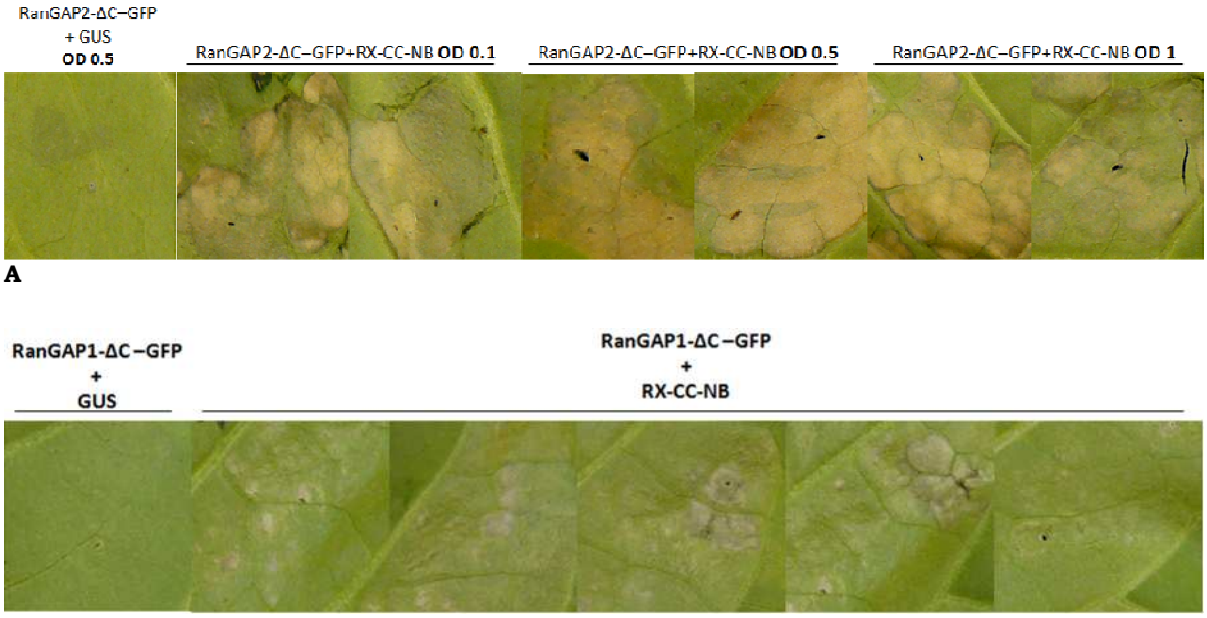

Figure 10. A) Results of coinfiltrations at different OD levels of RanGAP2-ΔC-GFP in *N. tabacum*. Although the best HR response can be seen in OD 0.5, similar responses were observed in OD 0.1 compared to OD 1. RanGAP2-ΔC-GFP coexpressed with GUS does not induce any response, establishing therefore a reliable negative control for the screen. B) Background reaction triggered by RanGAP1-ΔC-GFP + Rx-CC-NB in *N. Tabacum*. Different levels of response can be seen despite having the same OD of 0.5, making it difficult to assess a better response than the background.

3.2.3 Making of the mutant Library of the WPP domain of RanGAP2

From the pENTR-RanGAP2-ΔC-Δstop of point 3.2.1, a random mutagenesis reaction was performed using the GenMorph® II EZClone Domain Mutagenesis Kit (Figure 11), and following the procedure advised from the providers and described in methodology. The intended mutagenesis rate was to have between 0-4.5 mutations per construct. From the original 1.4 μg of input DNA, the PCR products were amplified to a 6.21 fold, according to the recommendations of the provider. The mutant fragments were cloned in the pENTR-D-TOPO vector by the EZ clone reaction, resulting in a RanGAP2-ΔC mutant library in this entry vector. The mutant fragments were subsequently introduced in the destination vector pBIN-KS-GFP (SOL2095) by the LR reaction. The resulting plasmids were transformed to

E. coli and grown over night in selective media. DNA was isolated from this overnight culture and was used to transform *A. tumefaciens* C58C1 + pCH32. The final library size was 2016 mutants in *A. tumefaciens*, accounting for 5.76 x coverage of the WPP domain. In this way, we estimated that the mutagenesis process would cover each base pair of the WPP domain, having the library covering the peptide 5.76 times. The library was then grown in 21 microtiter plates for three days. Afterwards, 20% glycerol stock were made and kept at -80°C. .

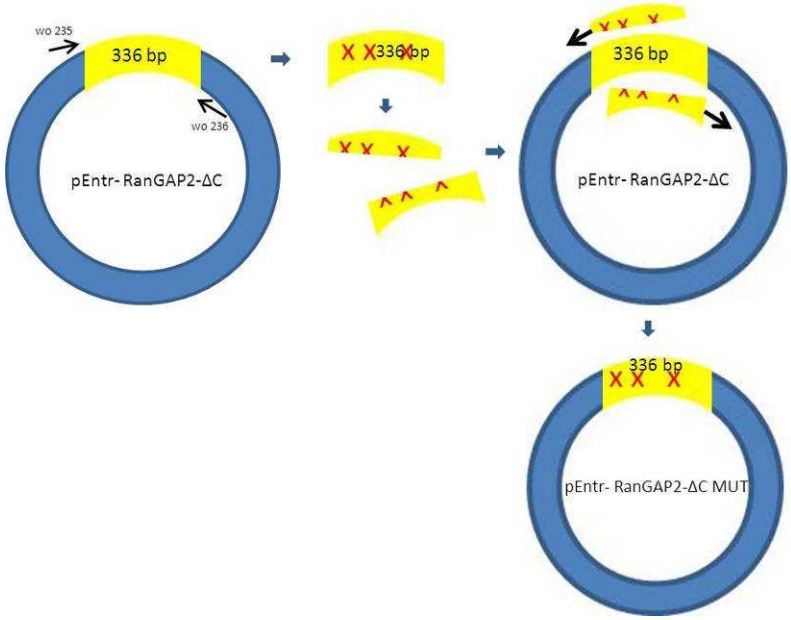

Figure 11. Random mutagenesis reaction using the GenMorph® II EZClone Domain Mutagenesis Kit. The coding region of the WPP domain (336 bp) from the pENTR-RanGAP2-ΔC-Δstop was amplified with the Mutazyme II® DNA polymerase by PCR using primers wo235 and 236. The PCR products were used as megaprimers in the EZ clone® PCR reaction in which the megaprimers annealed to the WPP domain of the original plasmid and a high fidelity polymerase amplified the entry vector.

From the resulting mutant colonies in *E. coli*, 20 were sequenced to analyse the overall performance of the kit. From the 20 samples tested, 65% of the clones were mutated with 1-3 mutations per construct. Mutations are biased to exchange bases between purines (A<->G) and pyrimidines (C<->T), the change between purine bases is called transition (TS) and the change between pyrimidines is called transversion (TV). However, according to the manufacturer, the enzyme mix contained in the kit reduces biased mutations (AligentTechnologies, 2009). The ratio of Ts/Tv registered in the 20 mutants sequenced was 1.4; which extrapolated

to each 100 clones, shows a slight bias of 10% towards T-C and A-G mutations, and vice-versa. From the sequencing results, in terms of incidence of mutations, we considered that the mutagenesis library of RanGAP2-ΔC was useful for screening. About 35% of the clones are WT. However, if we would change the mutagenesis conditions to decrease this number we would get a higher number of mutations per clone, which is not desirable. Detailed information about the mutation results is shown in table 4.

Table 4. Results of the mutated samples ordered for sequencing, data represents 65% of the complete batch.

Mutant	# of Mutations	# Deletions	AA lost	Ts/Tv
1	1		P	c-t
4	3		R,N,E	g-a;a-t;g-t
7	1		T	c-t
10	1		S	t-a
11	1		L	t-c
12	1		E	a-g
13	1		S	t-a
15	1		L	t-c
16	0	1	K	(FS)
17	2		A,F	g-a;t-a
19	2		T,Q	g-a;a-g
20	3		S,L,H	t-a;c-t;t-a

3.2.4 Standardization of procedure for culture and infiltration of mutants

The library was tested in *N. tabacum* for loss of HR when coexpressed with Rx-CC-NB-HA. In order to establish the protocol followed and described in methodology; we set in place a number of tests to analyze the potential variation in growth in microtiter plates. This was important for the culture plates that were used to make the glycerol stocks as well as the culture plates used for the screening. Too much variation in the growth could obscure the screening process.

We inoculated 12 mutants in a microtiter plate in 200 µl of selective media (K+Tet) at 28°C and 200 rpm. OD was measured during three days; glycerol was added and aliquots were taken each day to inoculate a second plate. With this procedure we determined the best incubation period for making both the glycerol stock plates and the working plates. Results showed that the best incubation time for making

glycerol stocks was 72hrs (Figure 12) and the best incubation time for working plates was 48hrs (Figure 13).

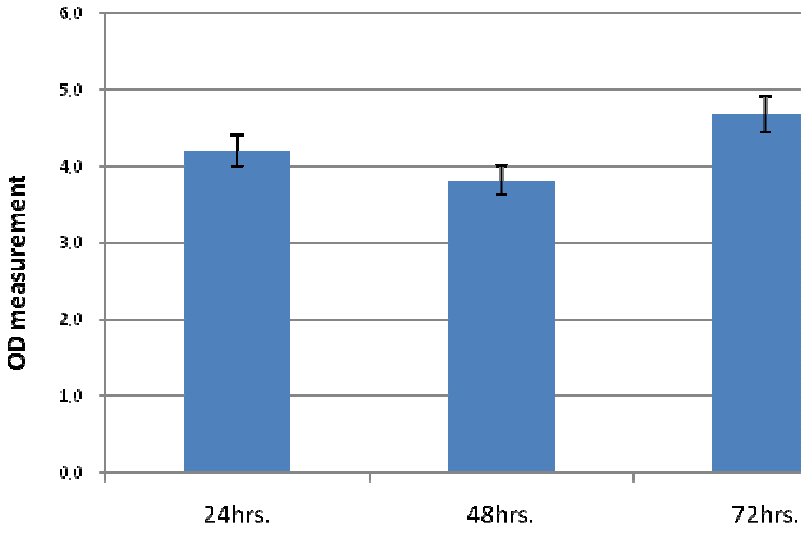

Figure 12. OD variation in three incubation points for adding glycerol. Data shows that 72 hrs of incubation gave a significant difference in growth.

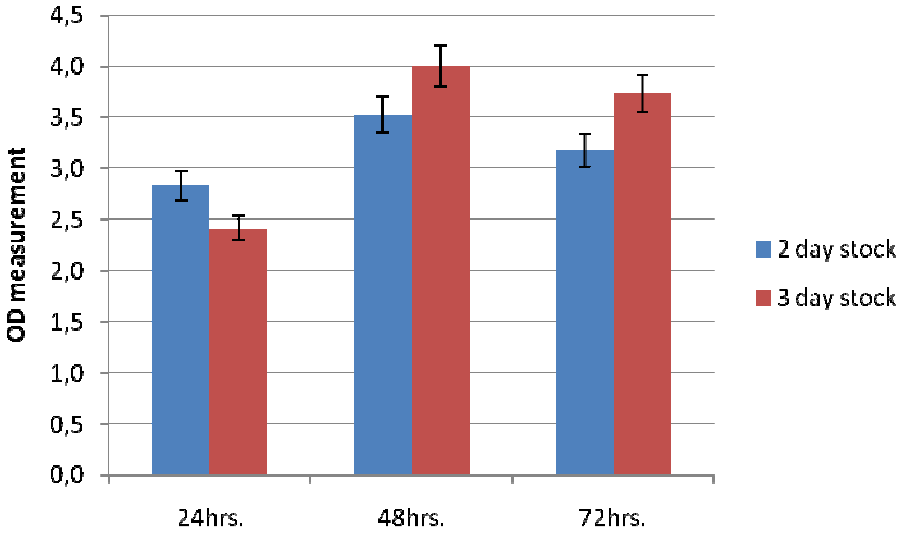

Figure 13. OD comparison between growth from two days and the three days stocks made from figure 12. The incubation time chosen for the working plates was 48hrs base on the data showed.

After 48 hrs of incubation at 28°C, the plates were centrifuged and the pellet resuspended in 200 µl of MMAi. OD was measured after centrifugation to test the potential loss of bacteria during the procedure, it was important to standardize the

concentration of bacteria for infiltration to avoid differences in the HR evaluation during the screening Results showed that in average; the final OD for infiltration is 1.3 in 100ul, with a standard deviation of 0.3.

3.3 Mutant Library screen

3.3.1 Mutants Screen in *planta*

Infiltrations in *N. tabacum* were performed according to methodology. Results given in this document correspond to 1250 mutants screened in two and half batches of 500 mutants per batch. The HR response was measured at 7 dpi. After evaluation of the the HR response at 7 dpi, those mutants that gave an HR response scored between 1 and 2 were considered for retest. The mutants chosen were evaluated to meet the criteria described in figure 14; and the overall performance of the leaf.

A

B

Figure 14. HR parameters followed for evaluating HR or the lack thereof. A) represents the sight from the top ofthe leaf. All frames show the different levels of HR phenotypes shown in the coinfiltration of mutant RanGAP2-ΔC-GFP + Rx-CC-NB-HA. B) represents the sight from the below the leaf. All frames are different phenotypes shown in the coinfiltration of RanGAP2-ΔC-GFP + Rx-CC-NB-HA.

Based on the criteria mentioned above, 51 mutants were found to be phenotypically located in 1 and 2. At the time of writing this report, from the 51 candidates, 27 from the second batch were retested once in *N. tabacum*. The 24 remaining candidates identified in the first batch, were co-infiltrated two more times. Each repetition was done in triplo. The complete list of mutants which have shown a phenotype so far in the study, are listed in Appendix 4.

As result of the two repetitions of the screen, out of the initial 24 mutants identified from the first batch, 13 showed consistently low levels of response (Figure 15). The

same HR parameters of figures 14 were followed in the repetitions. The final 13 mutants form batch 1 are listed in table 5.

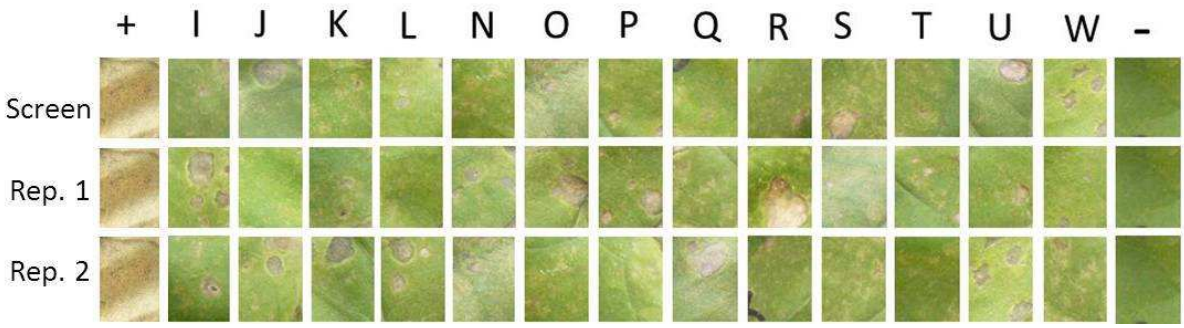

Figure 15. Repetition of agroinfiltrations for mutants that showed low levels of HR. Mutants were identified in the screen and revaluated in triplo in two different infiltrations in *N. tabacum* at OD 0.5. Pictures for repetition 1 and repetition 2 are average response from three samples tested in each repetition. Positive and negative controls are shown on the sides.

Table 5. Mutants from the first batch that have shown consistently a phenotype of loss of function The candidates were identified by letters.

Mutant	Code	Mutant	Code
B-E2	I	D-D3	Q
B-F8	J	D-E12	R
B-F12	K	D-G12	S
C-A5	L	E-A5	T
C-E5	N	E-A11	U
C-F12	O	E-F1	W
D-A3	P		

Mutants are identified by plate Id and position in the microtiter plate

The candidates from table 5 were sequenced, however, due to time limitations, only I, K, N, O, P, R and S were included in further CoIPs and Western blot analysis (see below).

3.3.2 Coimmunoprecipitation (CoIP) and Western blot immunoassays

We performed immunoassays to check whether the RanGAP2-ΔC-GFP protein has indeed lost the binding function to Rx-NB-CC-HA. Leaves tissue from a *N. bethamiana* plants coinfiltrated with Rx-CC-NB-HA plus WT RanGAP2-ΔC-GFP and RanGAP2-ΔC-GFP mutants was harvested at 3 dpi. Protein extractions were tested in a CoIP assay using GFP-trap® beads (Chromotek). Results obtained raised

interesting questions regarding levels of expression, stability of the proteins and the effect of the mutagenesis. All mutants showed a low degree of interaction, with the exception of mutants P and S whose mutations prevented the GFP fusion and therefore, were not pulled down. The lowest degree of interaction was given in mutant P probably related to expression levels and stability of the protein (Figure 17). Strikingly, mutants I, K, O and R showed a range of bands which are not corresponding to the expected size of the WT protein, these mutants were lacking the start codon (See 3.2.3) which suggests that the subproducts or degraded products of the WPP domain can still bind to Rx-CC-NB-HA. Results suggest that the key residues for the interaction with Rx are not located at the N-terminal part of the WPP. Coomasie brilliant blue (CBB) was used as loading control.

A western blot was also performed to check the protein levels *in planta* in absence of the Rx-CC , since the phenotype observed could be explained by a to lack of protein, particularly keeping in mind that Rx and the WPP are intrinsically related in stabilization, among others roles (Tameling et al., 2010). Results showed low levels of protein production in mutants N, P and S (Figure 16). These low levels of protein production are probably related to the lack of GFP in the protein fusion in mutants S and P (same reasoning as mentioned above), the faint bands detected at longer exposure could be due to production of free GFP. On the other hand, the lack of product in mutant N was probably given by sample manipulation.

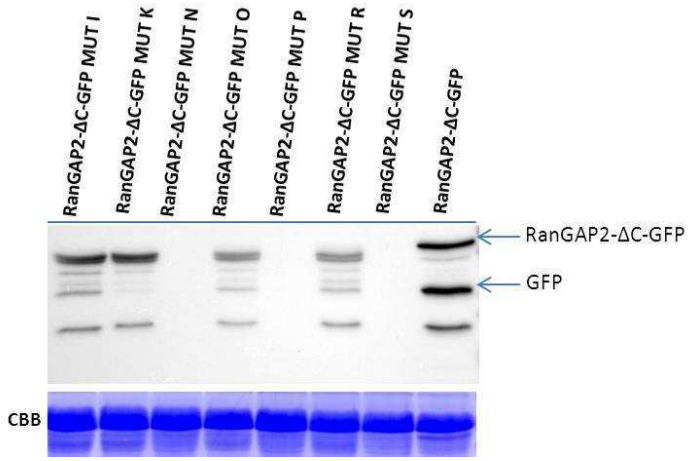

Figure 16. Protein levels of RanGAP2 ΔC-GFP mutants produced in *N. benthamiana*. Proteins were extracted from agroinfiltrated leaves at 3dpi. Proteins were purified on a SDS-PAGE. The immunoblot was analysed using GFP antibodies (α GFP). Faint bands were shown for P and S mutants at longer exposure. No band was shown for N. CBB was used as loading control.

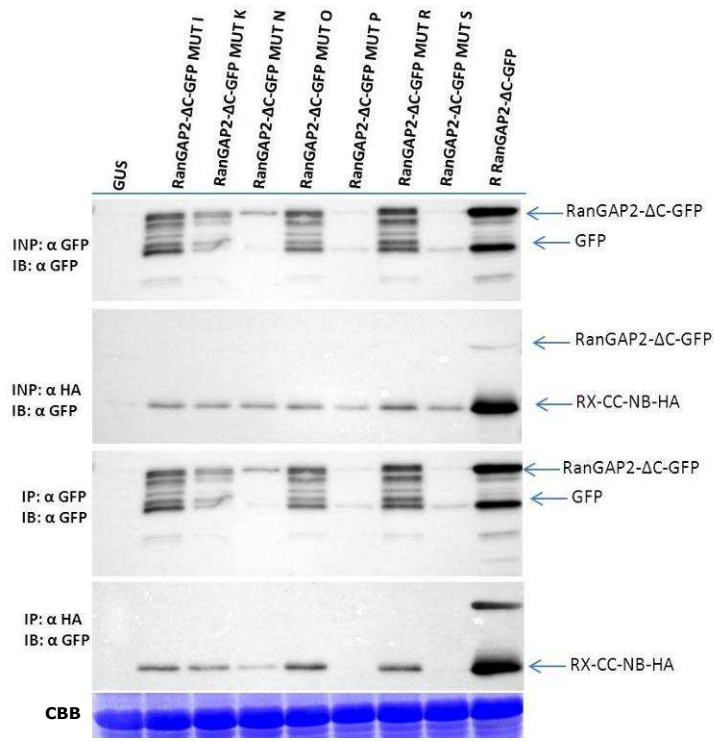

Figure 17. Coimmunoprecipitation assay (CoIP).Mutants proteins were transiently expressed in *N. bethamiana*. Proteins extracted from harvested tissues at 3dpi were purified in a SDS-PAGE and subjected to immunprecipitation with GFP Trap® beads. Immunoblots were analysed with GFP (α GFP) and HA (α HA) antibodies. Mutant proteins P and S could not be pulled down with Rx CC-NB-HA due to conformational difficulties. Mutants I, K, N, O and R were interacting with RX-CC-NB-HA. CBB was used as loading control.

3.3.3 Sequencing results and alignments.

Mutants from table 5 were sequenced and results were analyzed and summarized in table 6. Even though all mutants were sequenced, proteins I, K, N, O, P, R and S acquired a particular relevance due to the data provided in section 3.3.2.

The protein alignment (figure 18) showed that mutants I, K, O and R lost the start codon in their respective proteins. A mayor change was detected in mutant P where a frame shift near the middle of the sequence which affected the C terminal side of the protein. No mutations in the WPP motif were detected in the first group of mutants; however, the typical motif does not seem to play a relevant role in triggering HR upon signaling form Rx. (Peter Moffett, personal communication)

Table 6. Mutations found in the selected mutants from the first batch of candidates.

Mutant	# of Mutations	# Deletions	AA lost	AA gained
I	1		M*	V
J	2		A,D	T,C
K	2		M*,K	V,M
L	Seq. error+			
N	4		A,L,A,A	V,H,V,V
O	4		M*,I,F,L	V,N,Y,L
P	0	1 (A)	K	Frame Shift
Q	2		Q**,S	Q**,F
R	2		M*	V
S	2		V,C	M, TGA Stop
T	2		C,I	F,S
U	2		F,C	S,Y
W	1		N	S

*Methionine lost was the starting codon of the protein.

** Silent mutation

+ Sequence data from Mutant L was not reliable

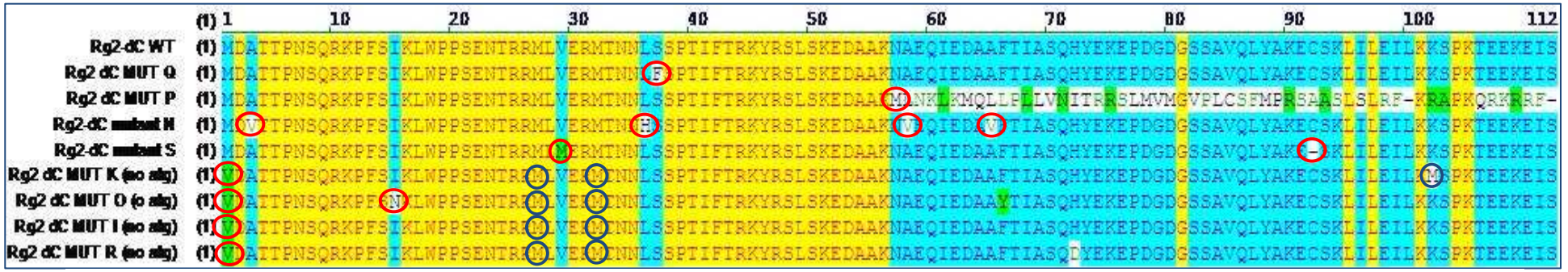

Figure 18 Protein alignment of mutants I, K, N, O, P, Q, R and S, fragments in light blue are the least conserved and in yellow the most conserved. The alignment was done in Vector NTI®. Circled in red are the mutations suffered in each protein. In blue alternative start methionines of mutants K, O, I and R

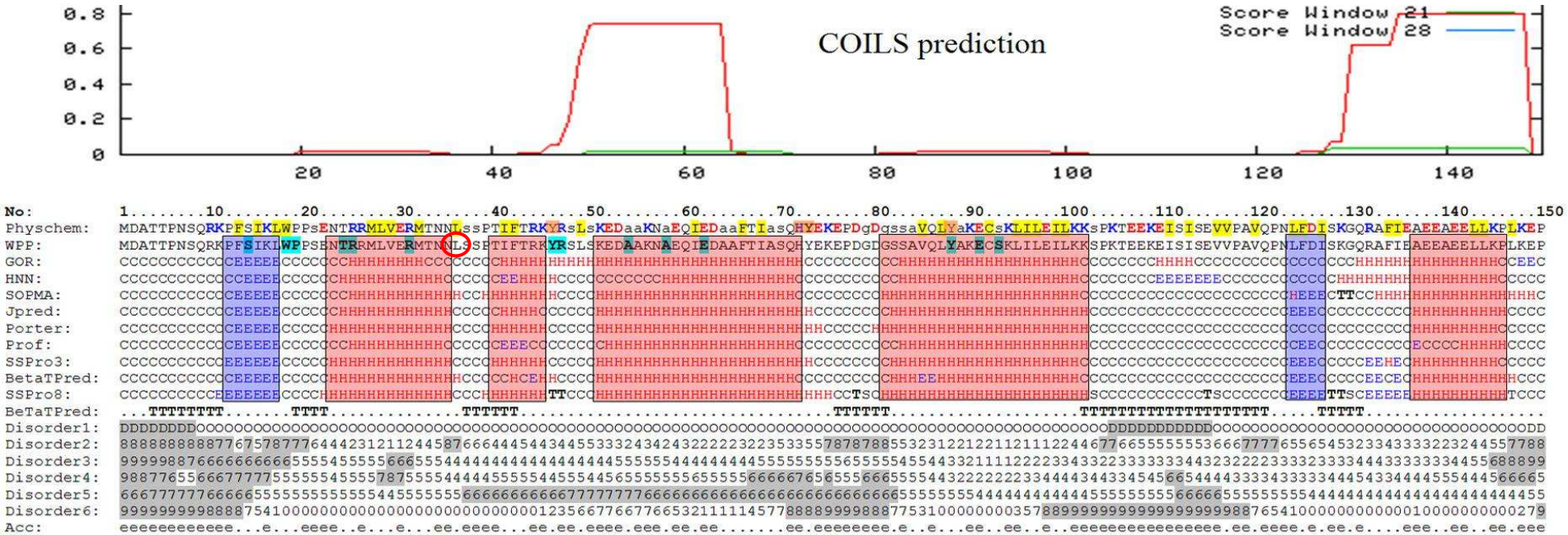

Figure 19 Secondary structure predictions for the WPP domain. Sections in pink are predictions for helixes, fragments in blue are predictions for strands . Alignment taken from Spiridion, 2010. In red the residue mutated in Mutant N localized at the end of a helix.

4. Discussion

4.1 Yeast two hybrid screen

Extensive research has been done to identify proteins involved in the signalling pathway triggered by Rx. Nonetheless, beside the interaction of RanGAP2 (Tameling and Baulcombe, 2007) and the chaperon chaperone complex SGT1-HSP90 (Boter et al., 2007), little is known about what other proteins involved in downstream signalling, binds to Rx. With the objective of searching for potential interactors of Rx, we performed yeast two hybrid screen with poor results.

The system chosen was the Matchmaker™ GAL4 Two-Hybrid System 3. This particular system provides a transcriptional assay for detecting proteins interaction. The main feature of the two hybrid system is to have the GAL4 transcription factor split in two different plasmids, when the GAL4 DNA-BD is in proximity with the AD of GAL4, the transcription is restored and the reporter genes –*ADE2, HIS3* and *LacZ* are expressed (Clontech Yeast Protocols Handbook, Madison, USA). Restoration of the GAL4 occurs when two proteins -that have an interaction- are fused to the bait DNA-BD and the prey AD. The interaction in vivo would put in proximity both GAL4 DNA-BD and AD.

Under this principle, we searched in a library of cDNAs coming from mRNA samples isolated from dying seedlings (Cf4/Avr4 tomato plants) and their parental lines (See 3.1) potential proteins that could have an interaction with baits 1001 and 1005 (Table 1). The objective was to identify proteins involved in downstream signalling of Rx.

4.1.1 Library screen

At the beginning of this thesis, 192 colonies from bait 1001 and 192 colonies from bait 1005 had been chosen from selective media and placed as glycerol stocks in minimum media -WL by Nora Ludwing. This was the result of a successful development of a CDNA library of dying seedlings (Cf4/Avr4 tomato plants) and their parental lines. The cDNA library was developed from mRNA isolated from dying seedlings CF-4/Avr4 using an Oligo(dT) priming method to eliminate poly dT regions and promote the presence of full length clones and 3'ends in the library. Moreover, the oligodT method plus the random priming strategy used aimed at achieving a greater representation of all portions of the different genes and bigger

size range of cDNAs in the library (Clontech Yeast Protocols Handbook, Madison, USA). This library was eventually transformed to yeast strain AH109 and mated with two baits; 1005 and 1001, both transformed to strain Y187. Both yeast strains lacked genes for synthesis of Adenine, Tryptophan, Leucine and Histidine.

The in the mating process, the baits and the prey library were expressed in two haploid yeast strains of opposite mating type. Once the two haploid are mated, they fuse into a diploid strain. Results were grown in –WL media in order to select for presence of both plasmids in the cotransformants. From bait 1001, 240 cotransformants were transferred to a master plate and replicated on selective media -HWL + 2.5 mM 3AT. Selective media –AHWL was not chosen because it showed too many colonies, proving to be not stringent enough. After 3 days of incubation a 30°C, 192 colonies out of 195 were placed in –WL as 25% glycerol stock.

From bait 1005, nearly 1200 colonies were transferred to master plates and replicated on selective media -AHWL. –HWL media was no chosen because it showed too many colonies; just like the previous case, proving to be not stringent enough. After 3 days of incubation at 30°C, 192 colonies out of 919 were placed in –WL as 25% glycerol stock.

At the start of my thesis, 384 colonies coming from glycerol stocks of both baits 1001 and 1005 were grown on –WL plates to select for presence of both plasmids. Since each bait had two microtiter plates, each was identified as B1 and B2, hence 1001/1005 B1 or B2.

The new master plates were transferred to selective media plates -AHWL and –HWL plus three different concentrations of 3-AT: 2,5mM, 10 mM and 15 mM. 3-AT is used to suppress leaky *HIS* reporter gene by competitively inhibiting the HIS3 gene product, although the sensitivity to the compound seem to be strain specific (MacDonald, 2001). The goal of the different concentrations of 3-AT was to increase stringency in the selection, however, in literature such stringency can be increased up to 50 mM of 3-AT (MacDonald, 2001).

The selective media plate –AHWL, selected for activation of the ADE2 gene, which upon activation of the GAL4 transcription factor, would trigger Adenine synthesis, allowing the yeast colony to survive in nutritional selective media lacking adenine.

The selection criterion for the colonies was to choose the biggest, healthiest and fastest growing colonies (Table 1). Evaluation of the colonies was done at 3 and 7 dpi. For the –AHWL culture, selection was supported mostly in data from 3 dpi, since the longest the yeast cells are able to survive, the more chances are that a colony would appear healthier as a collective effect.

Data collected from the selective media cultures was also pondered along with the results of the β galactosidase assay. All colonies were screened for activation of the *LacZ* reporter gene. Overlay assays of low melting point agarose containing 2.5 mg of X-Gal were performed in all colonies from both baits. When GAL4 transcription factor is reassembled by –theoretically- the interaction between bait and prey, the synthesis of β galactosidase is triggered. This assay verifies the sensitivity of the interaction by promoting degradation of X-Gal by the β galactosidase activity. When X-Gal is cleaved by the β galactosidase, a blue compound becomes visible, confirming the enzymatic activity. In the screen, evaluation of the LacZ reporter gene was done at three points in time (30 min, 60 min and 90min). The criteria followed for selecting colonies was choosing the better responsive colonies. According to data, the colonies that turned blue the fastest, and the strongest were selected as potential interactors. A similar criterion was followed for selecting candidates in nutritional selection media. As LacZ is a sensitivity assay, there are different levels of sensitivity for the test, the overlay assay chosen; however, is not the more sensitive. Lifting colonies assays and liquid assays with chemiluminescent substrates are usually the most sensitive procedures (MacDonald, 2001).

50 candidates listed in table 3 were selected for further testing. Data from the selective media cultures and the *LacZ* assays was analysed together with the DNA profiling described in 3.1.3. Clusters of colonies showing similar fragments in the prey vector identified by colony PCRs and enzymatic digestion patterns coming from digesting PCR products with Alu1 were analysed to estimate the possibility of having the same preys. Data of the selective media screen together with the DNA profiling of colonies was used in the selection of the 50 candidates.

In this way, all 50 candidates showed the best performance growing on selective media and each candidate had a different prey, according to the DNA profiling screen.

The final step for proving a positive interaction was to isolate the plasmids from the candidate clones, perform a second retransformation in a parallel set. In one set of cotransformants the isolated prey was co transformed with the original bait to a *PJ694a* yeast strain, repeating the same configuration as the selected colony. Although as a counterpart, the prey plasmid was also cotransformed with empty bait in the same yeast strain. Both cotransformants were tested again on selective media –AHWL; –HWL + 15mm 3AT; –HWL + 10mm and –WL to test for presence of both plasmids.

Retransformations with empty bait confirm the need of the bait for activation of the GAL4 transcription factor. However, as shown in figure 8, all 50 candidates selected during the screen showed no need of the bait for survival or, on the other hand, both transformants died on selective media. This data suggests that all selected colonies were either autoactive or the colony could have had a mutation which restored the adenine and histidine synthesis in the yeast cells.

On average, an estimated 70% of the yeast-two-hybrid procedures are successful for usable baits (MacDonald, 2001), However, the system is also well known for showing a common autoactive behaviour (Legrain et al., 2001).

Many possibilities can fit the phenotype shown in the colonies; however, the possibilities of autoactivity can be regarded from two choices: the bait or the prey are autoactive.

There are different reasons for which the bait was able to initiate transcription of the reporter genes by itself. One of them could be explained by an increased interactivity of the bait given by expression of fragments of a larger protein which is not folded in its natural conformation due to the lack of an intrinsic domain. If the fragments expressed had a cluster of exposed hydrophobic residues, this could the interactivity of the bait and interact with native unspecific yeast proteins gives the autoactivation feature (MacDonald, 2001).

A second possibility is the inclusion of the CC domain the 1001. CC regions are prone to have low affinity interactions with a multiple set of proteins and could have been an important factor in the screen using bait 1001. In general, baits containing fragments or complete sections CC domain have been considered more likely to give false positive results (MacDonald, 2001). This could also explain why bait 1001 showed more colonies identified as potential interactors (table 3).

Nevertheless, bait 1005 does not have a CC domain and still the negative result was the same as in bait 1001.

The prey plasmid can also trigger autoactivity, which would explain why cotransformants with empty bait were able to grow in nutritional selective media. Serebriiskii and Golemis (MacDonald, 2001) described a number of proteins frequently responsible for causing autoactivation. They are described as "sticky" proteins which have been identified to cause false positives in an indiscriminate number of baits. These proteins have been identified as subribosomal units, cytoesckeletal components or proteasome subunits. The main common feature associated to these kind of proteins is their broad interactivity, sticky proteins often have properties like exposed hydrophobic residues, charged patches (ribosomal subunits) or CC regions (cytoskeletal proteins or unspecific binding of heath-shock proteins)(MacDonald, 2001).

There are many open questions regarding the poor result obtained in the yeast-two-hybrid assay. Further attempts can be done taking in consideration some lessons learnt during this experiment. Enhanced *LacZ* activity has been reported in autoactive colonies, as well as indirect effects including altered growth rate, viability and cell permeability (MacDonald, 2001) which suggests that a change of criteria for selecting candidates may yield better results, since selecting constantly for the biggest, the healthiest or the fastest growing colonies may lead to select for autoactivators. However subjective this criteria may be, in this case some potential interactor could have been disregarded overshadowed by the performance of an autoactive colony.

A secondary suggestion could be to change the screening strategy. From this experiment the better performances can be omitted along with the worst performances, all remaining clones could be part of a large matrix approach study using the Protein Interaction Map strategy described in Legrain et al. (2001)

A final recommendation could be to develop a cDNA prey library from transgenic potato dying seedlings Rx/PVX-CP and repeat the experiment. Although Rx is able to trigger downstream signalling in tomato, the natural expression system could have an unexpected difference.

4.2 WPP random mutagenesis screen

The study of Rx as a model for exploring the complex mechanism of CC-NB-LRRs in triggering downstream signalling has had recent breakthroughs with the publication of the role of RanGAP2 as a retention factor, playing a pivotal role in the nucleocytoplasmic distribution of Rx in the cell (see sections 1.2 and 2) and localization of the sub domains of Rx (Tameling et al., 2010, Slootweg et al., 2010).

The subdomains of Rx have also shown to play an important role in regulating the function of the resistance in either the cytoplasm or the nucleus, since for instance, it is known that recognition of PVX occurs in the cytoplasm (Slootweg et al., 2010). By using confocal microscopy and luminescence assays, Slootweg et al. located in both the cytoplasm and the nucleus constructs of Rx-CC-NB-ARC-GFP and Rx-NB-ARC-GFP; whilst Rx-CC-GFP and full length Rx-GFP were localized mostly in the nucleus and the cytoplasm, respectively (Slootweg et al., 2010). This suggested that the CC domain accumulates in the nucleus and is required for nuclear localization. The mutant K176R (Slootweg et al., 2010) with a mutation in the P-loop region (the NB domain) of Rx inactivated the protein and disrupted the interaction between the CC domain and the rest of the protein, but not between the CC-NB domains and the LRR (Moffett et al., 2002, Rairdan et al., 2008). Moreover, expression of K176R localized mostly in the cytoplasm just as WT, however, expression of the mutant in a Rx-CC-NB-ARC-GFP construct (without the LRR domain) was fund in similar ratio in the nucleus and the cytoplasm, suggesting that the nuclear exclusion was determined by the LRR domain, and that the nucleotide bound conformation is needed for the nuclear localization of Rx (Slootweg et al., 2010).

With this information, the answer to elucidate how Rx triggers defence signalling may be behind a complex balance of localization of the Rx proteins in the cytoplasm and the nucleus, perhaps even quantitatively considering previous results with the tobacco N protein (a TIR-NB-LRR protein conferring resistance to Tobacco Mosaic Virus) showing a similar sub-localization as Rx and interacting with plant specific transcription factors, suggesting that the R protein could regulate nuclear events in conformation of the right complexes. In this regard barley MLA R protein has also been reported to function in the nucleus. (Shen et al., 2007, Slootweg et al., 2010, Burch-Smith et al., 2007).

Rx does not contain any known NLS, however, partitioning of the protein seem to work with RanGAP2 preventing Rx to enter the nucleus, and (Tameling et al., 2010), ironically, providing also linkage to the NE via the WPP domain (Tameling and Baulcombe, 2007). In such case, RanGAP2 would play a pivotal role if, considering that one way for Rx to enter the nucleus when activation is triggered could be via a complex cargo including third proteins like STG1 (frequently associated to R proteins and large complexes) which also has to use the GTPase trafficking system. In this line of thinking, a hypothetic conformation of the Rx activation (or transportation) complex could be related to an NLS protein which together with a chaperone complex (and probably more specific proteins), triggers conformational changes in the protein, since the sub-domains of the protein seem to have a role in localization. More specifically the LRR domain may play a role as the nuclear exclusion factor (Slootweg et al., 2010). However, questions remain about an extended role of RanGAP2 in the activation complex or the transportation into the nucleus, excluding the possibility of being an importer carrier, given its role as cytoplasmic retention factor (Tameling et al., 2010).

Based on the idea of unravelling the complexities and questions surrounding the role of RanGAP2, we set off to develop a random mutagenesis screen which would shed some light on finding what residues are essential in the binding surface of the WPP domain of RanGAP2 to interact with Rx-CC. In a secondary intention, the screen would help finding key residues to determine the modelling of the protein, since the WPP domain does not have a crystallized structure yet.

4.2.1 Development of the mutant library and screen *in planta*

Sub-clones of the C terminal WPP domain from both the RanGAP2 and RanGAP1 were fused to GFP in a pBIN-KS plasmid (RanGAP2-ΔC-GFP), and co-infiltrated with Rx-CC-NB to test stability and minimal OD concentrations for triggering HR; establishing a background in *Nicotiana tabacum* cv SR1 for evaluation of the mutants screen.

N. tabacum provided a reliable expression system with proven levels of response to the Rx model (Tameling et al., 2010). However, as shown in figure 10.b, determining gain of function above a surprising variable background noise in RanGAP1 was considered inconvenient, reason for which the gain of function of

this protein was cancelled. On the other hand, RanGAP2-ΔC-GFP proved to be a stable construct, according to the HR response recollected in figures 14 and 15.

With the background information established, we developed the mutant library from a pEntr-RanGAP2-ΔC plasmid. The strategy of the random mutagenesis library was to mutate 1-4 bases pairs in RanGAP2-ΔC to have a reasonable spectrum of mutations to analyze. Preferably close together in the same regions of the protein, in order to have a better understanding of the essential parts of the WPP sequence. As mentioned in results, the final coverage of the mutant library was 7.6x and 1250 mutants of the total library were included in this thesis as part of the final screen for loss of HR *in planta*.

4.2.2 Phenotypical loss of interaction

The RanGAP2-ΔC-GFP mutants which reported phenotype expressing levels of HR ranging between 1 and 2 of Figure 14 were selected to continue in further research (Figure 15). However, despite the robustness of the expression system, evaluation of agroinfiltrations required careful detail in assessing a standardized response, since there are variations in response levels between individuals, and even between two leafs of the same plant. These variations may have been influenced by environmental changes, including but not restricted to light, temperature and humidity.

Some isolated cases of contradictory data were found *in planta,* in which the response given by the controls and mutants alike was inconsistent, i.e. positive control WT RanGAP2-ΔC-GFP coexpressed with Rx-CC-NB-HA gave no HR or on the other hand, WT RanGAP2-ΔC-GFP coexpressed with GUS gave a response. On the contrary, the selected mutant candidates were identified in leaves showing multiple levels of response, from full HR to the negative control (Figure 15), indicating that a loss of response had been given, either by inactivating the protein because of a mutation, or, because low expression levels or low yields of transformation,

Since many factors could affect negatively or positively (ultra-sensitive leaves) a coinfiltration assay, the selected mutants were tested in two different assays and each assay was done in triplo. This step turns out to be crucial for having mutants whose genetic makeup can explain the phenotype shown.

4.2.3 Biological viability of the expressed mutant proteins

As shown in previous publications (Tameling et al., 2006) mutated proteins have to be tested for a compromised structure or destabilization in the cell in order to exclude conformational problems as the cause of the loss of interaction. However, a particular mutation like K176R, can indeed have a negative effect in the biological activity of the protein and in structure. Therefore, it becomes crucial to determine that the levels of expression of the novel protein are not influencing the phenotype shown; likewise the biological viability of the protein becomes important not only for the immediate connection to the HR response, but also in deciphering the structure of the protein.

From the immunoblots performed with RanGAP2-ΔC-GFP mutants I, K O, P, R and S and subsequent coimmunoprecipitations with GFP-Trap® beads, only mutants P and S showed no interaction between the Rx-CC-HA fusion and the RanGAP2-ΔC-GFP. However, it is worth mentioning that mutant P had a frame shift caused by a deletion from residue 54 onwards, meaning that the GFP protein, and therefore its tag, went missing. Explaining why the protein cannot be pulled down in either immunoblots. Moreover a similar situation is given in mutant S, whose mutation in residue 93 introduced a premature stop codon, preventing once again to fuse the GFP protein to the RanGAP2-ΔC protein,

On the other hand, the information given by sequencing shows that mutants K, O, I and R have lost of the initiation codon of the RanGAP2-ΔC-GFP. A mutation in this point would produce a truncated product, since translation will begin in any of the methionines found downstream in the plasmid sequence.

This situation would explain the appearance of multiple bands shown in both the coimmunoprecipitations and in the western blot for these mutants. It is not coincidental that this situation is given only in the four proteins that have lost their first ATG. Although, in principle, the same reasoning could also apply to the WT protein, which according to the alignment of Figure 18, may have two more methionines as initial codons, which, in case of activate translation in the same ORF as GFP, would give three bands of 41.6 KD, 38.5 KD and 37.9 KD corresponding to the wild type protein RanGAP2-ΔC-GFP; a protein translated from residue 27 and the protein translated from residue 33.

The pattern for alternative protein translation shown in the wild type is not the same as in mutants K, O, I, or R, which suggests synthesis of sub-products and

even partial degradation of such sub-products which must still be fused to the robust GFP protein.

Nonetheless, the CoIP data shows that RanGAP2-ΔC-GFP mutants K, O, I and R were successfully pulled down with an α:HA from a GFP blot, confirming interaction. This data gives interesting information, since a recent mutagenesis study in RanGAP proteins for determining residues influencing localization (Meier et al., 2010) showed that proteins with mutations in the WP residues of the WPP domain and the TR residues (corresponding to residues 25-26 of RanGAP2) are essential for targeting the NE (Meier et al., 2010). The WPP domain is located closer to the N terminal of the protein than the TR residues.

Strikingly, RanGAP2-ΔC-GFP mutants K, O, I and R were expressed, they interact with Rx-CC-NB-HA but in all the four cases, the next methionine after the mutated initial codon is located in residue 28 (Figure 18), meaning that the truncated proteins expressed in the cells are not localized to NE, and more importantly that the residues involved in targeting to the NE are not necessary for the Rx interaction.

With this information, the truncated products of K, O, I and R may become of significant importance in determining the regions of the WPP that are needed for interaction. By applying Edman degradation on these products, the residues identified in the process can be informative for further studies, given that the binding region is located within in the fragment. Residues located upstream the truncated peptide can be disregarded, since a downstream fragment of the protein is still binding to Rx-CC-NB-HA.

Interesting information can be provided from Mutant S, since the stop codon is located in the residue 93. However, to probe interaction of this mutant, antibodies against RanGAP2 should be developed. In case of a positive interaction, the binding surface of the WPP domain should be between residues 28 and 93. Although is a hypothesis based on information given by 8 mutants, a broader screen should yield more information.

It is also necessary to determine that the amount of protein produced was not influencing the CoIP results. In the Western blot assay, mutants N, P and S showed very low protein levels, longer exposure was needed to distinguish a faint band. These results suggest that the lack of interaction in the CoIP could have been due to the lack of protein in the immunoblot. Moreover, mutant N was not showing any

response from the GFP tag, suggesting a loss of sample, probably along the washing steps of the Western blot procedure.

Finally mutant N showed 4 mutations one of which can be important in terms of structure; however, a positive interaction in the CoIP shows that there is still binding of RanGAP2-ΔC-GFP with Rx-CC-NB-HA. This result raises questions about the level and stability of the protein, given that western blot has shown practically no protein production for mutant N, however the level of protein on the immunoblot could have been affected by sample manipulation. Notwithstanding, HR was not triggered or at least the biological activity of the mutant was tampered until certain level (Figure 15), whether the mutations played a role in delay or suppressions of response, is still an open question.

4.2.4 Impact of mutations in the protein structure

There is no secondary structure of WPP domain and no crystal structure for homology modelling is known to date. Nonetheless, there are scientists working on modelling RanGAP2 and its sub-domains (Meier et al., 2010) (Spiridion, unpublished data)

From the sequenced candidates, mutant N is the better fitting candidate for exploring the potential regions where mutations in the protein structure may have an important impact in determining the binding surface. Mutant N has 4 mutations one in particular is an H which is located on the border of the first predicted α helix (Figures 18 & 19).

The location of this mutation could potentially become important, since the objective of the experiment was to determine the binding surface of the WPP domain for interaction with Rx-CC, and although there may be exemptions, regions of exposed residues may have bigger chances to create phenotypical mutations. These kinds of exposed mutations in the structure can become essential in locating a cluster of mutations that may work as a constraint region in the ab initio modelling of the WPP domain.

The first residue lost in Mutant N was an Alanine at position 3 in a undetermined region. However, a more interesting candidate is the second residue lost a Leucine

in position 37. Leucine is a hydrophobic large residue which, in this case, was substituted by a Histidine; a large hydrophilic, semi aromatic residue with a particular preference to bind ion metals (Alberts, 2008).

The third and fourth mutations found in mutant N are close together in the predicted second helix; both changes were to a Valine. However, the third mutation, coincided with the mutation M12, described in Meier et al., (Meier et al., 2010). This residue is not essential for localization of RanGAP to the nuclear envelope, although no major characteristics are described for this mutation, except that is 4 residues away from a key residue in targeting to NE.

In an attempt to compare a template model with WT GAP2-ΔC-GFP and the mutant N, both sequences were analyzed using the Phyre server (Kelly, 2009). The template used by the system for both predictions was a human phd2 finger protein C. Albeit the alignment of the WT and the mutant N were approximated in the prediction of helixes and non-specific regions reported by Meier et al., none was accurate enough according to the template. While the WT GAP2-ΔC-GFP had a homology of only 25% with an e Value of 9.5; the mutant N had a homology of only 10% with an e Value of 17. (Kelly, 2009). This result supports the need for developing an accurate template for RanGAP2.

The information gathered in 4.2.3 can be useful in determining constraint regions for supporting an ab initio modelling approach of the RanGAP2 WPP domain by establishing the binding surface of WPP to Rx after residue 28. Nonetheless, further research of interesting mutants may give valuable information in the run for generating a model for RanGAP proteins.

5. Methodology

Table 7. List of primers used during experiments

Primer	Sequence
JV001	5'GGGATGTTTAATACCACTACAATGGATGA3'
J002	5'AAGTGAACTTGCGGGGTTTTTCAGTATCT3'
WO190	5'AGAATTCATGGCTTATGCTGCTGTTAC'
WO191	5'TGGATCCTACATGAGGCGCATGTGATGAG3'
WO198	5'AGAATTCATGGCTGAGAATATAATGGTTGGCCG3'
WO199	5'AGAATTCGAGAATATAATGGTTGGCCG3'
WO231	5'CACCGTCGACATGGATGCCACAAC3'
WO232	5'GGATCCTGAAATCTCCTTTTC3'
WO233	5'CACCGTCGACATGGATTCTGCAG3'
WO234	5'GGATCCTATTACACCTTCTG3'
WO235	5'GCCCCCTTCACCGTCGAC3'
WO236	5'GCGCCCACCCTTGGATCC3'

5.1 Plasmid constructs

The bait plasmid 1001 was created by amplifying by PCR (53°C annealing T., 1 min 72°C extension, 27 cycles) a 881 bp fragment from plasmid SOL19 (Tameling et al., 2010) encoding the CC-NB domains of RX. Products were obtained using domain specific primers wo190 and wo191 (see table 7) introducing EcoRI and BamHI restrictions sites. PCR products were purified with a QIAGEN® PCR Purification column. The EcoRI-BamHI genomic fragment was ligated into a pGEM®-T easy plasmid (Promega, Madison, USA). The fragment was digested from pGEM®-T easy using the corresponding restrictions enzymes, purified in a QIAGEN® PCR

Purification column and ligated into a EcoRiI-BamHI digested pGBKT7 plasmid. (SOL1001)(Nora Ludwing, unpublished results)

The bait plasmid 1005 was created by amplifying by PCR (51°C annealing, 1 min 72°C extension, 29 cycles) a 461 bp fragment from plasmid SOL19 (Tameling et al., 2010) encoding the NB domain of RX. Products were obtained using domain specific primers wo198 and wo199(see table 7) introducing EcoRI and BamHI restrictions sites, respectively. PCR products were purified with a QIAGEN® PCR Purification column (QIAGEN® Valencia, California, USA). The EcoRI-BamHI genomic fragment was ligated into a pGEM®-T easy plasmid (Promega, Madison, USA). The fragment was digested from pGEM®-T easy (Promega, Madison, USA) using the corresponding restrictions enzymes, purified in a QIAGEN® PCR Purification column and ligated into a EcoRiI-BamHI digested pGBKT7 yeast two hybrid plasmid (Clonetech, Mountain View, USA) labelled as SOL1001(Nora Ludwing, unpublished results)

The construct Sol3102 was created by amplifying by a high fidelity Phusion® Hot Start polymerase (Finnzymes, Vantaa, Finland) PCR (53°C annealing , 1 min 72°C extension, 30 cycles) a 336 bp fragment from plasmid SLDB3151 encoding the WPP domain of RanGap2. Products were obtained using domain specific primers wo231 and wo232 (see table 7). PCR products were purified with an Illustra GFX™ PCR DNA and Gel Band Purification Kit (Illustra GFX™, GE Healthcare, Munich, Germany). The genomic fragment was ligated into a pEntr D-Topo Gateway® Cloning vector (Invitrogen, California, USA). The plasmid was digested (EcoRI+BamHI) and sequenced (MWG Operon, Martinsride, Germany) to corroborate presence, orientation and fidelity of the fragment. The fragment was ligated by an LR reaction (Invitrogen, California, USA) into a pBin-KS-GFP destination vector labelled as SOL3102.

The construct Sol3101 was created by amplifying by a high fidelity Phusion® Hot Start polymerase (Finnzymes, Vantaa, Finland) PCR (53°C annealing T., 1 min 72°C extension, 30 cycles) a 321 bp fragment from plasmid SOL162 encoding the WPP domain of RanGap1. Products were obtained using domain specific primers wo233 and wo234 (see table 7). PCR products were purified with an Illustra GFX™ PCR DNA and Gel Band Purification Kit (Illustra GFX™, GE Healthcare, Munich, Germany). The genomic fragment was ligated into a pEntr D-Topo Gateway® Cloning vector (Invitrogen, California, USA). The plasmid was digested

(EcoRI+BamHI) and sequenced (MWG Operon, Martinsride, Germany) to corroborate presence, orientation and fidelity of the fragment. The fragment was ligated by an LR reaction (Invitrogen, California, USA) into a pBin-KS-GFP destination vector labelled as SOL3101.

5.2 Cf4/Avr4 dying seedlings cDNA Library

mRNA from seedlings of a cross between the transgenic Moneymaker (MM) Cf-4 line and the transgenic MM line carrying the cognate elicitor AVR4, along with mRNA of parental lines was extracted using a RNeasy™ Kit from QIAGEN® (QIAGEN® Valencia, California, USA) After being located at 22°C for 0 hrs, 3 hrs and 5 hrs. The first strand of cDNA was made using oligo(dT) primers on a total RNA~ 5.4µg according to the protocol PT3955-1 of the Matchmaker™ Library Construction & Screen kits (Clonetech, Mountain View, USA). A LD-PCR was performed for 4.5 reactions and 26 cycles. The cDNA with size 300-2000 bp was purified using Illustra GFX™ PCR DNA and Gel Band Purification Kit (Illustra GFX™, GE Healthcare, Munich, Germany). cDNA was recombined into a pGADT7-Rec vector following protocol PT3955 of the Matchmaker™ Library Construction & Screen kits (Clontech, Mountain View, USA) and transformed to yeast strain AH109.

5.3 Yeast Two-Hybrid

Two baits gene were expressed as a fusion to the GAL4 DNA-binding domain (DNA-BD), while the Cf4/Avr4 dying seedlings cDNA Library was expressed as a fusion to the GAL4 activation domain for detecting protein interactions in vivo in yeast. The cotransformed colonies were screened for Ade^+ and His^+ response in nutrition selective media SD/-WL, -AHWL and –HWL 3-AT: 2,5mM, 10 mM and 15 mM. Colonies were incubated at 30°C with evaluations at 3 and 7 dpi. β-Galactosidase assays were performed by overly assay and final selection of colonies was done for verification of false positives by performing retransformation with pairs of prey/original bait compared to prey/empty bait.

DNA profiling was performed by amplifying the prey inserts with primers JV001 and J002 by PCR (55°C annealing, 45 sec 72°C extension and 30 cycles) products were ran in a 1.1% agarose gel at 100 V. during 45 minutes. PCR products were

digested with ALUI overnight and ran in a 1.1% agarose gel at 100 V. during 45 minutes.

5.4 Transformation of yeast

Yeast strains AH109 and PJ694a were transformed by a simplified small scale LiAc protocol of the Matchmaker™ GAL4 Two-Hybrid System 3 & libraries User Manual (Clontech, Mountain View, USA). 50 ml of YPDA media was inoculated and incubated overnight at 30°C and 230 rpm until it reached an OD >1. Next day, fresh YPDA media was inoculated and incubated at 30°C and 230 rpm until it reached an OD~0.6. Yeast cells were centrifuged at 10000 rpm 10min, washed with MilliQ water and resuspended in 1.5ml of 1xTE/LiAc solution. Aliquotes of 50µl were taken in 1.5 ml tubes and centrifuged again. Supernatant was discarded and 0.6ml of PEG3400/LiAc solution was added. 0.1 µg of each plasmid was added along with 0.1 mg of salmon carrier DNA. Cells were vortexed and incubated 30 min at 30°C and 230 rpm. 70 µl of DMSO was added and cells were heat shocked for 15 min at 42°C, pelleted and resuspended again in 0.9% NaCl solution.

5.5 Random mutagenesis of the WPP domain

The random mutagenesis of the WPP domain was performed with the Genemorph II EZClone Domain Mutagenesis Kit (Agilent Technologies, La Jolla, California, USA). 175 ng of WPP domain contained in 1.4µg of pEntr D-RanGAP2-ΔC were mutated in a mutagenesis PCR reaction (57°C annealing, 1 min 72°C extension and 30 cycles) using a Mutazyme II® DNA polymerase and plasmids wo235 and wo236. This process was considered as the mutant megaprimer synthesis, resulting in the WPP amplification of mutated WPP domains. This mutants were used as primers (megaprimers) in an EZ clone® PCR reaction (60°C annealing, 6 min 68°C extension and 25 cycles) in which an EZ clone® enzyme mix was added to 50 ng of pEntr D-RanGAP2-ΔC and 250 ng of megaprimers. PCR products were pEntr-D-RanGAP2-ΔC-MUT vectors carrying the mutated WPP fragment.

5.6 Preparation of the Mutant Library

The mutated RanGAP2-ΔCs, carried in the pEntr-D-RanGAP2-ΔC-MUT vectors, were ligated into a pBIN-KS-GFP vector by an LR reaction. The pBIN-KS-RanGAP2-ΔC-GFP mutated were transformed in *A. tumefaciens* strain C58C1 + pCH32. Plated

in L selective media and incubated 48 hrs at 28°C. 2016 colonies were transferred to 21 microtiter plates, incubated in L selective media 72 hrs at 28°C and then added glycerol until 25% to make glycerol stocks stored at -80 °C.

5.7 Agroinfiltrations

Agrobacterium tumefaciens strain C58C1 carrying the helper plasmid pCH32 were transformed with binary vectors of interest (described in results) and transient expression was performed as described by Tameling et al. (2010). Bacteria were resuspended in MMAi containing 200 µM of acetosyringone. The final OD for suspension was 0.5 unless indicated otherwise. The infiltrations were placed under greenhouse conditions of 16 hrs of light at 21°C and 8 hrs of darkness at 19°C in a relative humidity of 75%.

5.8 Mutants preparation for agroinfiltration

Plates were centrifuged at 3600g for 6 minutes. Supernatant was poured off and let to dry. Pellets were resuspended in 100 MMAi containing 200 µM of acetosyringone. 100 µl of an Rx-CC-NB-HA OD 0.6 *A. tumefaciens* solution in MMAi was added to each sample of the microtiter plate.

5.9 Mutant library screen

Preparation of mutants in microtiter plates were agroinfiltrated in *N. tabacum* plants as described by Tameling et al. (2010). Distribution of the samples was as the same across all samples. Negative and positive controls were prepared as in 5.7, although, with a final OD of 0.8.

5.10 Immunoblots

RanGAP2-ΔC-GFP and Rx-CC-NB-HA proteins were transiently expressed in N. benthamiana leafs. Tissue was harvested at 3 dpi. grinded and proteins extracted in GTEN and 5mM DTT Buffer. CoIP samples were further centrifuged in a Sephadex® G-25 column at 2000 rpm per 2min. Proteins were pulled down using GFP Trap® beads and purified by SDS-PAGE. HA and GFP-Tag proteins were identified by immunoblotting (Tameling *et al.*, 2010) using α HA and α GFP antibodies and detected by peroxidase reaction. Membranes were stained with CBB R 250.

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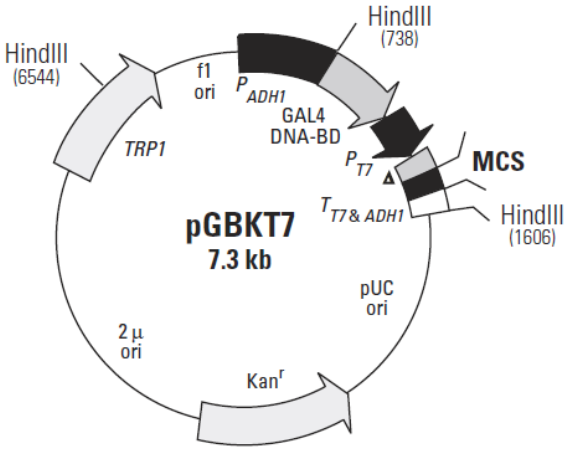
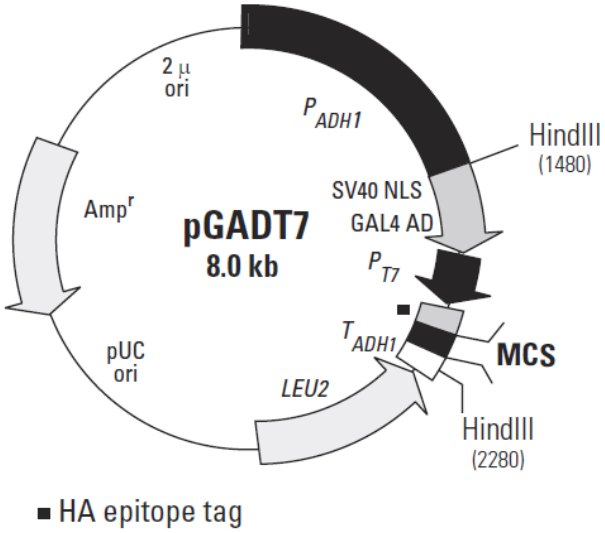
Appendices

1. Appendix 1 Map vectors pGBKT7 used in baits 1001 and 1005



Restriction map and multiple cloning site (MCS) of pGBKT7. Baits 1001 and 1005 were fused to aminoacids 1-147 of the GAL4 binding domain (DNA-BD). Taken from Clontech Yeast Protocols Handbook, Madison, USA.

2. Appendix 2 Map vector pGADT7



Restriction map and multiple cloning site (MCS) of pGADT7. Preys were fused to aminoacids 768-881 of the GAL4 activation domain (AD). Taken from Clontech Yeast Protocols Handbook, Madison, USA.

3. Appendix 3 Evaluation results Y2H

Complete analyses results of the yeast two hybrid library including nutritional selective media and DNA profiling results. Selected clones are highlighted in blue. (+)Positive growth. (/)Compromised performance (-) No growth

Code	Clone	Insert size	Band 1	Band 2	Band 3	# inserts	Autoact.	WL	AHWL	HWL 2.5	HWL 10	HWL 15	GAL Assay	Selected
05B1-D11	**D11**	2100	0	0				+	+	/	-	/	/	NO
05B2-B10	**B10**	2000	0	0				+	-	+	/	/	/	NO
05B1-F11	**F11**	1800	1100	300				+	+	/	- *	- *	/	NO
01B1-F1	**F1**	1800	0	0				+	+	+	+	+	+	YES
05B1-H12	**H12**	1500	500	400				+	+	-	-	-	/	NO
05B1-F12	**F12**	1500	400	200				+	+	+	/	/	-	NO
01B1-C4	**C4**	1500	0	0				+	/	+	+	+	/	NO
01B1-C8	**C8**	1500	0	0				+	/	+	+	+	-	NO
01B1-B9	**B9**	1400	800	600				+	+	+	+	+	-	NO
05B2-D8	**D8**	1400	600	400				+	+	+	+	+	+	YES
01B2-E7	**E7**	1400	400	200				+	+	+	+	+	+	YES
01B2-F2	**F2**	1400	0	0				+	/	/	+	/	/	NO
05B2-B7	**B7**	1300	800	500				+	+	-	/	-	+	NO
05B1-G2	**G2**	1300	600	300				+	+	+	+	+	+	YES
01B1-A12	**A12**	1300	600					+	+	+	+	/	+	NO
05B2-A10	**A10**	1200	900	300				+	+	/	-	/	+	NO
01B1-B4	**B4**	1200	900					+	+	+	+	+	-	NO
01B1-B12	**B12**	1200	900					+	/	+	+	+	-	NO
05B2-B11	**B11**	1200	900					+	+	+	/	-	+	NO
05B2-C8	**C8**	1200	900					+	+	+	+	/	+	NO

01B1-F12	**F12**	1200	800	400		+	+	+	+	+	-	NO
01B2-A10	**A10**	1200	700	500		+	+	+	+	+	/	NO
01B2-G9	**G9**	1200	700	500		+	+	/	+	+	/	NO
01B2-H9	**H9**	1200	700	500		+	+	+	+	+	/	NO
05B2-B12	**B12**	1200	700	500		+	+	+	+	+	/	NO
05B2-A8	**A8**	1200	700	400	100	+	+	+	/	-	/	NO
01B1-B1	**B1**	1200	700	200		+	+	+	+	+	/	NO
01B1-C11	**C11**	1200	600	500		+	+	+	+	+	-	NO
01B2-G5	**G5**	1200	600	400	200	+	+	+	-	/	+	NO
01B1-A9	**A9**	1200	600	400		+	+	+	+	+	-	NO
05B1-A2	**A2**	1200	600	300		+	+	+	+	+	/	NO
05B1-D7	**D7**	1200	600	300		+	+	+	+	+	- *	NO
01B1-A10	**A10**	1200	600			+	+	+	+	+	-	NO
01B2-A12	**A12**	1200	600			+	/	+	+	+	+	NO
01B2-B5	**B5**	1200	600			+	+	+	+	+	+	YES
05B2-F6	**F6**	1200	600			+	+	/	-	-	/	NO
05B1-A11	**A11**	1200	500	300		+	+	/	-	/	+	NO
05B1-B6	**B6**	1200	500	300		+	+	+	+	+	/	NO
01B2-E12	**E12**	1200	500	200		+	+	+	/	/	+	NO
05B2-D5	**D5**	1200	500			+	+	+	+	/	/	NO
01B1-G12	**G12**	1200	400			+	+	+	+	+	/	NO
01B1-E12	**E12**	1200	300			+	+	+	+	/	-	NO
01B2-E8	**E8**	1200	0	0		+	+	+	+	+	+	YES
01B2-F7	**F7**	1200	0	0		+	+	+	+	+	/	NO
01B2-F8	**F8**	1200	0	0		+	+	+	+	+	/	NO
05B2-E6	**E6**	1100	900			+	+	/	/	-	/	NO
05B2-G10	**G10**	1100	900			+	+	+	+	+	-	NO
05B2-A3	**A3**	1100	800	300		+	+	+	+	+	+	YES
05B2-H1	**H1**	1100	800	300		+	/	/	-	-	/	NO
01B1-D12	**D12**	1100	800			+	+	+	+	+	-	NO

05B2-C9	**C9**	1100	800				+	+	+	/	-	-	NO
01B1-D11	**D11**	1100	700	400			+	/	+	+	+	-	NO
01B2-F12	**F12**	1100	700	400			+	+	+	+	+	/	NO
01B2-G1	**G1**	1100	700	400			+	/	+	+	+	/	NO
05B1-D3	**D3**	1100	700	300			+	+	- *	- *	- *	- *	NO
05B1-C4	**C4**	1100	700	300			+	+	+	/	/	/	NO
05B2-C5	**C5**	1100	700				+	+	+	+	-	/	NO
01B2-E11	**E11**	1100	600	500			+	+	+	+	+	+	YES
05B1-A7	**A7**	1100	600	200			+	+	+	-	-	+	NO
01B2-B12	**B12**	1100	600				+	+	/	/	/	/	NO
05B1-E3	**E3**	1100	600				+	+	- *	- *	- *	- *	NO
05B2-H8	**H8**	1100	600			YES	+	/	/	-	-	/	NO
05B2-E3	**E3**	1100	500	300			+	+	/	+	+	+	NO
05B2-E1	**E1**	1100	500	100			+	+	+	+	+	+	YES
05B1-B5	**B5**	1100	500				+	+	+	/	/	+	NO
05B2-H11	**H11**	1100	500				+	+	+	/	/	-	NO
01B1-E5	**E5**	1100	400	300			+	+	+	+	+	/	NO
01B1-H3	**H3**	1100	400	300			+	+	+	/	+	/	NO
05B1-H5	**H5**	1100	400	200			+	+	+	+	+	+	YES
01B1-E11	**E11**	1100	0	0			+	+	+	+	+	-	NO
01B1-H9	**H9**	1100	0	0		YES	+	+	+	/	/	/	NO
01B2-F6	**F6**	1100	0	0			+	+	+	+	+	+	YES
01B2-H12	**H12**	1100	0	0			+	+	+	+	/	/	NO
05B1-G4	**G4**	1000	1000				+	+	+	+	+	+	YES
05B2-D10	**D10**	1000	1000				+	+	/	-	-	/	NO
05B1-B1	**B1**	1000	800	200			+	+	+	/	/	/	NO
05B2-B9	**B9**	1000	800	200			+	+	+	/	/	-	NO
01B1-B8	**B8**	1000	800				+	+	+	+	+	-	NO
01B2-D10	**D10**	1000	800				+	+	+	+	+	+	YES
01B2-C9?	**C9?**	1000	700	400			+	+	+	+	+	+	YES

01B1-E7	**E7**	1000	700	300				+	/	+	+	/	-	NO
01B1-F7	**F7**	1000	700	300				+	+	+	+	+	/	NO
01B2-H11	**H11**	1000	700	300				+	+	+	-	-	/	NO
05B1-D5	**D5**	1000	700	200				+	+	-	/	-	- *	NO
05B2-B6	**B6**	1000	700	200				+	+	+	+	-	-	NO
01B1-B10	**B10**	1000	600	400				+	+	+	+	+	-	NO
01B1-B11	**B11**	1000	600	400				+	/	+	+	+	-	NO
01B1-C6	**C6**	1000	600	400				+	/	+	+	+	/	NO
01B1-C9	**C9**	1000	600	400				+	+	+	+	+	/	NO
01B1-D1	**D1**	1000	600	400				+	+	+	+	+	/	NO
01B1-D3	**D3**	1000	600	400				+	+	+	+	+	/	NO
01B1-E10	**E10**	1000	600	400				+	+	+	/	/	-	NO
01B1-F9	**F9**	1000	600	400				+	-	+	+	+	/	NO
01B2-B3	**B3**	1000	600	400				+	+	+	+	+	-	NO
01B2-B9	**B9**	1000	600	400				+	+	+	+	+	+	NO
01B2-D12	**D12**	1000	600	400				+	+	+	+	+	+	YES
01B2-G10	**G10**	1000	600	400				+	+	+	+	+	+	NO
01B2-H5	**H5**	1000	600	400				+	+	+	+	+	+	NO
05B1-B4	**B4**	1000	600	400				+	+	/	/	-	-	NO
05B2-G5?	**G5?**	1000	600	300	200		YES	+	/	+	+	+	-	NO
01B1-D2	**D2**	1000	600	300				+	+	+	+	+	/	NO
01B2-H7	**H7**	1000	600	300				+	+	+	+	+	+	YES
05B1-D8	**D8**	1000	600	300				+	+	-	/	-	- *	NO
05B2-G9	**G9**	1000	600	200				+	/	+	/	/	/	NO
05B1-E9	**E9**	1000	600					+	+	/	- *	- *	/	NO
05B1-F8	**F8**	1000	600					+	+	+	/	/	/	NO
01B1-A2	**A2**	1000	500	400				+	+	+	+	+	/	NO
01B1-H11	**H11**	1000	500	400				+	+	+	+	+	+	YES
01B1-F3	**F3**	1000	500	300	200			+	+	+	-	-	-	NO
01B1-F6	**F6**	1000	500	300	200			+	+	+	/	/	/	NO

01B1-C10	**C10**	1000	500	300			+	+	+	+	+	+	YES
01B1-G11	**G11**	1000	500	300			+	+	+	+	/	/	NO
01B2-F5	**F5**	1000	500	300			+	+	+	/	-	/	NO
05B1-E8	**E8**	1000	500	300			+	+	/	- *	- *	- *	NO
05B1-H3	**H3**	1000	500	300			+	+	+	/	+	/	NO
05B2-F2	**F2**	1000	500	300			+	/	+	+	+	/	NO
05B2-G1	**G1**	1000	500	300			+	+	+	-	+	/	NO
01B1-D7	**D7**	1000	500	200			+	+	+	+	+	-	NO
05B1-H8	**H8**	1000	500	200			+	+	+	/	/	/	NO
01B1-D10	**D10**	1000	500				+	-	+	+	+	/	NO
01B1-G5	**G5**	1000	500				+	+	+	/	/	/	NO
01B1-G8	**G8**	1000	500				+	+	+	+	+	/	NO
01B1-H8	**H8**	1000	500				+	-	+	+	-	/	NO
01B2-C2	**C2**	1000	500				+	+	+	+	+	/	NO
01B2-E2	**E2**	1000	500				+	/	+	+	+	/	NO
01B2-E5	**E5**	1000	500				+	+	+	+	+	/	NO
05B1-G11	**G11**	1000	500				+	+	/	/	+	-	NO
05B1-H11	**H11**	1000	500				+	+	/	-	-	/	NO
05B2-G6	**G6**	1000	500				+	+	/	/	-	-	NO
05B2-G8	**G8**	1000	500				+	+	+	+	+	+	YES
05B2-H2	**H2**	1000	500				+	+	+	+	/	+	NO
05B2-H6	**H6**	1000	500				+	+	+	/	-	-	NO
05B2-H10	**H10**	1000	500				+	/	+	/	-	-	NO
01B1-B2	**B2**	1000	400	600			+	+	+	+	+	+	YES
05B1-B12	**B12**	1000	400	300		YES	+	+	+	+	+	/	NO
01B1-G3	**G3**	1000	400	200			+	+	+	-	-	-	NO
01B1-H6	**H6**	1000	400	200		**YES**	+	+	+	+	+	+	NO
01B2-B10	**B10**	1000	400	200		YES	+	+	+	+	+	+	NO
05B1-B8	**B8**	1000	400	200		YES	+	+	+	+	+	+	NO
05B2-F8	**F8**	1000	400	200			+	+	+	+	+	/	NO

01B1-E6	**E6**	1000	300			+	+	+	+	+	/	NO
01B1-F11	**F11**	1000	300			+	+	+	+	/	-	NO
01B2-A7	**A7**	1000	300			+	+	+	+	+	+	YES
05B1-G7	**G7**	1000	300			+	+	+	/	/	/	NO
05B2-F10	**F10**	1000	300			+	+	-	-	-	+	NO
01B2-G8	**G8**	1000	0	0		+	+	+	+	+	+	YES
01B2-B7	**B7**	900	900		2	+	+	+	+	+	+	NO
05B1-B9	**B9**	900	900			+	+	+	+	+	/	NO
05B2-A12	**A12**	900	900			+	+	+	+	+	/	NO
05B2-C7	**C7**	900	900			+	+	/	/	-	/	NO
05B2-G11	**G11**	900	900			+	+	+	+	+	-	NO
01B2-G2	**G2**	900	800			+	+	+	+	/	+	NO
01B2-C5?	**C5?**	900	700	500		+	+	+	+	+	/	NO
01B2-D4?	D4?	900	700	500		+	+	+	+	+	/	NO
05B1-D12	**D12**	900	700	200		+	+	+	+	/	+	NO
05B2-C11	**C11**	900	700	200		+	+	+	/	-	-	NO
05B2-A11	**A11**	900	700	200		+	/	+	/	/	-	NO
01B1-A7	**A7**	900	700			+	+	+	+	+	+	YES
05B1-D10	**D10**	900	700			+	+	+	+	+	+	YES
05B2-D7	**D7**	900	700			+	+	+	+	+	-	NO
01B2-G6	**G6**	900	600	400		+	+	+	+	+	/	NO
01B1-B7	**B7**	900	600	300		+	+	+	+	+	/	NO
01B1-C12	**C12**	900	600	300		+	+ w	+	+	+	+	YES
01B1-G4	**G4**	900	600	300		+	+	+	+	+	/	NO
01B2-A11	**A11**	900	600	300		+	+	+	+	+	+	YES
01B2-B11	**B11**	900	600	300		+	+	+	-	/	/	NO
01B2-C6	**C6**	900	600	300		+	+	+	+	+	+	NO
01B2-C7	**C7**	900	600	300		+	+	+	+	+	+	YES
01B2-C10	**C10**	900	600	300		+	+	+	+	+	+	NO
01B2-D2	**D2**	900	600	300		+	+	+	+	+	+	NO

01B2-G4	**G4**	900	600	300		+	+	+	+	+	+	NO
01B2-G11	**G11**	900	600	300		+	+	+	+	+	+	NO
05B1-E10	**E10**	900	600	300		+	+	/	- *	- *	/	NO
05B2-D4	**D4**	900	600	300		+	+	+	+	+	/	NO
05B1-C1	**C1**	900	600	200		+	+	+	/	/	-	NO
05B1-C2	**C2**	900	600	200		+	+	+	+	/	-	NO
05B1-C11	**C11**	900	600			+	+	+	/	+	/	NO
05B1-F4	**F4**	900	600			+	+	+	+	+	+	YES
01B1-A6	**A6**	900	500	400		+	+	+	+	+	+	YES
01B2-C11	**C11**	900	500	400		+	+	+	+	+	+	NO
01B2-D7	**D7**	900	500	400		+	+	+	+	+	+	NO
01B2-D8	**D8**	900	500	400		+	+	+	+	+	+	NO
01B2-D9	**D9**	900	500	400		+	+	+	+	+	+	NO
01B2-E6	**E6**	900	500	400		+	+	+	+	+	/	NO
01B2-E9	**E9**	900	500	400		+	+	+	+	+	+	NO
01B2-E10	**E10**	900	500	400		+	+	+	+	+	+	NO
01B2-F1	**F1**	900	500	400		+	+	+	+	+	+	NO
01B2-F3	**F3**	900	500	400		+	+	+	+	+	+	NO
01B2-F9	**F9**	900	500	400		+	+	+	+	+	/	NO
01B2-F11	**F11**	900	500	400		+	+	+	+	+	/	NO
01B2-H4	**H4**	900	500	400		+	+	+	+	/	/	NO
01B2-D11	**D11**	900	500	300	100	+	+	+	+	+	+	YES
01B1-A1	**A1**	900	500	300		+	+	+	+	+	+	YES
01B1-C3	**C3**	900	500	300		+	+	+	-	-	/	NO
01B1-C5	**C5**	900	500	300		+	/	+	+	+	+	NO
01B1-C7	**C7**	900	500	300		+	+	+	+	+	+	YES
01B1-D6	**D6**	900	500	300		+	/	+	+	+	/	NO
01B1-D8	**D8**	900	500	300		+	/	+	+	+	-	NO
01B1-F8	**F8**	900	500	300		+	+	+	-	-	-	NO
01B2-A9	**A9**	900	500	300		+	+	+	-	/	+	NO

05B1-H4	**H4**	900	500	300			+	+	/	- *	- *	/	NO
05B2-G12	**G12**	900	500	300			+	+	+	+	+	/	NO
05B1-H9	**H9**	900	500	200			+	+	+	- *	- *	/	NO
01B1-B5	**B5**	900	500				+	+	+	+	+	+	YES
01B1-D5	**D5**	900	500				+	+	+	/	/	/	NO
01B1-E1	**E1**	900	500				+	+	+	+	+	/	NO
05B2-H5	**H5**	900	500				+	+	+	/	/	/	NO
01B1-G7	**G7**	900	400	300	200		+	+	+	-	-	-	NO
01B1-H5	**H5**	900	400	300	200		+	+	+	/	/	-	NO
05B1-A4	**A4**	900	400	200			+	+	+	/	-	/	NO
01B1-G9	**G9**	900	400				+	/	+	+	+	/	NO
05B1-A5	**A5**	900	400				+	+	+	/	-	/	NO
05B1-A9	**A9**	900	400				+	+	/	- *	- *	/	NO
05B1-D1	**D1**	900	400				+	+	/	/	/	- *	NO
05B2-C10	**C10**	900	400				+	+	+	-	-	-	NO
05B2-F5	**F5**	900	400				+	+	+	+	+	/	NO
05B2-F9	**F9**	900	400				+	/	+	/	+	-	NO
05B2-G7	**G7**	900	400				+	/	+	+	+	-	NO
05B2-B1	**B1**	900	300	200			+	+	+	+	+	/	NO
05B2-H9	**H9**	900	300	200			+	/	+	+	+	/	NO
01B1-E2	**E2**	900	300				+	+	+	+	+	/	NO
01B1-F10	**F10**	900	300				+	+	+	+	/	+	NO
01B2-F4	**F4**	900	300				+	+	+	+	+	/	NO
05B1-G12	**G12**	900	300				+	+	-	-	-	/	NO
01B2-D3	**D3**	900	0	0			+	+	/	+	+	/	NO
05B1-G9	**G9**	900	0				+	+	/	-	-	+	NO
01B1-D4	**D4**	800	800				+	/	+	+	+	/	NO
05B1-D6	**D6**	800	800				+	+	+	+	+	- *	NO
05B1-D9	**D9**	800	800				+	+	/	/	/	/	NO
05B2-B4	**B4**	800	800				+	+	/	/	-	-	NO

05B2-C6	**C6**	800	800		+	+	+	+	-	/	NO
05B2-D6	**D6**	800	800		+	+	+	/	/	+	NO
05B1-B3	**B3**	800	700		+	+	+	+	+	/	NO
05B1-B7	**B7**	800	700		+	+	+	+	+	+	YES
05B1-G6	**G6**	800	700		+	+	/	/	/	/	NO
05B2-E11	**E11**	800	700		+	+	+	+	+	/	NO
05B2-F1	**F1**	800	700		+	+	+	+	+	-	NO
05B2-H4	**H4**	800	700		+	+	+	+	+	-	NO
01B1-A11	**A11**	800	600	200	+	+	+	+	+	/	NO
01B1-A4	**A4**	800	600		+	+	+	+	+	/	NO
05B1-A6	**A6**	800	600		+	+	+	+	+	/	NO
05B1-E11	**E11**	800	600		+	+	+	+	/	+	NO
05B1-F10	**F10**	800	600		+	+	+	+	+	+	YES
05B2-F11	**F11**	800	600		+	+	+	+	+	/	NO
01B1-E4	**E4**	800	500	300	+	+	+	+	+	/	NO
01B1-G1	**G1**	800	500	300	+	+	/	+	+	-	NO
01B1-G2	**G2**	800	500	300	+	+	+	-	-	/	NO
01B1-H2	**H2**	800	500	300	+	+	+	+	+	/	NO
01B2-C4	**C4**	800	500	300	+	+	+	+	+	+	NO
01B2-A1	**A1**	800	500	300	+	+	+	+	+	+	NO
01B2-C1	**C1**	800	500	300	+	+	+	+	+	/	NO
01B2-D1	**D1**	800	500	300	+	+	+	+	+	+	NO
01B2-E1	**E1**	800	500	300	+	+	+	+	+	+	NO
01B2-E3	**E3**	800	500	300	+	+	+	+	+	/	NO
01B2-E4	**E4**	800	500	300	+	+	+	+	+	+	NO
01B2-G12	**G12**	800	500	300	+	/	/	-	-	+	NO
01B2-H1	**H1**	800	500	300	+	+	+	+	+	+	YES
05B1-E2	**E2**	800	500	300	+	+	/ *	- *	- *	-	NO
05B1-H7	**H7**	800	500	300	+	+	/	- *	- *	/	NO
05B2-D11	**D11**	800	500	300	+	+	+	+	/	/	NO

05B2-F12	**F12**	800	500	300		+	+	+	+	+	/	NO
01B1-H4	**H4**	800	500	200		+	+	+	/	+	-	NO
05B2-G2	**G2**	800	500	100		+	+	+	+	+	/	NO
01B1-A5	**A5**	800	500			+	+	+	+	+	-	NO
01B2-F10	**F10**	800	500			+	+	+	+	+	/	NO
05B1-C3	**C3**	800	500			+	+	+	/	/	/	NO
01B1-C2	**C2**	800	400	300		+	+	+	+	+	+	YES
05B1-E1	**E1**	800	400	300		+	+	+	- *	- *	- *	NO
05B1-F1	**F1**	800	400	300		+	+	/	- *	- *	/	NO
05B2-A7	**A7**	800	400	300		+	+	+	+	+	+	YES
05B2-H12	**H12**	800	400	300		+	+	/	+	+	-	NO
01B1-F2	**F2**	800	400	200		+	+	+	-	-	/	NO
01B1-F4	**F4**	800	400	200		+	+	+	+	+	-	NO
05B1-A10	**A10**	800	400	200		+	+	+	-	-	/	NO
05B2-B2	**B2**	800	400	200		+	+	+	+	/	/	NO
05B2-B3	**B3**	800	400	200		+	+	+	+	/	/	NO
01B1-H10	**H10**	800	400			+	+	+	+	+	/	NO
01B2-H10	**H10**	800	400			+	+	+	/	/	/	NO
05B1-A3	**A3**	800	400			+	+	+	/	-	/	NO
05B1-C12	**C12**	800	400			+	+	+	-	-	/	NO
05B1-D2	**D2**	800	400			+	+	+	+	+	- *	NO
05B1-E7	**E7**	800	400			+	+	+	-	-	- *	NO
05B1-H10	**H10**	800	400			+	+	/	-	/	/	NO
05B2-A6	**A6**	800	400			+	+	+	+	/	-	NO
05B2-E9	**E9**	800	400			+	/	+	/	+	-	NO
05B2-F7	**F7**	800	400			+	+	+	+	+	/	NO
05B1-E6	**E6**	800	300			+	+	+	/	/	- *	NO
05B2-E4	**E4**	800	200			+	+	+	+	+	+	YES
01B1-A8	**A8**	700	700			+	+	+	+	+	-	NO
01B2-C12	**C12**	700	700			+	+	+	+	+	+	YES

05B1-C9	**C9**	700	700			+	+	+	+	/	/	NO
05B1-E12	**E12**	700	700			+	+	+	/	/	- *	NO
05B1-G3	**G3**	700	700			+	+	/ *	/	/	/	NO
05B1-H6	**H6**	700	700			+	+	+	/	/	/	NO
05B2-A2	**A2**	700	700			+	+	+	+	+	/	NO
05B2-A4	**A4**	700	700			+	+	+	+	+	/	NO
05B2-B5	**B5**	700	700			+	+	+	+	+	-	NO
05B2-D9	**D9**	700	700			+	+	+	/	/	/	NO
05B2-E5	**E5**	700	700			+	+	+	+	+	/	NO
05B2-F4	**F4**	700	700			+	+	+	+	+	-	NO
05B1-G10	**G10**	700	600	300		+	+	+	+	/	/	NO
05B1-F2	**F2**	700	600			+	+	-	- *	- *	-	NO
05B1-F7	**F7**	700	600			+	+	+	+	/	+	NO
01B2-A5	**A5**	700	500	200		+	+	+	/	+	/	NO
05B2-H7	**H7**	700	500	200		+	/	+	/	-	-	NO
01B2-B1	**B1**	700	500			+	+	+	+	+	/	NO
01B2-A3	**A3**	700	400	300		+	+	+	+	+	+	YES
05B2-D1	**D1**	700	400	300		+	/	/	+	+	-	NO
01B2-A2	**A2**	700	400	200	100	+	+	+	+	+	+	YES
05B1-D4	**D4**	700	400			+	+	+	- *	- *	- *	NO
05B1-F5	**F5**	700	400			+	+	- *	/	/	-	NO
05B1-H2	**H2**	700	300	200		+	+	+	+	+	+	YES
01B2-H3	**H3**	700	300			+	+	/	+	/	-	NO
05B1-F3	**F3**	700	300			+	+	- *	- *	- *	/	NO
05B1-H1	**H1**	700	300			+	+	+	- *	- *	+	NO
05B2-E8	**E8**	700	300			+	/	/	+	+	+	NO
05B2-F3?	**F3?**	600	600	300		+	/	/	/	/	-	NO
01B1-B6	**B6**	600	600			+	+	+	/	/	+	NO
01B1-C1	**C1**	600	600			+	+	+	+	+	+	YES
01B1-E8	**E8**	600	600			+	/	+	+	+	-	NO

01B1-E9	**E9**	600	600	+	+	+	/	+	-	NO
01B2-B4	**B4**	600	600	+	+	+	+	+	/	NO
01B2-G3	**G3**	600	600	+	+	+	-	-	-	NO
01B2-H2	**H2**	600	600	+	+	+	+	/	/	NO
01B2-H8	**H8**	600	600	+	+	/	/	-	+	NO
05B1-C8	**C8**	600	600	+	+	/	/	/	/	NO
05B1-F6	**F6**	600	600	+	+	/	/	-	-	NO
05B1-F9	**F9**	600	600	+	+	+	- *	- *	+	NO
05B2-D3	**D3**	600	600	+	+	+	+	+	/	NO
05B2-E7	**E7**	600	600	+	+	+	+	+	-	NO
05B1-B10	**B10**	600	500	+	+	+	/	-	/	NO
05B2-C4	**C4**	600	500	+	+	/	-	-	/	NO
05B1-A8	**A8**	600	400	+	+	+	/	/	+	NO
05B1-A12	**A12**	600	400	+	+	+	+	+	+	YES
05B1-E5	**E5**	600	300	+	+	/	- *	- *	- *	NO
05B2-A1	**A1**	600	300	+	+	/	-	-	/	NO
05B2-E10	**E10**	600	300	+	+	+	+	+	-	NO
05B2-C1	**C1**	600	300	+	+	/	-	-	/	NO
01B1-H7	**H7**	500	500	+	/	+	+	+	-	NO
01B2-A6	**A6**	500	500	+	+	+	+	+	+	YES
01B2-C3	**C3**	500	500	+	+	+	+	+	/	NO
05B1-A1	**A1**	500	500	+	+	+	+	+	/	NO
05B1-B2	**B2**	500	500	+	+	+	+	+	/	NO
05B1-C6	**C6**	500	500	+	+	+	+	+	/	NO
05B1-C7	**C7**	500	500	+	+	+	+	+	+	YES
05B1-E4	**E4**	500	500	+	+	-	- *	- *	- *	NO
05B1-G1	**G1**	500	500	+	/	- *	- *	- *	/	NO
05B1-G8	**G8**	500	500	+	+	+	/	/	+	NO
05B2-A9	**A9**	500	500	+	+	+	+	+	-	NO
05B2-B8	**B8**	500	500	+	+	+	+	+	-	NO

05B2-C12	**C12**	500	500				+	+	+	+	/	-	NO
05B2-D2	**D2**	500	500				+	+	+	+	+	-	NO
05B2-G4	**G4**	500	500				+	/	+	+	+	-	NO
05B2-A5	**A5**	500	400				+	/	/	-	-	-	NO
01B1-F5	**F5**	400	400				+	+	+	+	/	/	NO
05B1-C10	**C10**	400	400				+	+	+	+	+	+	YES
01B1-B3	**B3**	0	900	800	300	**2**	+	/	+	+	+	/	NO
01B1-H1	**H1**	0	900	800		**2**	+	+	+	+	+	/	NO
01B2-G7	**G7**	0	800	600		2	+	+	+	+	+	+	NO
05B2-D12	**D12**	0	800	500		2	+	+	+	+	+	+	NO
05B1-B11	**B11**	0	800	400		2	+	+	+	+	+	+	NO
05B1-C5	**C5**	0	800	400		2	+	+	+	+	+	/	NO
01B1-A3	**A3**	0	600	500		**2**	+	+	+	+	+	/	NO
01B2-B8	**B8**	0	600	400		2	+	+	+	+	+	+	NO
01B1-H12	**H12**	0	600			**2**	+	+	+	+	+	-	NO
05B2-C2	**C2**	0	500	300		2	+	+	+	+	+	+	NO
05B1-G5	**G5**	0	500	200		2	+	+	+	+	+	+	NO
05B2-E2	**E2**	0	500			2	+	/	+	-	/	/	NO
05B2-C3	**C3**	0	500			2	+	+	+	+	+	+	NO
05B2-G3	**G3**	0	500			2	+	+	+	+	+	-	NO
05B2-H3	**H3**	0	500			2	+	+	+	+	+	-	NO
01B2-A4	**A4**	0	400	300		2	+	+	+	+	+	/	NO
01B2-B6	**B6**	0	400	300		2	+	+	+	/	/	+	NO
01B2-B2	**B2**	0	300	200		2	+	+	+	+	+	+	NO
01B2-A8	**A8**	0	300			2	+	+	+	/	/	+	NO
01B2-H6	**H6**	0	300				+	+	+	+	+	/	NO
01B1-D9	**D9**	**0**	**0**	**0**			+	/	+	+	+	/	NO
01B1-E3	**E3**	0	0	0			+	+	+	/	+	/	NO
01B1-G6	**G6**	0	0	0			+	-	+	+	+	+	NO
01B1-G10	**G10**	**0**	0	0			+	/	+	+	/	/	NO

01B2-C8	**C8**	0	0	0		+	+	+	+	+	+	NO
01B2-D5	**D5**	0	0	0		+	+	+	+	+	/	NO
01B2-D6	**D6**	0	0	0		+	+	+	+	+	+	NO
05B2-E12	**E12**	0	0	0		+	+	-	-	-	/	NO

4. Appendix 4 Mutants Library screen results for loss of interaction

Complete analyses results of the mutant library screen for loss of HR. Selected clones highlighted in yellow were re tested. Evaluation scale is 0-5, being 5 full HR.

Plate A									Neg Ctrl	Pos Ctrl	Leaf (U/L)	LOW OD
12	3	3	1	1	4	5	5	5	0		L	
11	1	3	3	5	5	2	2	5	0		U	
10	1	1	1	4	1	1	3	3	0		L	
9	2	3	1	3	1	3	3	4	0		U	
8	2	1	5	5	2	3	3	5	1		U	
7	5	5	5	5	5	4	5	4	0		L	
6	2	1	1	2	2	3	2	3	0.5		U	
5	4	3	3	3	3	4	4	3	4		L	
4	5	5	5	5	5	5	4	5	4		L	
3	1	3	4	3	3	4	3	3	0.5		U	
2	2	3	2	3	3	3	3	5	0		L	
1	4	2	3	3	2	4	3	3	1		U	
	A	B	C	D	E	F	G	H				

Plate B									Neg Ctrl	Pos Ctrl	Leaf (U/L)	LOW OD
12	3	3	4	2	4	1	5	4	0		U	
11	1	1	2	2	3	2	1	1	0		L	
10	2	2	4	3	2	4	4	2	0		U	
9	2	2	3	2	1	2	2	2	0		L	

8	**0.5**	**3**	**3**	**3**	**2**	**1**	**3**	**2**	0.5		U	
7	**1**	**1**	**1**	**1**	**3**	**2**	**1**	**4**	0.5		L	
6	**1**	**1**	**2**	**2**	**2**	**1**	**2**	**2**	0		L	
5	**1**	**2**	**4**	**2**	**4**	**3**	**3**	**2**	0.5		U	
4	**1**	**2**	**2**	**4**	**3**	**3**	**3**	**3**	0		U	
3	**2**	**2**	**2**	**2**	**2X**	**2**	**2**	**1**	0		L	1
2	**3**	**3**	**5**	**4**	**0.5**	**4**	**4**	**2**	0		U	
1	**1**	**2**	**2**	**1**	**2**	**3**	**2**	**2**	0		L	
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**				

Plate C									Neg Ctrl	Pos Ctrl	Leaf (U/L)	LOW OD
12	**4**	**5**	**4**	**4**	**3**	**1**	**2**	**3**	1		U	
11	**3**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	5		L	
10	**3**	**2**	**1**	**1**	**1**	**1**	**2**	**2**	0		L	
9	**1**	**5**	**4**	**3**	**4**	**4**	**3**	**3**	0		U	
8	**1**	**1**	**2**	**4**	**2**	**2**	**3**	**2**	5		L	
7	**3**	**3**	**4**	**2**	**4**	**3**	**2**	**2**	1		U	
6	**1**	**1**	**1**	**2**	**2**	**1**	**1**	**2**	0		L	
5	**1**	**4**	**4**	**3**	**1**	**3**	**4**	**3**	0		U	
4	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	0		L	
3	**4**	**3**	**2**	**2**	**3**	**2**	**4**	**3**			U	
2	**2**	**3**	**4**	**4**	**4**	**4**	**4x**	**4**	0		U	1
1	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**	0		L	
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**				

Plate D									Neg Ctrl	Pos Ctrl	Leaf (U/L)	LOW OD
12	**3**	**4**	**5**	**5**	**1**	**4**	**1**	**3**	0	5	U	
11	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**3**	0		L	
10	**1**	**1**	**2**	**3**	**4**	**1**	**3**	**2**	0		L	

9	**3**	**5**	**3**	**5**	**2**	**3**	**4**	**3**	0	3	U	
8	**2**	**3**	**2**	**5**	**3**	**3**	**3**	**5**			L	
7	**1**	**3**	**2**	**4**	**1**	**1**	**3**	**5**		5	U	
6	**1**	**1**	**2**	**5**	**2**	**2**	**2**	**2**	0		U	
5	**2**	**2**	**5**	**2**	**2**	**2**	**2**	**3**	0		L	
4	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**			L	
3	**1**	**4**	**5**	**0.5**	**4**	**4**	**5**	**5**	0		U	
2	**2**	**4**	**4**	**5**	**5**	**5**	**5**	**3**	0		L	
1	**3**	**2**	**4**	**5**	**3**	**1**	**2**	**4**			U	
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**				

Plate E

									Neg Ctrl	Pos Ctrl	Leaf (U/L)	LOW OD
12	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**	0		L	
11	**1**	**2**	**3**	**4**	**5**	**5**	**5**	**2**	0		U	
10	**3**	**3**	**3**	**2**	**2**	**2**	**3**	**3**	3		U	
9	**4**	**3**	**4**	**1**	**2**	**5**	**3**	**2**	0		L	
8	**5**	**5**	**3**	**3**	**4**	**5**	**3**	**1**	0	5	U	
7	**2**	**1**	**1**	**2**	**2**	**1**	**2**	**1**	0		L	
6	**2**	**1**	**2**	**2**	**2**	**2**	**2**	**2**	0		L	
5	**0.5**	**5**	**2**	**2**	**4**	**4**	**4**	**4**	0		U	
4	**5**	**5**	**5**	**5**	**3**	**2**	**5**	**5**	0		U	
3	**2**	**1**	**2**	**2**	**1**	**2**	**2**	**2**	3		L	
2	**2**	**1**	**1**	**1**	**1**	**2X**	**2X**	**2X**			L	3
1	**4**	**4**	**5**	**4**	**3**	**1**	**5**	**3**	0		U	
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**				

Plate F

									Neg Ctrl	Pos Ctrl	Leaf (U/L)	LOW OD
12	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	5	5	L	
11	**3**	**5X**	**3**	**2**	**3**	**2**	**2**	**2**	0		U	1

10	**5**	**3**	**3**	**4**	**4**	**2**	**2**	**1**	0		U	
9	**2**	**1**	**1**	**2**	**2**	**1**	**1**	**3**	0		L	
8	**1**	**1**	**2**	**5**	**1**	**1**	**1**	**1**	1		U	
7	**4**	**4**	**1**	**2**	**3**	**2**	**2**	**1**	4		L	
6	**1**	**1**	**3**	**1**	**2**	**1**	**1**	**2**	2		L	
5	**2**	**3**	**5x**	**5x**	**3**	**2**	**2**	**4**	0		U	2
4	**3**	**2**	**2**	**5x**	**4**	**4**	**4**	**1**	0	5	U	2
3	**4**	**4**	**4**	**5x**	**1**	**4**	**4**	**1**	1		L	1
2	**1**	**2**	**5x**	**5x**	**5x**	**5x**	**5X**	**1**	0		U	5
1	**1**	**3**	**1**	**3**	**1**	**1**	**1X**	**3**	0		L	1
	A	B	C	D	E	F	G	H				

Plate G									Neg Ctrl	Pos Ctrl	Leaf (U/L)	LOW OD
12	**1**	**3**	**4**	**2**	**1**	**4**	**1**	**2**	0.5		U	
11	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	0		L	
10	**1**	**2**	**4**	**4**	**2**	**1**	**4**	**3**	0		U	
9	**5**	**4**	**2**	**2**	**2**	**2**	**2**	**2**	5		L	
8	**4**	**4**	**3**	**2**	**4**	**3**	**4**	**4**	0		U	
7	**2**	**2**	**1**	**3**	**1**	**2**	**2**	**1**	1		L	
6	**1**	**3**	**2**	**2**	**3**	**4**	**3**	**3**	1		U	
5	**3**	**2X**	**1**	**1**	**1**	**1**	**2**	**1**	0		L	1
4	**3**	**2**	**4**	**1**	**1**	**4**	**3**	**3**	0	1	U	
3	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	0		L	
2	**3**	**2**	**2**	**3**	**1**	**1**	**3**	**3**	5		L	
1	**1**	**1**	**2**	**2**	**3**	**1**	**4**	**3**	0		U	
	A	B	C	D	E	F	G	H				

Plate H									Neg Ctrl	Pos Ctrl	Leaf (U/L)	LOW OD
12	**3**	**2**	**1**	**2**	**2**	**2**	**1**	**2**	0		L	

11	**3**	**3**	**2**	**3**	**3**	**3**	**4**	**2**	0		U	
10	**3**	**3**	**5X**	**2**	**2**	**3**	**3**	**1**	0		U	1
9	**2**	**3**	**1**	**1**	**2**	**1**	**2X**	**2**	1		L	1
8	**1**	**2**	**1X**	**1**	**1**	**1**	**1**	**1**	1		L	1
7	**2**	**2**	**1**	**1**	**3**	**3**	**4X**	**2**	0		U	1
6	**3**	**1**	**1X**	**4X**	**2**	**3**	**3**	**1**	0		U	2
5	**1**	**1**	**1X**	**1**	**2X**	**3**	**2X**	**4**	0		L	3
4	**4**	**5**	**1**	**2**	**2**	**3**	**4**	**1**	0		U	
3	**5**	**1**	**1**	**1**	**5**	**3**	**3**	**1**	5		L	
2	**2**	**2**	**2**	**1**	**3**	**3**	**3**	**3**	0		U	
1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	0		L	
	A	B	C	D	E	F	G	H				

Plate I

									Neg Ctrl	Pos Ctrl	Leaf (U/L)	LOW OD
12	**1**	**2**	**2**	**2**	**1**	**4**	**1**	**4**	0		U	
11	**2**	**3**	**3**	**1**	**1**	**2**	**2**	**2**	1		L	
10	**3**	**1**	**2**	**2**	**3**	**4**	**2**	**1**	0		U	
9	**1**	**2**	**1**	**1**	**1**	**1**	**2**	**2**	0		L	
8	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	0		L	
7	**3**	**3**	**3**	**2**	**1**	**3**	**3**	**1**	0		U	
6	**2**	**3**	**1**	**1**	**2**	**2**	**1**	**2**	1		L	
5	**3**	**4**	**1**	**2**	**3**	**4**	**4**	**4**	0		U	
4	**1**	**1**	**1**	**1**	**1X**	**2**	**1**	**3**	0		L	1
3	**1**	**4**	**4**	**3**	**1**	**3**	**2**	**1**	0		U	
2	**2**	**4**	**5**	**1**	**2**	**2X**	**3**	**4**	0.5		U	1
1	**1**	**4X**	**3X**	**1**	**3**	**2**	**2**	**1**	0		L	2
	A	B	C	D	E	F	G	H				

Plate J									Neg Ctrl	Pos Ctrl	Leaf (U/L)	LOW OD
12	**2**	**4**	**5**	**5**	**2**	**1**	**5**	**5**	0		U	
11	**4**	**3X**	**3**	**1**	**1**	**2**	**2**	**2**	0.5		L	1
10	**4**	**2**	**4**	**4**	**2**	**2**	**4**	**2**	0		U	
9	**2**	**1**	**1**	**1**	**2X**	**3**	**1**	**1**	0		L	1
8	**4**	**4**	**4**	**3**	**4**	**3**	**4**	**2**	0		U	
7	**2**	**2**	**1**	**1**	**1**	**2**	**1**	**2**	0		L	
6	**2**	**1**	**1**	**1**	**3X**	**2**	**1**	**1**	0		L	1
5	**3**	**4**	**4**	**3**	**2**	**1**	**4**	**4**	0		U	
4	**4**	**4**	**1**	**4**	**4**	**5**	**5**	**5**	0		U	
3	**1**	**1**	**4X**	**1**	**2**	**1**	**2**	**1**	1		L	1
2	**3**	**1**	**5X**	**5X**	**5X**	**5X**	**1X**	**4**	0		U	5
1	**2**	**1**	**1**	**2**	**2**	**2**	**2**	**1**	1		L	
	A	B	C	D	E	F	G	H				

Plate K									Neg Ctrl	Pos Ctrl	Leaf (U/L)	LOW OD
12	**1**	**1**	**1**	**2**	**1**	**2**	**1**	**2**		5	U	
11	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**		3	U	
10	**3**	**2**	**1**	**2**	**2**	**2**	**2**	**2**		5	U	
9	**3**	**1**	**3**	**4**	**5**	**3**	**1**	**1**		5	U	
8	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	0	5	U	
7	**3**	**5**	**1**	**1**	**3**	**2**	**1**	**2**		2	U	
6	**5**	**3**	**2**	**1**	**5**	**4**	**2**	**2**		5	U	
5	**3**	**4**	**4**	**3**	**4**	**4**	**4**	**3**		5	U	
4	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	0	1	U	
3	**2**	**3**	**1**	**1**	**1**	**1**	**1**	**1**		3	U	
2	**2**	**1**	**1**	**1**	**1**	**2**	**2**	**1**		3	U	

1	**4**	**3**	**3**	**1**	**1**	**1**	**1**	**1**		2	U	
	A	B	C	D	E	F	G	H				

Plate L									Neg Ctrl	Pos Ctrl	Leaf (U/L)	LOW OD
12	**4**	**3**	**5**	**3**	**5**	**2**	**2**	**2**		5	U	
11	**1**	**2**	**3**	**5**	**5**	**2**	**2**	**2**		5	U	
10	**1**	**2**	**1**	**2**	**1**	**1**	**2**	**2**		5	U	
9	**1**	**2**	**2**	**2**	**1**	**2**	**1**	**1**		5	U	
8	**1**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	0	5	U	
7										5	U	
6	**1**	**3**	**3**	**3**	**2**	**3**	**3**	**3**	0	5	U	
5	**1**	**2**	**2**	**2**	**2**	**2**	**2**	**2**		5	U	
4	**3**	**1**	**2**	**4**	**2**	**3**	**3**	**1**		5	U	
3	**3**	**2**	**2**	**2**	**1**	**2**	**2**	**1**		5	U	
2	**1**	**2**	**2**	**2**	**1**	**2**	**2**	**2**		5	U	
1	**3**	**4**	**4**	**4**	**4**	**3**	**3**	**2**		5	U	
	A	B	C	D	E	F	G	H				

Plate M									Neg Ctrl	Pos Ctrl	Leaf (U/L)	LOW OD
12	**2**	**3**	**3**	**2**	**2**	**2**	**3**	**3**		5	U	
11	**3**	**1**	**2**	**2**	**2**	**3**	**2**	**2**		5	U	
10	**2**	**2**	**2**	**2**	**2**	**1**	**2**	**2**		5	U	
9	**2**	**2**	**3**	**1**	**2**	**2**	**3**	**1**	0	5	U	
8	**1**	**1**	**1**	**1**	**2**	**2**	**2**	**2**		5	U	
7	**3**	**2**	**1**	**1**	**2**	**2**	**2**	**1**	0	5	U	
6	**3**	**2**	**2**	**3**	**2**	**2**	**2**	**3**		5	U	
5	**2**	**4**	**2**	**3**	**1**	**2**	**3**	**3**		5	U	
4	**2**	**2**	**1**	**2**	**3**	**2**	**2**	**2**		5	U	
3	**2**	**2**	**2**	**1**	**1**	**2**	**1**	**2**		5	U	

2	**2**	**2**	**2**	**2**	**2**	**1**	**2**	**1**		5	U	
1	**4**	**1**	**1**	**2**	**1**	**2**	**2**	**2**		3	U	
	A	B	C	D	E	F	G	H				