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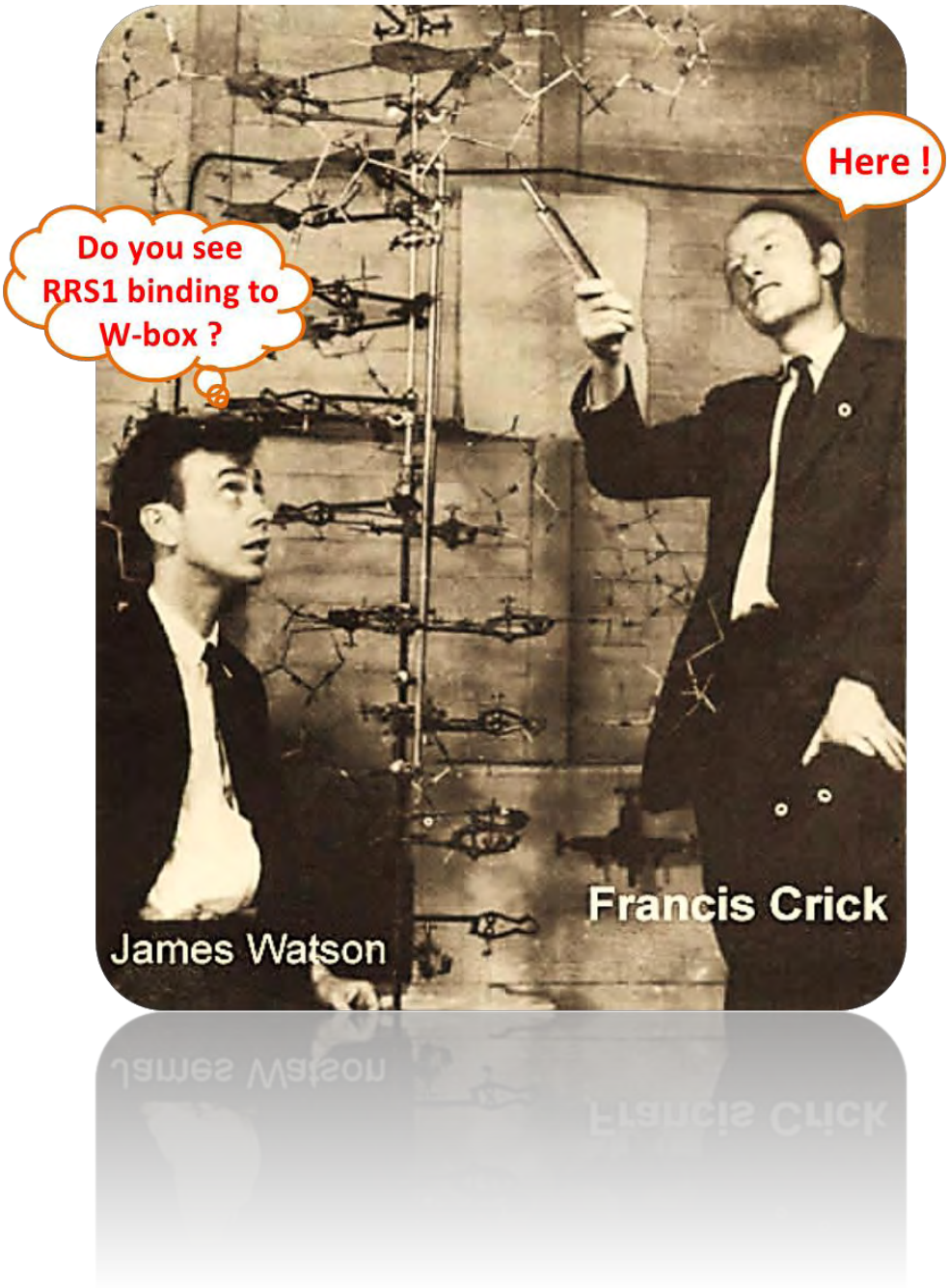
Here !
Do you see RRS1 binding to W-box ?
James Watson
Francis Crick

# Acknowledgements

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To them I dedicate this thesis.

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# ABBREVIATIONS

| | |
|---|---|
| **ABA** | abscisic acid |
| **ADP** | adenosine diphosphate |
| **ARF** | ADP-ribosylation factor |
| **BAK1** | brassinosteroid insensitive 1-ssociated receptor kinase 1 |
| **BCA** | biological control agent |
| **BEAF** | boundary element-associated factor |
| **BIK1** | botrytis-Induced Kinase 1 |
| **bZIP** | basic Leucine Zipper |
| **CaM** | calmodulin |
| **CBP60g** | calmodulin binding protein 60-like.g |
| **CC** | coiled-coil |
| **cDNA** | complementary deoxyribonuleic acid |
| **CERK1** | chitin elicitor receptor kinase1 |
| **cGMP** | cyclic guanosine monophosphate |
| **ChIP** | chromatin immunoprecipitation |
| **CKs** | cytokinins |
| **Col-0** | *Arabidopsis thaliana* ecotype Columbia 0 |
| **CRT1** | compromised for recognition of Turnip Crinkle Virus |
| **CWDEs** | cell wall-degrading enzymes |
| **DamID** | DNA adenine methyltransferase identification |
| **DNA** | deoxyribonuleic acid |
| **DREF** | DNA replication-related element binding factor |
| **EAR** | ERF-associated amphiphilic repression |
| **EDS1** | enhanced disease suceptibility 1 |
| **EF–Tu** | elongation factor Tu |
| **EFR** | EF-Tu receptor |
| **EMSA** | electrophoretic mobility shift assay |
| **EPS** | extracellular polysaccharide |

| | |
|---|---|
| **ERF** | ethylene responsive transcription factor |
| **ET** | ethylene |
| **ETI** | effector friggered immunity |
| **ETS** | effector triggered susceptibility |
| **FARM** | fragments associated to RRS1-R driven Methylation |
| **flg22** | 22-amino acid motif of the bacterial flagellin |
| **FLS2** | flagellin sensing 2 |
| **GAs** | gibberellins |
| **GEF** | guanine nucleotide exchange factor |
| **HR** | hypersensitive response |
| **HLH** | helix-loop-helix domain |
| **hrc** | hrp-conserved |
| **hrp** | hypersensitive response and pathogenicity |
| **ICS1** | isochorismate synthase 1 |
| **IkB** | inhibitor of kB |
| **JA** | jasmonic acid |
| **KO** | knockout |
| **kb** | kilobase |
| **LHP1** | like heterochromatin protein 1 |
| **LPS** | lipopolysaccharides |
| **LRR** | leucine rich repeats |
| **LSD1** | lesion simulating disease resistance 1 |
| **MAMPs** | microbe associated molecular patterns |
| **MAP** | mitogen activated protein |
| **MPK** | mitogen activated protein kinase |
| **NB-ARC** | nucleotide-binding adaptor shared by Apaf1, and CED4 |
| **NB-LRR** | nucleotides binding leucine rich repeats |
| **NBS** | nucleotides binding site |
| **Nd-1** | *Arabidopsis thaliana* ecotype Niederzens 1 |
| **NDR1** | non race-specific disease resistance 1 |
| **NF-kB** | nuclear factor kB |

| | |
|---|---|
| **NLS** | nuclear localization signal |
| **NO** | nitric oxide |
| **NPR1** | nonexpressor of pathogenesis-related genes 1 |
| **PAD4** | phytoalexin deficient 4 |
| **PAL** | phenylalanine-ammonia-lyase |
| **PAMP** | pathogen associated molecular patterns |
| **PBS1** | avrPphB susceptible 1 |
| **PBL** | PBS1-like |
| **PCR** | polymerase chain reaction |
| **PGN** | peptidoglycan |
| **Pop** | *pseudomonas* outer protein |
| **Pop P2** | an *R.solanacearum* type III effector |
| **PR1** | pathogenesis-related gene 1 |
| **PRR** | pattern recognition receptors |
| **PTI** | PAMP triggered immunity |
| **PSII** | photosystem II |
| **pv.** | pathovar |
| **R** | resistance |
| **RIN4** | RPM1-interacting protein 4 |
| **RLK** | receptor-like kinase |
| **RLP** | receptor-like proteins |
| **RNA** | ribonucleic acid |
| **RNAPII** | RNA polymerase II |
| **ROS** | reactive oxygen species |
| **RPM1** | resistance to *Pseudomonas syringae pv maculicola* 1 |
| **RPS** | resistance to *Pseudomonas syringae* |
| **RRS1-R** | Resistance to *Ralstonia solanacearum* |
| **SA** | salicylic acid |
| **SAG101** | senescence associated gene 101 |
| **SAR** | systemic acquired resistance |
| **SARD1** | systemic acquired resistance deficient 1 |

| | |
|---|---|
| **SNC1** | suppressor of npr1-1, constitutive 1 |
| **SPL6** | squamosa promoter binding protein-like 6 |
| **SR1** | signal responsive 1 |
| **STAND** | signal transduction ATPases with numerous domains |
| **SUMO** | small ubiquitin-like modifier |
| **TAL** | transcription activator-like |
| **T3E** | type III effector |
| **T3SS** | type III secretion system |
| **TF** | transcription factor |
| **TGA** | TGACG-sequence-specific DNA-binding protein |
| **TGN/EE** | trans-Golgi network/early endosome |
| **TIR** | Toll/interleukin-1 receptor |
| **TMV** | Tobacco mosaic virus |
| **TPR1** | topless-related 1 |
| **UPR** | Unfolded Protein Response |
| **UV** | ultra-violet |
| **vir** | Virulence |
| **Yop** | *Yersinia* outer protein |
| **WAT1** | walls are thin |

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# PREFACE

## The thesis is presented in 7 sections.

1- Chapter 1: The background and motivation for the project is outlined in the introduction.

2-Chapter 2 presents prerequisite to take on *in vivo* transcription factor binding sites characterization: The aim of this chapter was to determine the expression profile of *RRS1* genes *in planta* and to test the DNA binding properties of RRS1-R and RRS1-S in vitro.

3-Chapter 3 presents the set-up of the DamID approach in order to determine *in vivo* binding sites of RRS1-R and RRS1-S proteins. It focuses on the identification of RRS1-R binding sites.

4-Chapter 4 contains a publication as a first co-author in "Plant Signaling & Behaviour". It is devoted to a side project consisting in reviewing what is known on the molecular mechanisms occurring when bacterial hrp mutants are used as biocontrol agents in protection against bacterial diseases. These mechanisms were particularly well described in the context of the interaction of *Ralstonia solanacearum* (*R. solanacearum*) and *Arabidopsis thaliana* (*A. thaliana*).

5-In chapter 5, the results are discussed in terms of perspectives and opening questions.

6-Material and Methods are described in the chapter 6.

7-References are listed in chapter 7.

# Chapter I

# General Introduction

## 1.1 **Plant immunity: efficiency and limits**

Plants have several basic needs for survival. They require light, water, air, minerals and nutrients. They also need to be able to reproduce in order to ensure survival of species. Climate disorders, unfavorable geology, pathogen attacks are some of the main threats to plants. Adaptations which develop over time and generations as a response to the ever challenging environment allow an organism to reduce competition for space and nutrients, reduce predation and increase resistance to pathogens. Adaptive traits are particularly well developed in plants that are sessile organisms, and this is highlighted by the extraordinary phenotypic plasticity of plants depending of their growing conditions.

Relationships between plants and neighboring microbes (bacteria, fungi, and oomycetes, nematodes) lasts all along plant life, from seeds falling down on the soil to the death of developed plants. Even if some interactions with these microbes benefit plant development, others affect plant fitness. Therefore, plants are engaged in a battle against many surrounding pathogens and have developed numerous and sophisticated strategies to face these aggressions. These defense mechanisms consist of pre-formed structures, toxic compound production and induced immune reactions. The immune system of a plant is indeed very efficient to resist to the vast majority of pathogens; when all genotypes of a plant species are resistant to all strains of a pathogen, the term non-host resistance is used. Reversely, in cases of host interactions, some pathogens are virulent on some plant genotypes whereas some plant cultivars can resist only to certain strains of a pathogen species. In this context, an interaction is called compatible when the plant is susceptible to a pathogen and develops disease, whereas it is incompatible when the pathogen growth is rapidly limited in the plant which is then resistant.

Plants trigger immune responses to pathogens via a two-layer surveillance system. The first layer is composed of extracellular receptors, or Pattern Recognition Receptors (PRR). These receptors detect pathogens outside plant cells and induce a nonspecific resistance called PTI, for PAMP (Pathogen Associated Molecular Patterns) Triggered Immunity. Indeed, to be able to colonize efficiently plants, pathogens have developed several strategies that enable them to escape the host resistance. Among others strategies, they

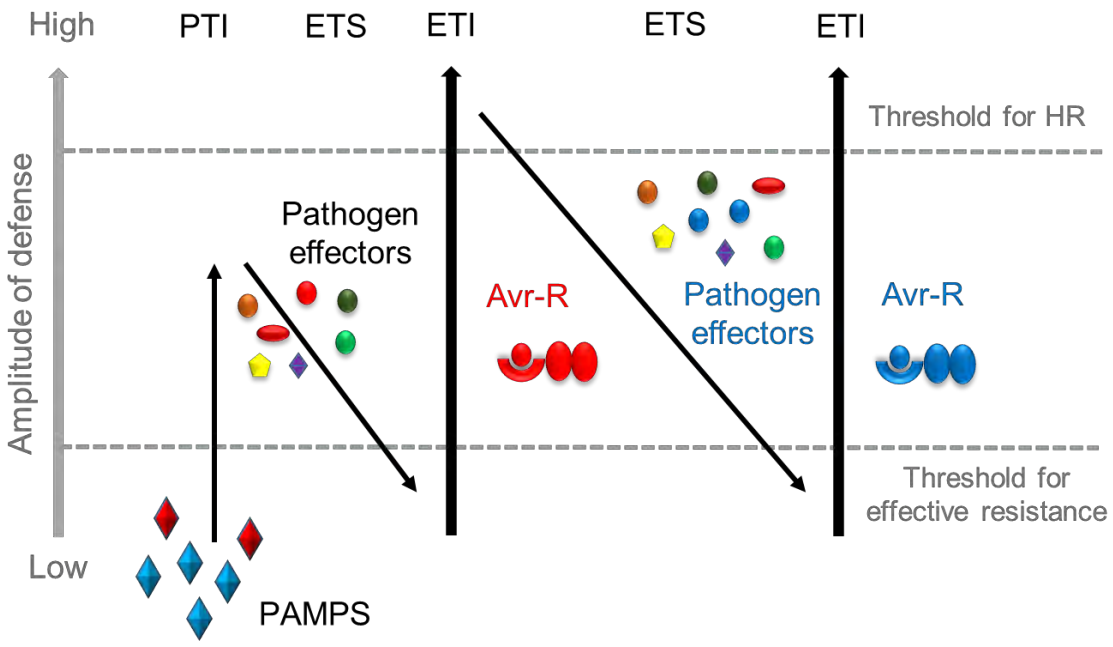



**Figure C1-1.** A zigzag model of the plant immune system.

Pathogen-associated molecular patterns (PAMPs) are detected via plant transmembrane pattern-recognition receptor (PRRs) to trigger basal resistance (PTI). Then, pathogens inject effectors into plant cells. If plants do not recognize effectors, these latter one will interfere with PTI, resulting in effector-triggered susceptibility (ETS). Finally, an effector is specifically recognized by an R protein and the effector triggered immunity is established. PTI is often accompanied with the induction of hypersensitive response leading to a rapid cell death (HR). In last phase, pathogen isolates without a protein effector (red) are selected, and perhaps gained new effectors (in blue), which allows pathogens to suppress ETI. Selection will then again favour the acquisition of a new plant R protein (NB-LRR alleles) that can recognize the newly acquired effectors, resulting again in ETI. (Adapted from Jones and Dangl, 2006)

produce proteins, named effectors, which have the capacity to inactivate plant defense systems and cause disease (ETS for effector triggered susceptibility). A second layer of immunity intervenes then in plant defense, thanks to receptors, or nucleotide binding leucine rich repeats proteins (NB-LRR) that are encoded by Resistance genes (R genes). These receptors recognize specifically some pathogen effectors, named avirulence (Avr) protein, and this recognition triggers an induced and specific resistance, also named ETI (Effector Triggered Immunity). Such a co-evolution of host plants and pathogenic microbes resulting in a highly adaptive and rapidly evolving immune system is illustrated by the zigzag model (Dangl e Jones, 2001). (Figure C1-1) (Tiffin e Moeller, 2006). Indeed, the host range of a pathogen can evolve rapidly due for example, to its capacity to synthesize a host-specific toxin or to generate a "novel" effector by mutation or gene transfer from a related organism (Friedman e Baker, 2007).

### 1.1.1 Basal defense, a first layer of resistance involved in the recognition of conserved microbial patterns

On the cell surface, plant express PRR. These receptors perceive highly conserved molecular signatures of microbes, referred to as Microbe Associated Molecular Patterns (MAMPs) or, when focusing more on pathogenic microbes, PAMPs, for Pathogen Associated Molecular Patterns. So far, most characterized PRRs in plants belong either to the family of receptor-like kinase (RLKs) which have an LRR or LysM extracellular receptor domain and an intracellular kinase domain, or to the family of receptor-like proteins (RLP) which do not possess a kinase domain. Typical examples of PAMPs are bacterial flagellin (flg22), elongation factor Tu (EF–Tu), sulfated peptide Ax21, peptidoglycan (PGN), lipopolysaccharides (LPS), fungal cell wall polysaccharides, chitin, and oomycete glucans (Akerley, Cotter e Miller, 1995; Felix *et al.*, 1999; Dow, Newman e Von Roepenack, 2000; Gust *et al.*, 2007; Erbs *et al.*, 2008). (Figure C1-2) Interaction of PRRs with their corresponding PAMPs initiates a battery of defenses responses, such as the induction of MAP (mitogen-activated protein) kinase (MAPK) signaling, production of reactive oxygen species (ROS), callose deposition at the site of infection and transcriptional activation of defense-related gene.

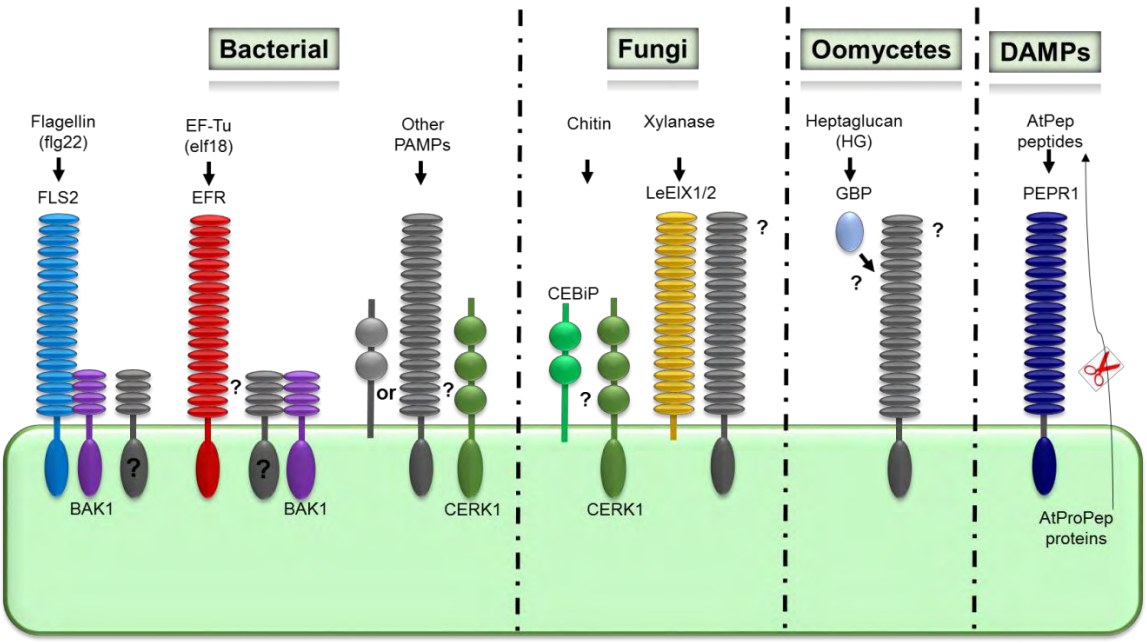



**Figure C1-2.** Pattern recognition receptors (PRRs) and signaling adaptors in plants. Bacterial flagellin (flg22) and elongation factor EF-Tu (elf18) are recognized by the Arabidopsis LRR-RKs FLS2 and EFR receptors, respectively. FLS2, and potentially EFR, form a complex with BAK1 and maybe other SERK proteins. The Arabidopsis LysM-RK CERK1 mediates recognition of an unknown PAMP in plant immunity and is also required for chitin responses. The chitin high-affinity-binding site in rice corresponds to CEBiP. In tomato, the RLPs LeEIX1/2 recognizes xylanase and triggers signaling. In legumes, the soluble glucan-binding protein (GBP) directly binds oomycetal heptaglucan. The Arabidopsis LRR-RK PEPR1 recognizes the endogenous AtPep peptides that act as Damage-associated molecular pattern molecules (DAMPs). (Adapted from Zipfel, 2009)

The best analyzed plant responses to PAMPs are based on the recognition of bacterial flagellin and bacterial elongation factor Tu by the RLKs FLS2 (Flagellin sensing 2) and EFR (EF-Tu receptor) respectively. The 22 amino acid peptide (flg22) corresponding to the highly conserved amino terminus of flagellin is sufficient to trigger immune responses in *Arabidopsis thaliana* (*A. thaliana*), tomato, tobacco and barley (Felix *et al.*, 1999; Peck *et al.*, 2001; Taguchi *et al.*, 2003; Chinchilla *et al.*, 2006; Hann e Rathjen, 2007; Shen *et al.*, 2007). An N-acetylated peptide comprising the first 18 amino acids, of EF-Tu, termed elf18, is fully active to trigger PTI (Kunze *et al.*, 2004). Treatment of *A. thaliana* seedlings with elf18 or flg22 induces a common set of responses including whole genome reprogrammation (Zipfel *et al.*, 2006). Perception of fungal chitin oligosaccharides by the LysM-RLK CERK1 (Chitin Elicitor Receptor Kinase1) is also well documented. N-acetylchitooctaose $(GlcNAc)_8$ induces also PTI in plants as attested by ROS production (Kaku *et al.*, 2006; Miya *et al.*, 2007).

### 1.1.2 A second layer of specific resistance is mediated by resistance proteins

Plants evolved specific R genes sensing pathogen-derived effectors in order to cope with host adapted pathogens, which inject effectors within the plant cell to escape the first extracellular layer of immunity (Dangl e Jones, 2001). These R genes encode NB-LRR proteins structurally related to animal NLR proteins (Nucleotide-binding oligomerization domain-NOD- and LRR containing proteins). The Nucleotide-Binding domain is also known as the NB-ARC (Nucleotide-Binding adaptor shared by Apaf1, and CED4) domain. NB-ARC proteins form a subclass of the STAND super family (signal transduction ATPases with numerous domains), a class of molecular switches that are involved in a variety of processes, including immunity, apoptosis (e.g. Apaf1 and CED4) and transcriptional regulation (Danot *et al.*, 2009). STAND proteins have a modular architecture allowing them to function simultaneously as sensor, switch and response factor. NB-LRR proteins can be further divided into two main subclasses depending on their N-terminal domain (Meyers *et al.*, 2003) (Figure C1-3). One class comprising the TIR-NB-LRR receptors, has homology to the Drosophila Toll and human Interleukin-1 receptor intracellular signaling domains. The second class, CC-NB-LRR receptors, possesses a

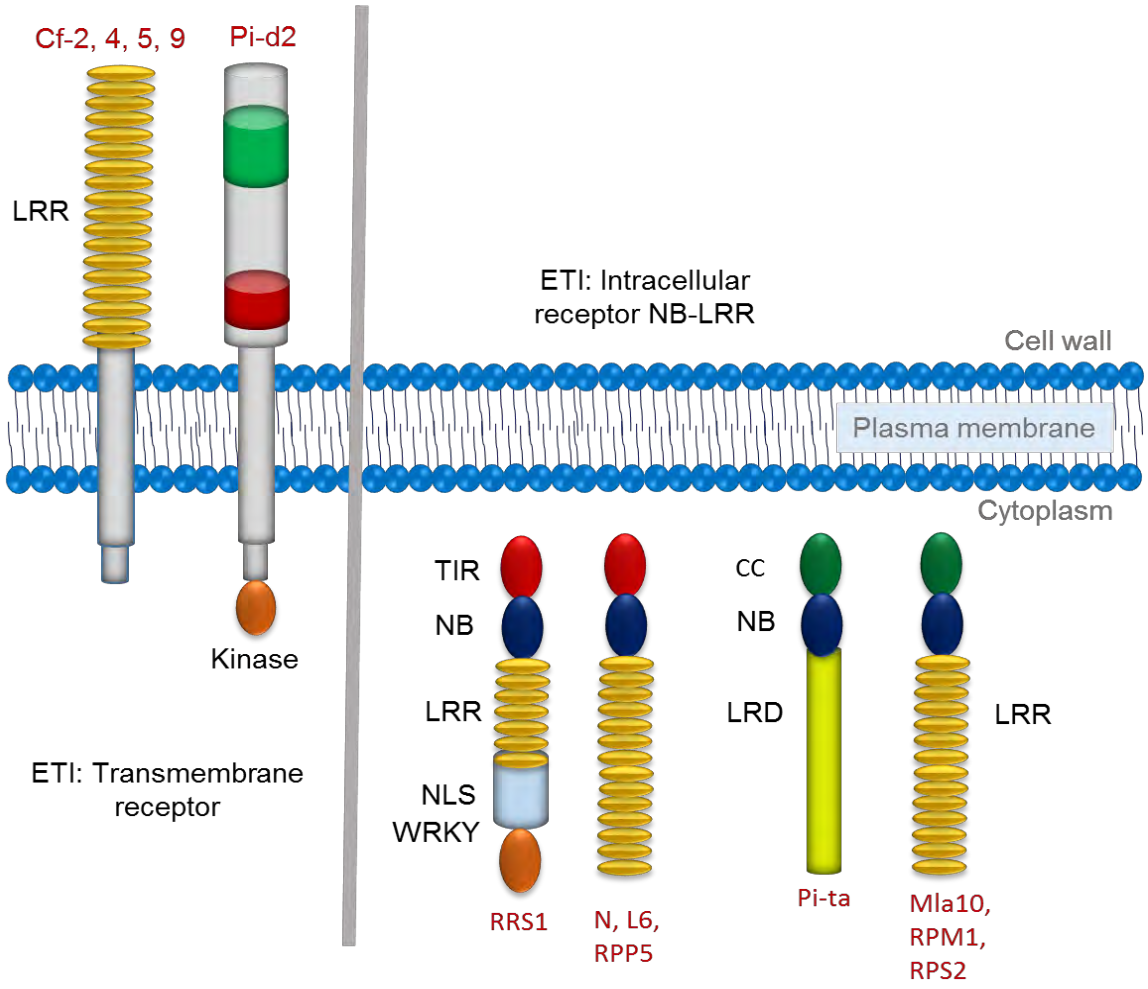



**Figure C1-3**. Different classes of R protein and their cellular location.

Schematic representation of the various domains of R proteins acting either as transmembrane receptors or intracellular NB-LRR receptors. The predicted domains of R proteins are presented as follows: CC (Coiled-Coil); TIR (Toll and Interleukin 1 Receptor-like motif); NB (Nucleotide binding); LRD (Leucine-Rich Domain); LRR (Leucine-Rich Repeat); NLS (Nuclear Localization Signal); WRKY transcription factors. (Adapted from Hammond-Kosack KE and Parker JE, 2003)

predicted coiled-coil (CC). R-genes have been isolated from a variety of plants. A collection of plant R genes is available through the PRG data base which register about 112 manually curated R genes in 29 different species (Sanseverino *et al.*, 2013). Some main plant R genes and Avr associated genes are shown in Table C1-1.

### 1.1.3 Signaling pathways at a glance

MAPK cascades constitute the main signaling pathway from PRRs to downstream components in PTI (Tena, Boudsocq e Sheen, 2011; Hamel *et al.*, 2012). In ETI, signaling pathways from the TIR-NB-LRR and CC-NB-LRR receptors require the CRT1 ATPase (compromised for recognition of Turnip Crinkle Virus) general factor, reported to serve as facilitating the activation of receptors (Kang *et al.*, 2012). Most characterized CC-NB-LRRs then recruit the plasma membrane-associated protein NDR1 (NON-RACE SPECIFIC DISEASE RESISTANCE-1 (Century, Holub e Staskawicz, 1995; Knepper, Savory e Day, 2011) whereas all TIR-NB-LRRs, and one CC-type NB-LRR receptor (HRT), require nucleocytoplasmic EDS1 (Enhanced Disease Suceptibility1) and its partners PAD4 (Phytoalexin Deficient 4) and SAG101 (Senescence associated gene 101) for signal transduction (Venugopal *et al.*, 2009).

Downstream of both PTI or ETI activation, diverse plant hormones act as central players in the triggering of the plant immune signaling network (Howe e Jander, 2008; Bari e Jones, 2009; Pieterse *et al.*, 2009; Katagiri e Tsuda, 2010). Salicylic acid (SA) and jasmonic acid (JA) and their derivatives are recognized as major defense hormones (Browse, 2009; Vlot, Dempsey e Klessig, 2009). Accumulation of SA is important for resistance to biotrophic pathogens. By contrast, JA cooperates with ethylene (ET) to regulate resistance to necrotrophic pathogens. However, the hormones (ET) (Vlot, Dempsey e Klessig, 2009), abscisic acid (ABA) (Ton, J., Flors, V. e Mauch-Mani, B., 2009), gibberellins (GAs) (Navarro *et al.*, 2008), auxins (Kazan e Manners, 2009), cytokinins (CKs) (Walters e Mcroberts, 2006), brassinosteroids (Nakashita *et al.*, 2003), and nitric oxide (NO) (Moreau *et al.*, 2010) function as well as modulators of the plant immune signaling network and add another layer of regulation. Changes in hormone concentration or sensitivity triggered during pathogenic interactions mediate a whole range of adaptive plant responses, often at

**Table C1-1.** AVR/ R couples identified in plant/pathogen interaction studies

| Avr gene | NB-LRR | Host/gene name |
|---|---|---|
| *ATR13* | *RPP13* | *A. thaliana* RPP13 (Recognition of *peronospora parasitica* 13); ATP binding |
| *AvrB, AvrRPP1A* | *RPP1* | *A. thaliana* RPP1 (Recognition of *peronospora parasitica* 1) |
| *AvrPphB* | *RPS5* | *A. thaliana* RPS5 (Resistant to *P.syringae* 5) (RPS5) |
| *AvrRpm1* | *RPM1* | *A. thaliana* RPM1 (Resistant to *P.syringae* pv maculicola 1); protein binding |
| *AvrRPP4* | *RPP4* | *A. thaliana* RPP4 (Recognition of *peronospora parasitica* 4) |
| *AvrRPP5* | *RPP5* | *A. thaliana* RPP5 (Recognition of *peronospora parasitica* 5) |
| *AvrRPP8* | *RPP8* | *A. thaliana* RPP8 (Recognition of *peronospora parasitica* 8) |
| *AvrRps4* | *Rps4* | *A. thaliana* RPS4 (Resistant to *P.syringae* 4) |
| *AvrRpt2* | *Rps2* | *A. thaliana* RPS2 (Resistant to *P.syringae* 2) |
| *Coat protein* | *HRT* | *A. thaliana* viral resistance protein (HRT) gene |
| *Coat protein* | *RCY1* | *A. thaliana* RCY1 gene for R-protein |
| *Pop P2* | *RRS1* | *A. thaliana* RRS1 (Resistant to *R.solanacearum* 1) |
| *AvreBs3* | *Bs3* | *Capsicum annuum* cultivar ECW-30R Bs3 (Bs3) gene |
| *AvreBs2* | *Bs2* | *Capsicum chacoense* disease resistance protein BS2 |
| *AVRA* | *MLA10* | *Hordeum vulgare* MLA10 (Mla10) |
| *AvreRpg1* | *RPG1* | *Hordeum vulgare subsp. vulgare* RPG1 gene, Rpg1-Swiss Hv 489 allele |
| *Avr3* | *Dm3 (RGC2B)* | *Lactuca sativa* resistance protein RGC2B (RGC2B) gene |
| *AvrM* | *M* | *Linum usitatissimum* rust resistance protein M gene |
| *AyrL* | *L6* | *Linum usitatissimum* alternatively spliced rust resistance (L6) gene |
| *AvrN AvrL567* | *N* | *Nicotiana glutinosa* virus resistance (N) gene |
| *AvreXa1* | *XA1* | *Oryza* sativa mRNA for XA1 |
| *AvreXa21* | *xa21* | *Oryza sativa Indica* Group Xa21 gene for receptor kinase-like protein, cultivar:II you 8220 |
| *Avr-Pita* | *Pi-ta* | *Oryza sativa* (japonica cultivar-group) pi-ta protein (Pi-ta) gene |
| *Coat protein* | *Rx2* | *Solanum acaule* Rx2.ac15 gene for NBS-LRR protein, exons 1-3 |
| *Avrblb1* | *Rpi-blb1* | *Solanum bulbocastanum* putative disease resistant protein RGA2 gene |
| *Avr1* | *R1* | *Solanum demissum* late blight resistance protein (R1) gene |
| *Avr4* | *Cf-4* | *Lycopersicon hirsutum* Cf-4 resistance gene cluster |
| *30 kD movement protein* | *Tm-2a* | *Lycopersicon esculentum* ToMV-resistance locus, Tm-2^2 resistance allele |
| *Avr1* | *I-2* | *Lycopersicon esculentum* disease resistance protein I2 (I2) gene |
| *Replicase* | *Tm-2* | *Lycopersicon esculentum* ToMV-resistance locus, Tm-2 resistance allele |
| *Avr5* | *Cf-5* | *Lycopersicon esculentum* disease resistance protein (Cf-5) gene |
| *Avr2* | *Cf-2* | *Lycopersicon pimpinellifolium* leucine rich repeat protein Cf-2.1 gene |
| *Avr9* | *Cf-9* | *Lycopersicon pimpinellifolium* Cf-9 resistance gene cluster |
| *AvrPto* | *Pto* | *Lycopersicon pimpinellifolium Rio* Grande-PtoR protein kinase |
| *Avr3a* | *R3a* | *Solanum tuberosum* potato late blight resistance protein R3a gene |
| *Coat protein* | *Rx* | *Solanum tuberosum* rx gene |
| *Nla proteae* | *RY-1* | *Solanum tuberosum subsp. andigena* ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19 |
| *AvrRP1-D* | *Rp1-D* | Zea mays rust resistance protein (Rp1-D) gene |

the cost of growth and development (Walters e Heil, 2007). Antagonistic and synergistic interactions between diverse hormone signal transduction pathways provides the plant with a powerful capacity to finely tune its immune response according to the invader encountered and to utilize its resources in a cost-efficient manner (Mundy, Nielsen e Brodersen, 2006; Jaillais e Chory, 2010). Transcriptome analyses are powerful tools to decipher the changes accompanying plant defense and transcription dynamics is emerging as an important theme in plant resistance or susceptibility establishment.

This introduction aims at the description of pathogens attack strategies and plant mechanisms known to play a role in induced immunity, highlighting more specifically the steps of pathogen perception and transcriptional changes associated to pathogen attacks.

## 1.2 Pathogen effectors: two sided coins.

Based on their lifestyles, phytopathogenic microbes can be divided into biotrophs, hemibiotrophs, and necrotrophs. While biotrophs feed on living cells and actively maintain host cell viability, necrotrophs kill host cells before feeding on dead tissues. Hemibiotrophs adopt an early biotrophic phase followed by a necrotrophic phase. In this chapter, we will focus on pathogens with a biotrophic step in their infectious cycle.

Pathogens can deliver effectors into the apoplast or directly inside the host cell. Apoplastic effectors include cell wall-degrading enzymes (CWDEs), toxins, and various cysteine-rich proteins. CWDEs and toxins are important virulence factors for necrotrophs but are thought to play a less important role for biotrophs and hemibiotrophs (Barras, Vangijsegem e Chatterjee, 1994; Cantu *et al.*, 2008). Intracellular effectors are secreted mainly by biotrophic and hemibiotrophic pathogens. These intracellular effectors will retain our attention in this introduction.

Bacterial pathogens can deliver these effectors into the host cell through different secretion systems (type III, type IV, and type VI secretion systems). Among these, the type III secretion system (T3SS) plays a crucial role for several plant bacterial pathogens such as *Pseudomonas syringae* (*P. syringae*), *Xanthomonas spp*., and *Ralstonia solanacearum* (*R. solanacearum*). The N terminus of type III effectors (T3Es) displays biased amino acid

composition, which serves as a signal recognized by the type III secretion machinery (Collmer *et al.*, 2002; Vinatzer, Jelenska e Greenberg, 2005).

Some biotrophs (e.g., *Blumeria graminis*) or hemibiotrophs (e.g., *Phytophthora infestans*) fungal and oomycete pathogens develop into the host cell a specialized structure called haustorium (Kamoun, 2006; Horbach *et al.*, 2011). Haustoria are not only responsible for nutrient uptake but also represent the main site of effector secretion (Mendgen and Hahn, 2002). Intracellular effectors of fungal and oomycete pathogens are presumably targeted to extracellular spaces by their N-terminal signal peptide before being translocated into the plant cell. How these effectors are translocated into the plant cell is an active area of investigation. (Dou e Zhou, 2012).

Extensive genome sequencing programs coupled with robust computational predictions of sequence motifs characteristic of effector proteins, allowed the description of complete sets of putative T3Es for a significant number of bacterial pathogens and the identification of their host targets is therefore well documented. We are going to concentrate on bacterial T3Es from different phytopathogenic bacteria in order to present the various cellular and molecular processes which they target.

### 1.2.1 Effectors and their mode of action

Plant components targeted by effectors are located in several plant cell compartments including, plasma membrane, nucleus, chloroplast or vesicle compartments in the cytoplasm. Even if host components are manipulated by effectors to favor disease, as previously mentioned, this manipulation in ETI can also provide an efficient alarm mechanism that can turn on plant immunity. Molecular mechanisms are tangled in different ways for ETS and ETI. Intricate molecular interactions need then to be deciphered in order to pinpoint important clues of the interaction outcome. Furthermore, some plant resistance-signaling components appear to be targeted by multiple bacterial effectors and a growing body of evidence supports the idea that plants have evolved a highly sophisticated surveillance system, involving molecular sensors that are able to perceive multiple effector activities. These observations may reflect the molecular co-evolution between host plants and invading bacteria. Some illustrative examples have been chosen to present our current

knowledge about the molecular dialogue resulting from the co-evolution of pathogens and host plants (Deslandes e Rivas, 2012).

## 1.2.2 Examples of effectors targeting plasma membrane components

### 1.2.2.1 AvrPto and AvrPtoB target multiple plant kinases

AvrPto and AvrPtoB, two effectors from *Pseudomonas syingae* (*P. syringae*) have been shown to suppress ETI or PTI by targeting multiple plant kinases. AvrPto was indeed reported to interact with the kinase domain of FLS2 and EFR and appeared to block PAMP signaling by inhibiting their kinase activity. AvrPtoB may also interact with the kinase domain of FLS2 and thereby facilitate its degradation by the proteasome. In addition, both AvrPto and AvrPtoB induce ETI in tomato by interacting with the intracellular serine/threonine kinase Pto in concert with the NB-LRR protein PRF. Furthermore, AvrPtoB is recognized by Fen, another kinase from the Pto family. This leads to effector triggered susceptibility (ETS) through the degradation of Fen and the suppression of ETI.

### 1.2.2.2 AvrB, AvrRpm1, AvrRpt2, and HopF2 target RIN4

Multiples effectors of *P. syringae* were shown to suppress PTI in *A. thaliana* through the targeting of RIN4 (RPM1-interacting protein 4), a key regulator of plant immunity that provides a link between PTI, ETI and ETS responses. Phosphorylation of RIN4 by AvrB results in the blocking of PTI when the resistance protein RPM1 is not present or in ETI in the presence of RPM1 that will sense the RIN4 phosphorylation. This is also the case for another effector of *P. syringae*, AvrRpm1. AvrRpt2 is a third effector from *P. syringae* and is a cysteine protease that will also target RIN4 and disrupt both ETI by abrogating detection by RPM1 of RIN 4 modifications induced by other effectors and PTI. Moreover RIN4 has been identified as a virulence target of another *P. syringae* effector (Figure C1-4).

### 1.2.2.3 AvrPphB targets PBS1 and related kinases

In *A. thaliana*, the *P. syringae* T3E AvrPphB targets and cleaves the RLCK (receptor-like cytoplasmic kinase) PBS1, which triggers ETI responses when the NB-LRR R protein

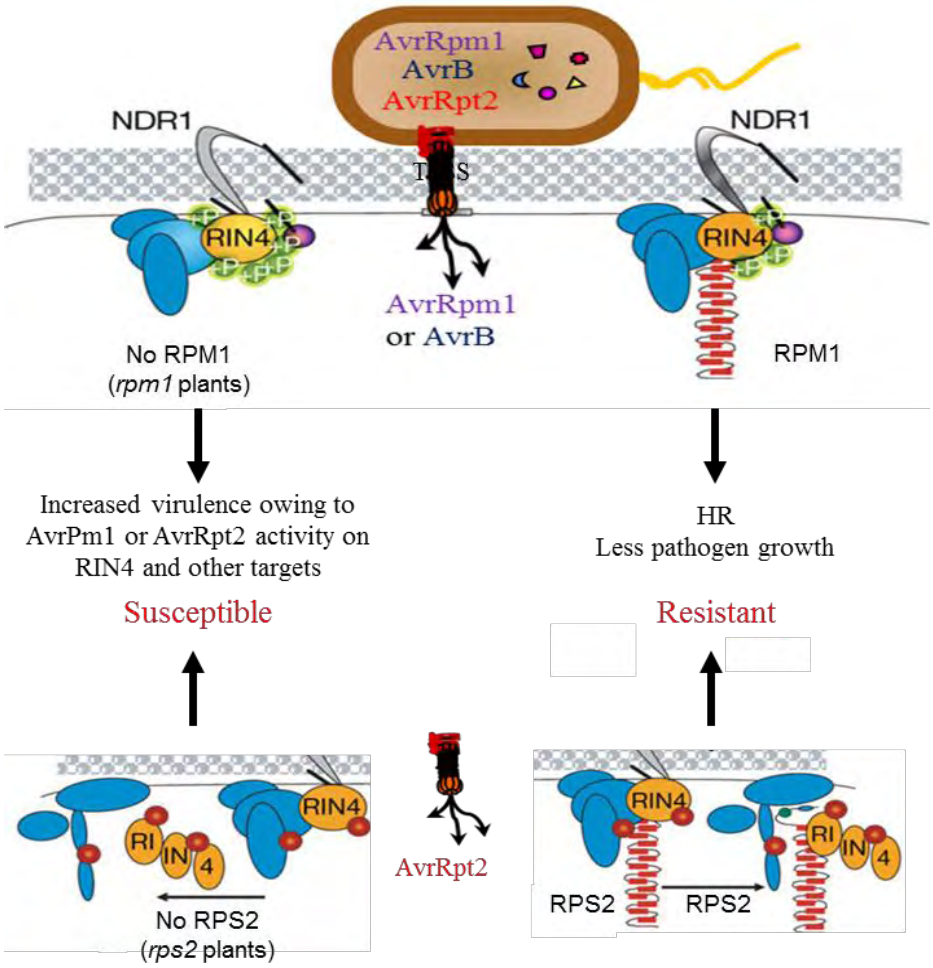



**Figure C1-4.** The Arabidopsis RPM1, a plasma membrane NB-LRR protein is activated by either AvrRpm1 or AvrB effector. AvrRpm1 enhances the virulence of some P. syringae strains on Arabidopsis as does AvrB on soybeans. Both proteins are delivered into cells by the type III secretion system and targeted to the plasma membrane. One of their targets is RIN4, which is phosphorylated (+P), and activates RPM1. In the absence of RPM1, AvrRpm1 and AvrB presumably act on RIN4 and other targets to contribute to virulence. RPS2 is a plasma membrane NB-LRR protein and is activated by the AvrRpt2 cysteine protease. AvrRpt2 also targets RIN4. Cleavage of RIN4 by AvrRpt2 leads to RPS2-mediated ETI. In the absence of RPS2, AvrRpt2 may cleave RIN4 and other targets as part of its virulence function. Light blue motives represent RAPs (Rin-4 associated proteins) and unidentified proteins. (Adapted from Jones and Dangl, 2006)

RPS5, which guards PBS1, is present (Shao *et al.*, 2003; Ade *et al.*, 2007). Interestingly, AvrPphB is also capable to inhibit PTI signaling by cleaving a number of PBS1-like (PBL) kinases, including BIK1 and PBL1 that play a general role as integrators of immune signaling responses triggered by multiple immune receptors (Zhang, J. *et al.*, 2010) (Figure C1-5).

## 1.2.3 Examples of effectors targeting chloroplast components

### 1.2.3.1 HopI1 targets Hsp70

HopI1 proteins from *P. syringae* are localized in chloroplasts where salicylic acid (SA), an important hormone for immune signaling, is synthesized. This effector suppresses SA accumulation as well as the related plant defences and affects thylakoid stack structure within chloroplasts (Jelenska *et al.*, 2007). HopI1 is a virulence factor required for bacterial growth and symptom development in a variety of crop plants. HopI1 forms large complexes in association with an heat shock protein, Hsp70 and recruits cytosolic Hsp70 to chloroplasts (Jelenska, Van Hal e Greenberg, 2010). At high temperature, the Hsp70 pool appears to be diverted to deal with heat stress functions at the expense of the defense response. In agreement with this observation, HopI1 is dispensable for virulence at high temperature suggesting that this effector reduces plant defenses by subverting an Hsp70 defense-promoting function (Jelenska, Van Hal e Greenberg, 2010). Finally, Hsp70 is essential for mediating HopI1 virulence in response to a non-pathogenic strain of *P. syringae*, supporting the idea that Hsp70 plays a role in basal resistance.

### 1.2.3.2 HopN1 targets PsbQ

HopN1, a cysteine protease T3E protein from *P. syringae*, is associated with the hypersensitive response (HR), that prevents spreading of the pathogen, in non-host tobacco plants and disease in host tomato plants (López-Solanilla *et al.*, 2004; Rodríguez-Herva *et al.*, 2012). In tomato, HopN1 co-localizes in chloroplastic thylakoids with PsbQ, a member of the oxygen evolving complex of Photosystem II (PSII), and is able to degrade PsbQ, thereby inhibiting the PSII activity in chloroplast preparations. Interestingly, PsbQ induces ROS production and programmed cell death upon infection (Rodríguez-Herva *et al.*, 2012).

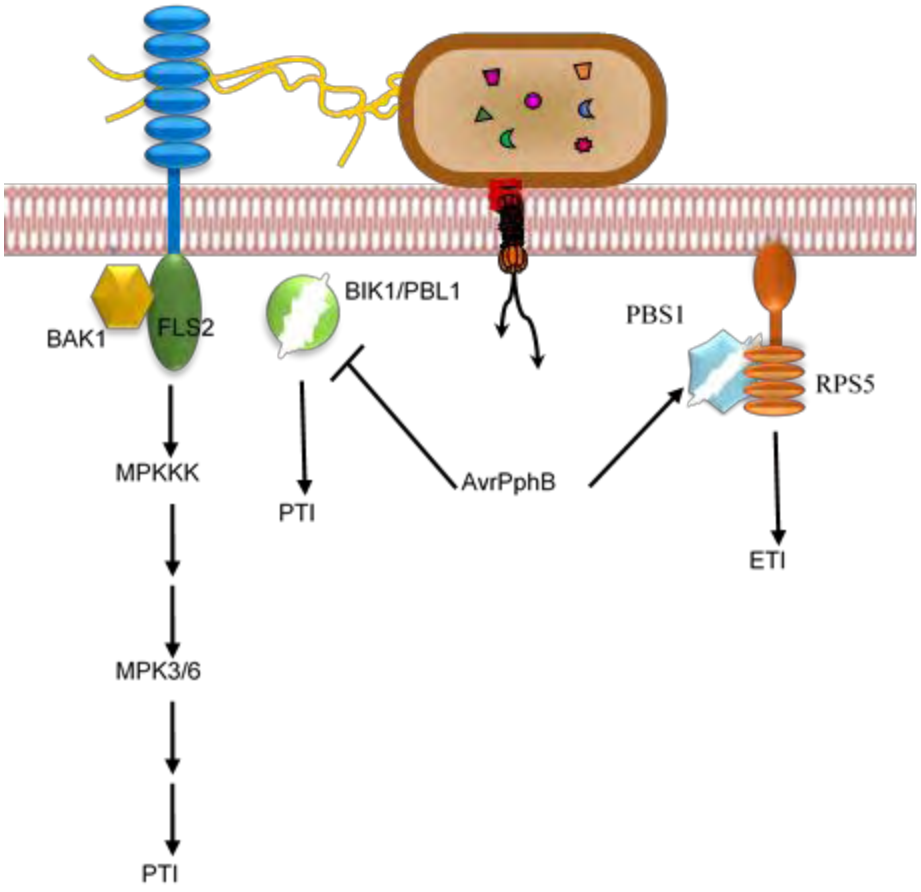



**Figure C1-5.** Schematic representation depicting the virulence and avirulence function of the bacterial cysteine protease AvrPphB. The AvrPphB effector is delivered into plant cells by *P. syringae* via the type III secretion system. Multiple innate immune signaling pathways are targeted by AvrPphB including: PTI, via the cleavage of BIK1 kinase; ETI, via the cleavage of the kinase PBS1, guarded by the resistance protein RPS5. (Adapted from Porteret *et al*, 2012)

These results highlight a general role of PsbQ in maintaining photosynthetic activity during infection by plant pathogens and underline the contribution of the photosynthetic pathway during plant defense responses. The identification of PsbQ as a natural target of HopN1 uncovers a virulence strategy aimed at the subvertion of host defenses by repressing the generation of potentially harmful ROS.

## 1.2.4 Examples of effectors targeting vesicle trafficking

### 1.2.4.1 HopM1 targets AtMIN7

The *P. syringae* virulence protein HopM1 accumulates in the trans-Golgi network/early endosome (TGN/EE) of host cells where it interacts with and mediates degradation of AtMIN7 (*A. thaliana* HopM1 Interactor7) by the host 26S proteasome (Nomura *et al.*, 2006; Nomura *et al.*, 2011). AtMIN7 belongs to the adenosine diphosphate (ADP) ribosylation factor (ARF) guanine nucleotide exchange factor (GEF) protein family, whose members are key components of vesicle trafficking and may play a role in plant immunity by mediating callose deposition on the plant cell wall. This illustrates a strategy of suppression of cell wall-associated host defense, thereby promoting bacterial infection (Nomura *et al.*, 2006) (Figure C1-6).

Interestingly, AtMIN7 is required not only for PTI, but also for ETI and SA-regulated immunity (Nomura *et al.*, 2011). Indeed, activation of ETI by three different *P. syringae* effectors (AvrRpt2, AvrPphB and HopA1) blocks HopM1-dependent AtMIN7 destabilization without affecting translocation of HopM1 into the host cell. Thus, blocking pathogen-mediated degradation of AtMIN7 in the TGN/EE is a critical step of the establishment of ETI. Furthermore, this finding provides an illustration of a mechanism by which plants are able to re-establish pathogen resistance during ETI in the context of pathogen suppression of ETI-associated components via effector proteins. Indeed, this work suggests a competition between HopM1-mediated degradation of AtMIN7 and plant defence-induced AtMIN7 stabilization inside the plant cell.

## 1.2.5 Examples of effectors targeting MAPK signaling

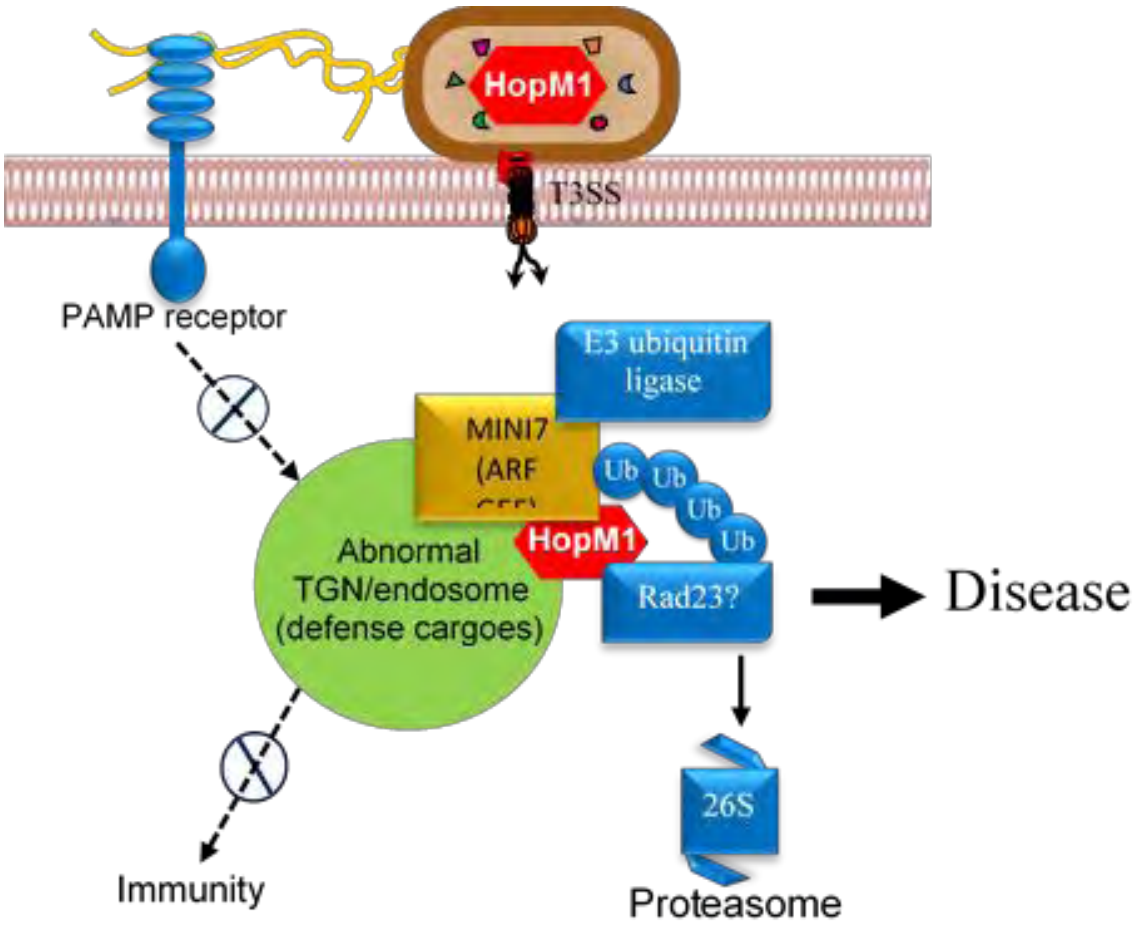



**Figure C1-6.** HopM1 effector manipulates components of a putative TGN/endosome-associated proteasome degradation machinery. (In blue: E3 ubiquitin ligase; Ub, ubiquitin; Rad23; and 26S proteasome) in order to remove the ARF-GEF protein MIN7, leading in dysfunctional TGN/endosomes, immune suppression, and disease. (Adapted from website of Sheng Yang HE Lab)

Targeting of MAPK signaling is a conserved virulence strategy used by a wide range of animal and bacterial pathogens. As a result of the inhibition of PAMP-induced phosphorylation of MKKs, an effector interferes with PAMP-triggered defenses and promotes pathogen virulence inside the plant. In *A. thaliana*, at least two MAPK signaling cascades are activated upon PAMP perception. The first one involves MPK3 and MPK6, whereas the second one leads to the activation of MPK4, which was previously described to be able to negatively regulate PTI through modulation of multiple hormone pathways (Petersen *et al.*, 2000) and requires the MAP kinase kinases MEKK1 and MKK1/MKK2 (Nakagami *et al.*, 2006; Suarez-Rodriguez *et al.*, 2007; Gao *et al.*, 2008; Qiu, Zhou, *et al.*, 2008).

#### 1.2.5.1 HopAI1 targets MPK3, MPK4 and MPK6

The *P. syringae* effector HopAI1 displays phosphothreonine lyase activity that results in dephosphorylation of MAP kinases MPK3 and MPK6 in a way that prevents their rephosphorylation. HopAI1-mediated inactivation of MAPK proteins results in the suppression of PAMP-induced gene transcription and cell wall-associated host defenses (Zhang, J. *et al.*, 2007).

#### 1.2.5.2 HopF2 targets MKK5

HopF2 is able to suppress PTI signaling through the attenuation of multiple MAP kinase kinases (MKKs). For example, HopF2 ADP-ribosylates MKK5 *in vitro* and inhibits its kinase activity (Wang *et al.*, 2010). Beyond its ability to interfere with MAPK signaling, HopF2 expression diminishes flagellin-induced phosphorylation of BIK1, whose activation results in the phosphorylation of the FLS2/BAK1 receptor complex (Wu *et al.*, 2011).

#### 1.2.5.3 AvrB targets RAR1 and MPK4

AvrB was shown to enhance plant susceptibility to *P. syringae* by perturbing jasmonic acid (JA) hormone signaling when the resistance protein RPM1 is absent (Shang *et al.*, 2006). In addition to its ability to induce RIN4 phosphorylation (see above), AvrB enhances also phosphorylation of MPK4. MPK4 was shown to be able to interact with and

phosphorylate RIN4 (Cui *et al.*, 2010), which negatively regulates resistance to *P. syringae*, and positively modulate JA responses. Thus, AvrB may induce plant susceptibility by promoting MPK4-mediated perturbation of hormone signaling (Cui *et al.*, 2010). Indeed, AvrB mediates suppression of PTI responses through its interaction with RAR1, a cochaperone of HSP90 that is required for ETI signaling. (Shang *et al.*, 2006). Interestingly, both RAR1 and HSP90 are required for AvrB-induced plant susceptibility and up regulation of JA responses (Shang *et al.*, 2006).

## 1.2.6 Example of effectors targeting nuclear components

### 1.2.6.1 TAL effectors target plant promoters

TAL (Transcription Activator-Like) effectors are virulence determinants found in plant pathogenic *Xanthomonas spp.* and *R. solanacearum*. These effectors present a central DNA-binding domain and a C-terminal region comprising nuclear localization signals (NLSs) and an acidic activation domain typical of transcription factors (TFs) (Scholze e Boch, 2011). According to their architecture, TAL effectors mimic eukaryotic TFs and are able to activate transcription in the plant nucleus after binding to their host target promoters (Kay *et al.*, 2007; Römer *et al.*, 2007).

The role of most TAL effectors in virulence is still rather elucidated. Some of them may be involved in the activation of genes encoding sugar transporters. Indeed, the *X. oryzae* pv. *oryzae* (*Xoo*) TAL effector PthXo1 induces expression of OsSWEET11 that is defined as a susceptibility gene because its expression facilitates rice infection by *Xoo*. OsSWEET11 has been proposed to mediate sugar efflux in plants in order to feed bacteria, although its ability to transport sugars remains to be demonstrated (Yang, Sugio e White, 2006). A second study suggests that OsSWEET11 may act as a copper transporter in the plasma membrane in order to decrease the copper content of the xylem sap and facilitate vascular infection by *Xoo* (Yuan *et al.*, 2010). Interestingly, distinct TAL effectors appear to target different types of functionally interchangeable SWEET genes. For example, two additional TAL effectors from *Xoo* (AvrXa7 and PthXo3) (Antony *et al.*, 2010) as well as the TalC protein from strain BAI3 of the African *Xoo* strain (Yu *et al.*, 2011) trigger induction of OsSWEET14 expression.

AvrBs3 from *Xanthomonas campestris* pv. *vesicatoria*, one of the best characterized TAL proteins, induces cellular hypertrophy in susceptible pepper (*Capsicum annuum*) varieties, which probably promotes bacterial proliferation and dispersal (Marois, Van Den Ackerveken e Bonas, 2002). AvrBs3 directly binds to a conserved element (called UPA box) in the UPA20 promoter and induces the expression of UPA20, a gene encoding a basic helix-loop-helix-type TF that acts as a master regulator of cell enlargement (Kay *et al.*, 2007). Notably, resistant pepper varieties evolved to deceive TAL recognition specificities in order to trap the effector and trigger defence responses. Indeed, the promoter of the pepper resistance gene Bs3, which encodes a protein homologous to flavine-dependent mono-oxygenases, contains a UPA box that is recognized and bound by AvrBs3, resulting in transcription of Bs3. As a result, HR (instead of mesophyll hypertrophy) is triggered (Römer *et al.*, 2007; Römer *et al.*, 2009).

#### 1.2.6.2 XopD targets AtMYB30

XopD from *Xanthomonas campestris* is a modular T3E targeted to the nucleus of host cells. XopD exhibits small ubiquitin-like modifier (SUMO) protease activity thanks to the presence of a cysteine protease domain at its C-terminus (Hotson *et al.*, 2003). In addition, two tandem repeated transcriptional repressor EAR (ERF-associated Amphiphillic Repression) motifs confer to XopD the ability to repress transcription of defence- and senescence-related plant genes (Kim *et al.*, 2008). Finally, an intact helix-loop-helix domain (HLH) is necessary for XopD nuclear targeting (Canonne *et al.*, 2011) and the ability to display non-specific DNA-binding (Kim *et al.*, 2008). It has been suggested that a XopD N-terminal domain of unknown function may confer specificity for DNA-binding, but this hypothesis remains to be demonstrated (Canonne *et al.*, 2010). A recent study showed that XopD from strain B100 of *X. campestris* pv. *campestris* is able to target the R2R3-type MYB TF AtMYB30 in *A. thaliana* (Canonne *et al.*, 2011), a positive regulator of plant defence and cell death associated responses (Raffaele *et al.*, 2008).

#### 1.2.6.3 HopAI1 and AvrRps4 target EDS1

The *P. syringae* effector AvrRps4 is recognized by the Toll-interleukin-1 receptor- (TIR)-NB-LRR protein RPS4 in *A. thaliana* (Gassmann, Hinsch e Staskawicz, 1999). RPS4-

mediated immune responses require EDS1, a lipase-like protein considered as a crucial regulator of immunity. EDS1 nucleo-cytoplasmic shuttling and coordination of its cytoplasmic and nuclear activities are required for immunity-related transcriptional reprogramming (Garc á *et al.*, 2010). Like EDS1, RPS4 and AvrRps4 display a nucleo-cytoplasmic distribution. Depending on its subcellular localization, AvrRps4 was shown to trigger distinct, but coordinated defence-related responses. Indeed, restriction of bacterial growth relies on AvrRps4 nuclear localization whereas programmed cell death and transcriptional reprogramming of defence related-genes require nucleo-cytoplasmic pools of the bacterial effector (Heidrich *et al.*, 2011). Consistent with the fact that nuclear pools of EDS1 and RPS4 are also essential for AvrRps4-triggered immunity (Wirthmueller *et al.*, 2007; Garc á *et al.*, 2010), EDS1-AvrRps4 and EDS1-RPS4 complexes have been recently detected within the nucleus (Heidrich *et al.*, 2011). Taken together, these data indicate that EDS1 associates with RPS4 to form an RPS4-EDS1 receptor signaling module that is able to shuttle between the cytoplasm and the nucleus and intercept AvrRps4.

## 1.3 Effector recognition mechanisms

Recognition of an effector by an R protein constitutes the first step of ETI activation, in agreement with the “gene for gene” theory proposed by Flor in 1942. Current evidence suggests that NB-LRRs behave as molecular switches that are in an auto-inhibited but primed conformation by intra-molecular interactions between the different domains and associations with co-factors (Collier e Moffett, 2009; Lukasik e Takken, 2009). Release from inhibition by an effector triggers a series of conformational changes that allows the R protein to activate downstream defenses (Collier e Moffett, 2009; Lukasik e Takken, 2009; Takken e Goverse, 2012). Four different molecular models have been proposed to explain different recognition processes of Avr proteins by R proteins (Figure C1-7).

### 1.3.1 Ligand-receptor model

Initially, it was thought that products of R genes act as receptors by directly interacting with the products of Avr genes (Keen, 1990). This ligand-receptor model was supported by the fact that some Avr gene products are small and co-localize with R gene products. Indeed, direct binding of a few R-Avr combinations was found, consistent with a receptor-

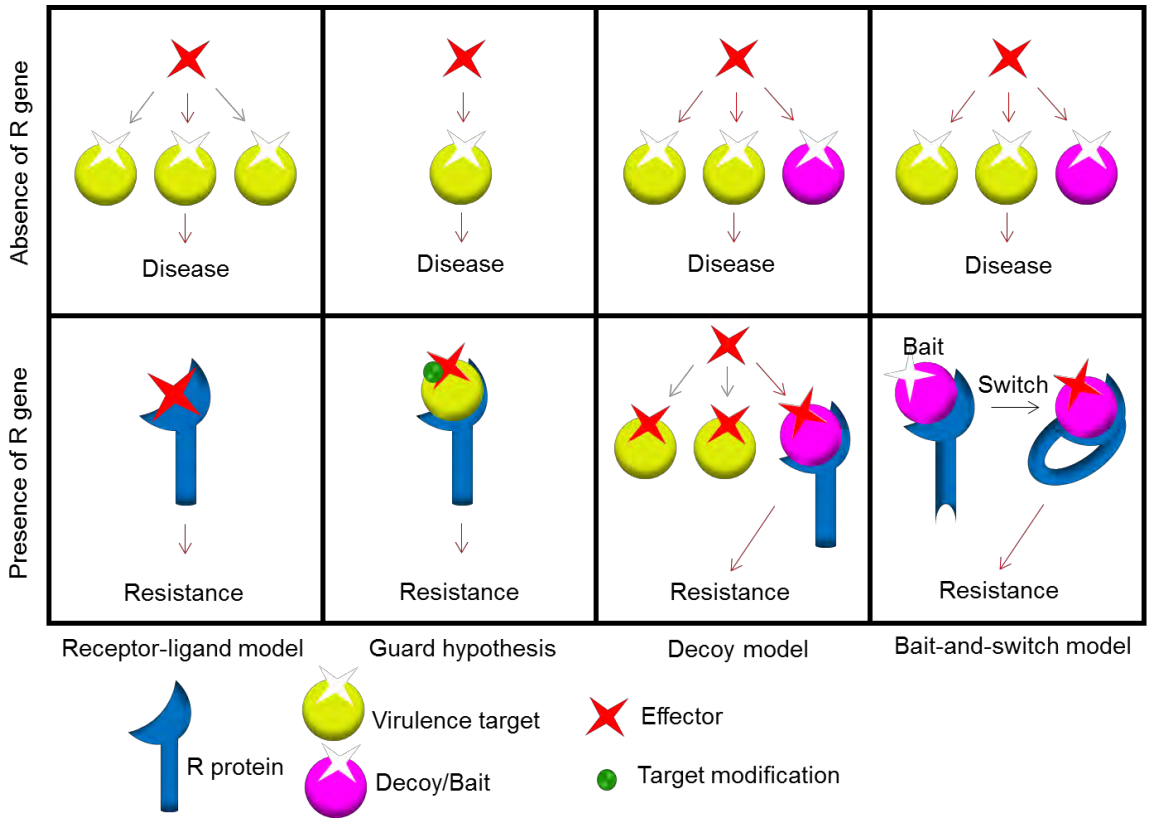



**Figure C1-7.** Proposed models for effector recognition.

The various models are represented schematically: guard hypothesis; Decoy model; bait and switch models. The interaction is represented either in the absence of R gene (top), or presence of R gene (bottom). (Adapted from Hann and Boller, 2012)

ligand mode of action (Jia *et al.*, 2000; Deslandes *et al.*, 2003; Dodds *et al.*, 2006; Ueda, Yamaguchi e Sano, 2006). However, for a number of R-Avr combinations, physical interactions have not been detected, and perception in these cases is thought to be indirect.

### 1.3.2 Guard model

The idea that effectors have specific targets in the host inspired another indirect mechanism of effectors recognition by R proteins. The guard model predicts that R proteins act by monitoring (guarding) the effector target and that modification of this target by the effector results in the activation of the R protein, which triggers disease resistance in the host (Van Der Biezen e Jones, 1998; Dangl e Jones, 2001).

The Guard model was originally proposed to explain the perception of *P. syringae* AvrPto by the tomato proteins Pto and Prf (Van Der Biezen e Jones, 1998) and was later generalized to other models (Dangl e Jones, 2001). Classical examples of these presumed guardees are *A. thaliana* RIN4 and PBS1 and tomato RCR3 and Pto (Jones e Dangl, 2006). The indirect effector perception mechanism postulated by the guard model explains how multiple effectors could be perceived by a single R protein, thus enabling a relatively small R gene repertoire to perceive a broad diversity of pathogens (Dangl e Jones, 2001). Support for the guard model has been accumulating with the identification of a number of guarded effector targets (see below).

### 1.3.3 Decoy model

The decoy concept is based upon the observation that some host targets of effectors act as decoys to detect pathogen effectors via R proteins (Zhou e Chai, 2008; Zipfel e Rathjen, 2008). This concept emerged as many alterations of the decoy by effectors did not result in enhanced pathogen fitness in plants that lack the R protein and triggered innate immunity in plants that carry the R protein. In addition, this model is compatible with the fact that many pathogen effectors have multiple targets in the host.

### 1.3.4 Bait and switch Model

This model proposes that the use of recognition cofactors as baits is a mechanism employed by R proteins to sense Avr proteins and activate a molecular switch that results in the induction of resistance responses. As such, the bait and switch model provides a mechanistic explanation of how NB-LRR proteins translate pathogen recognition into defense activation (Collier e Moffett, 2009).

### 1.4 Recognition through pairs of resistance proteins.

Another concept arose from an increasing number of reports on dual NB-LRR genes conferring resistance to pathogens. However, in this mechanism, the function of each one of the R protein is not established yet (Figure C1-8).

The first evidence that a pair of NB-LRR genes function together in disease resistance against a pathogen isolate was the finding that both RPP2A and RPP2B R proteins are required for disease resistance to an oomycete pathogen isolate (Sinapidou *et al.*, 2004). Characterization of N-NRG1 and RPM1-TAO1 revealed that disease resistance to viral and bacterial pathogens expressing a single avr product (p50-*Tobacco Mosaic Virus*, AvrB- *P. syringae*, respectively) can be mediated by an NB-LRR pair encoding proteins of the TIR and CC subclasses (Peart *et al.*, 2005; Eitas, Nimchuk e Dangl, 2008). The existence of CC-NB-LRR-encoding gene pairs mediating disease resistance to fungal pathogens came from the identification of Lr10-RGA2 and Pi5-1-Pi5-2 (Lee *et al.*, 2009; Loutre *et al.*, 2009). Finally, characterization of Pikm1-TS and Pikm2-TS demonstrated that two NB-LRR genes encoding non-TIR domains are required for disease resistance against a fungal pathogen isolate (Ashikawa *et al.*, 2008). Recent investigation of RRS1 and RPS4 demonstrated that this TIR-NB-LRR pair is required for disease resistance against multiple pathogen isolates (Gassmann, Hinsch e Staskawicz, 1999; Deslandes *et al.*, 2002; Narusaka *et al.*, 2009). In this case, heterodimerization of the TIR domains of both proteins was demonstrated by immunoprecipitation (Williams et al., XV Congress on Molecular Plant-Microbe Interactions, Kyoto, 2011, Japan).

## 1.5 Regulation of plant gene transcription: beyond perception, a major step in immune response

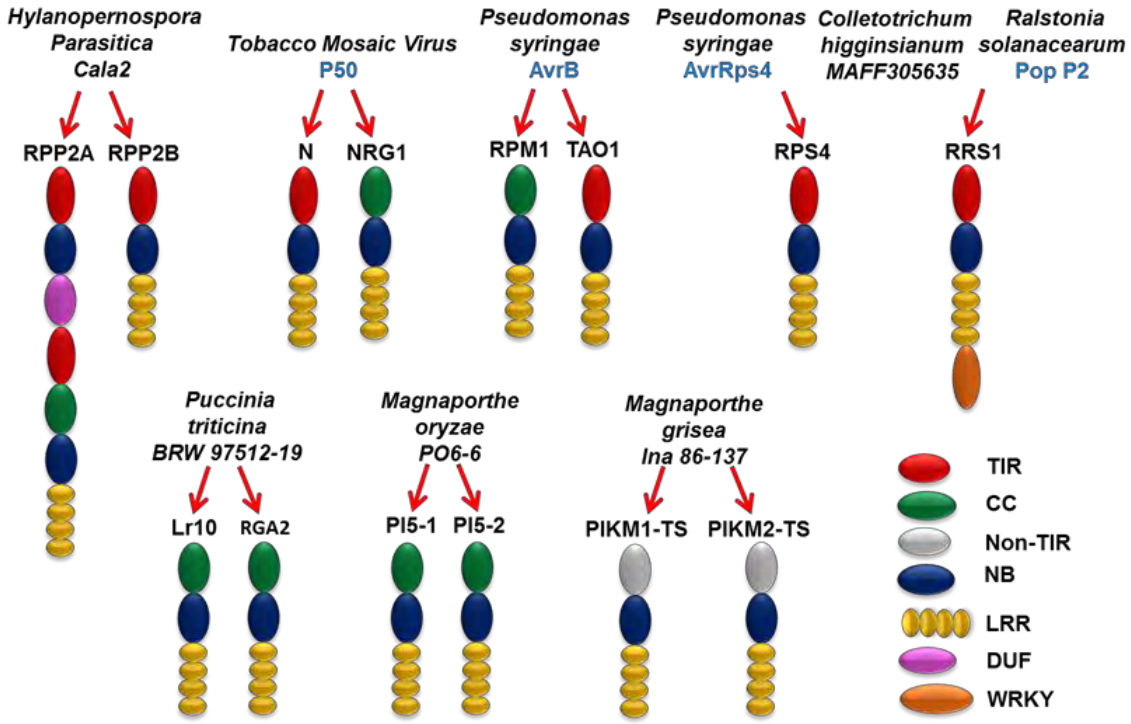



**Figure C1-8.** Schematic representation of the domain structure of NB-LRR proteins and pathogen isolates (black italic).

Top row: R proteins NB-LRR identified in Arabidopsis and Tobacco. The Avr gene products are represented with blue lettering. Bottom row: NB-LRR proteins present in wheat and rice. The various pathogen isolates are represented with black italic lettering. (Adapted from Eitas and Dangl, 2010)

Following perception, signal transduction pathways result, in the end, in the induced production of defense proteins that directly or indirectly inhibit pathogen proliferation. Many transcription factors (TFs) are involved in the various defense pathways leading to these responses. On overview of the most important classes of transcription factors, (AP/ERF, MYB, MYC, bZIP and WRKY transcription factor families) engaged in plant defense is available in a review from van Verk and collaborators (Van Verk, 2009). In the last few years, the diversity of transcription factor families involved in defense increased largely. For example, TCP and NAC transcription factors appeared to be important actors in these processes (Muktar *et al.*, 2011; Nuruzzaman, Sharoni e Kikuchi, 2013). Although this study is not exhaustive, the following examples will highlight important mechanisms by which TFs contribute to plant immunity. Emphasis will be related to this study.

### 1.5.1 Hormonal control mediated through transcriptional regulations: What is known about salicylic acid signaling pathways

Transcription regulation, related to hormonal immunity control, is exemplified by using salicylic acid (SA), as this hormone is an important player in induced defense of the plant against invading biotrophic pathogens.

Several levels of transcriptional regulation have been described concerning the mode of action of SA (hormone synthesis or downstream pathways). In A. thaliana, the EDS1/PAD4 couple controls SA biosynthesis and is essential for the activation of the SA signaling (Wiermer, Feys e Parker, 2005; Wang *et al.*, 2008). The observation that EDS1 and PAD4 expression are induced by SA suggests the existence of a feedback loop that amplifies the signal.

It was shown that transcription of EDS1 and PAD4 is negatively regulated by SR1 ,a Ca2+/calmodulin-dependent binding transcription factor, leading to a pathogen-controlled accumulation of SA (Du *et al.*, 2009). SID2 constitutes another critical component in the biosynthesis of SA in response to biotic challenges; *SID2* encodes indeed an isochorismate synthase (ICS1) capable of catalysing the formation of isochorismate, the SA precursor from chorismate (Wildermuth *et al.*, 2001). Several transcriptional regulators influence *SID2* expression. Positive regulators, such as WRKY28 which binds to the *SID2* promoter

and induces *SID2* expression in transfection assays, have been identified. Two other genes involved in *SID2* regulation are *CBP60g* and *SARD1*. CBP60g is a member of a family of calmodulin (CaM) binding proteins identified as being strongly induced in response to MAMPs treatment. Chromatin immunoprecipitation experiments established that, following pathogen attack, the binding of these proteins is increased in *ICS1* promoters and they were demonstrated as potent activators of ICS1 transcription (Wang *et al.*, 2009; Zhang, Y. *et al.*, 2010). Negative regulators of *SID2* expression have also been characterized; EIN3, a key transcription factor involved in the ethylene signal transduction pathway (ref) is capable of binding to the *SID2* promoter and combined mutations of *ein3* and of its close homolog *eil1, a EIN3*-related transcription factor gene, lead to elevated *SID2* expression, SA accumulation and increased resistance to bacterial infection (Chen *et al.*, 2009). Similarly, three related NAC transcription factors (ANAC019, ANAC055 and ANAC072) were found to inhibit *SID2* expression, SA accumulation and resistance to bacterial infection (Zheng *et al.*, 2012).

The transcriptional regulator NPR1 (NONEXPRESSOR of PR GENES 1), is a major actor in the downstream pathway, and controls approximately 95% of SA-dependent genes (Wang, Amornsiripanitch e Dong, 2006). In the absence of SA, NPR1 is localized in the cytoplasm, where it forms multimers. SA treatment induces a redox change in the cell, leading to the dissociation of the NPR1 complex and migration of NPR1 monomers into the nucleus where they behave as positive regulators (Kinkema, Fan e Dong, 2000; Mou, Fan e Dong, 2003; Tada *et al.*, 2008). Once inside the nucleus, NPR1 binds indeed to TGA transcription factors, enhancing their binding to SA-responsive promoters. Upon SA treatment, NPR1 is phosphorylated in the nucleus and this modification facilitates the interaction between NPR1 and CULLIN, a hydrophobic protein providing a scaffold for ubiquitin ligases (E3). This interaction enhances NPR1 degradation required for the full induction of target genes (Spoel *et al.*, 2009). A recent study has indicated that NPR3 and NPR4, two NPR1-like proteins, act as SA receptors and regulate NPR1 functions (Fu *et al.*, 2012). NPR1 and TGAs directly regulate *PATHOGENESIS-RELATED 1* (*PR1*) expression, which results in PR1 protein production and secretion into the apoplast, where this protein exerts its antimicrobial activity on proliferating pathogens. NPR1 also

positively regulates TBF1 (a TL1-binding factor) expression and, in turn, TBF1 promotes SA-dependent *BiP2* expression. The BiP2 protein prevents activation of the Unfolded Protein Response (UPR) in the absence of biotic stress. Another actor, nitric oxid (NO) initiates SA biosynthesis and nitrosylates key cysteines on TGA- class transcription factors to aid in the initiation of SA-dependent gene expression. Against this, *S*- nitrosylation of NPR1 promotes the NPR1 oligomerization within the cytoplasm to reduce TGA activation (Mur *et al.*, 2013).

A complex interplay between different hormone systems contributes to the fine tuning of SA biosynthesis/signaling. For example, an antagonism between SA-JA/ET exists and several transcriptional regulators play key roles in this process. SA has a negative effect on the accumulation of the AP2/ERF-type transcription factor ORA59 (for OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF domain protein59), an important factor in the JA signaling pathway (Van Der Does *et al.*, 2013).

### 1.5.2 Immunity triggered by several PAMPS or by PTI and ETI overlaps at the level of transcriptome responses

Analyses of *A. thaliana* transcriptional responses triggered by various MAMPs are very similar in the early stages after treatment with flg22, elf26, and chitin, suggesting that the induced PTI responses are similar (Wan *et al.*, 2008). In contrast, late responses to oligo-galacturonides (degradation products of the plant cell wall typically produced by fungal pathogens) and flg22 diverged (Denoux *et al.*, 2008). This may allow plants to ensure the appropriate immune response according to the nature of the pathogen.

Genome-wide transcriptional profiling and analysis of various signaling mutants in *A. thaliana* suggest the existence of a highly overlapping signaling network in PTI and ETI (Figure C1-9) (Tsuda *et al.*, 2009). A significant overlap between genes induced by flg22 and genes induced by effector recognition was also observed (Navarro *et al.*, 2004). Accordingly, several transcription factors were identified as regulators both of ETI and PTI. For instance, OsWRKY62 is a negative regulator of basal and Xa21-mediated defense against *Xanthomonas oryzae* pv. *oryzae* in rice (Peng *et al.*, 2008). MYB6, a barley

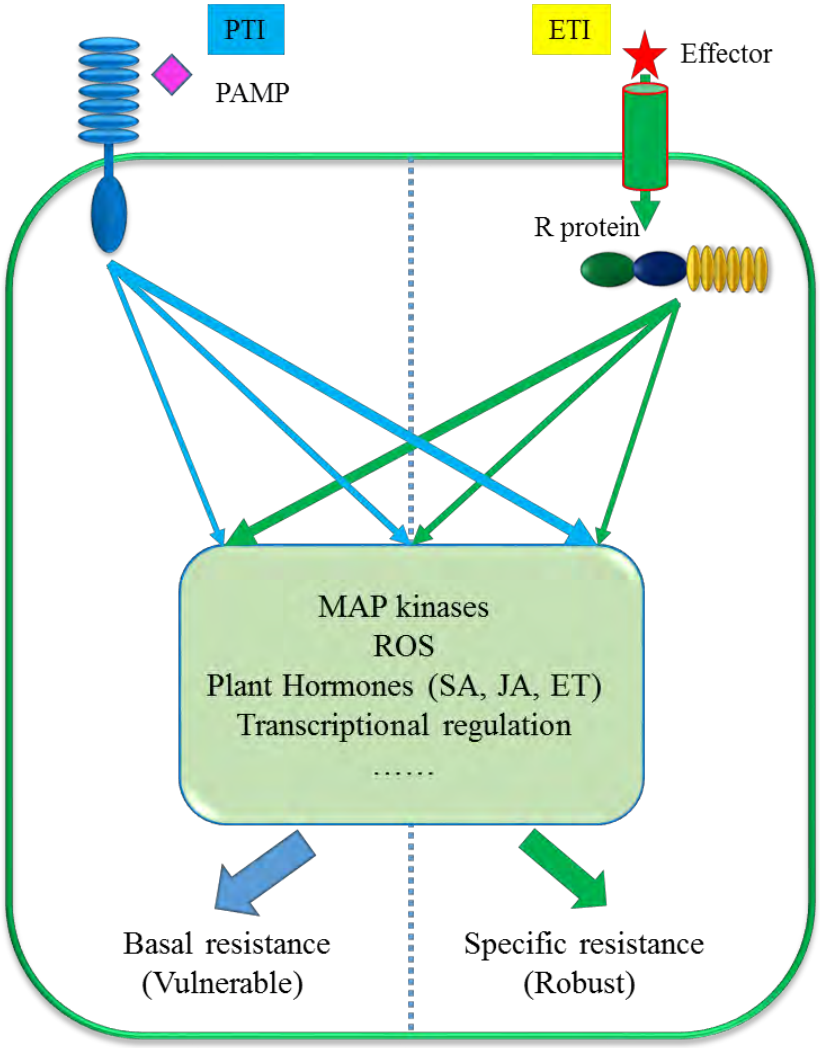



**Figure C1-9.** Illustration of the overlapping signaling in PTI and ETI.

The recognition of the MAMP by a PRR triggers basal resistance (PTI) and leads to the activation of a signaling pathway which affects plant gene transcription. Then, pathogen injects effectors into the cell. An effector is detected by an R protein, which triggers strong immune responses (ETI). Plant gene transcription is also altered. In addition, transcriptional profiling suggests an overlap between the signaling pathways of PTI and ETI. (Adapted from Tsuda and Katagiri, 2010)

transcription factor was also identified as a positive regulator implicated both in basal and specific resistance (Chang *et al.*, 2013).

If we consider that PTI evolved before ETI (Abramovitch, Anderson e Martin, 2006), the overlap of transcription reprogramming between both types of immunity suggests that, while acquisition of a new class of recognition molecules, R proteins, was necessary for evolution of ETI, ETI adapted the rest of the immune machinery mostly from a pre-existing PTI machinery. In addition this fact underlines the importance of gene transcriptional regulation in setting up the plant response (Tsuda e Katagiri, 2010). Indeed, in ETI, there are several examples in Tobacco, in *A. thaliana* or in barley, of NB-LRR proteins directly interacting with transcription factors or cofactors to regulate gene expression (Zhu *et al.*, 2010; Chang *et al.*, 2013; Padmanabhan *et al.*, 2013). This suggests that beyond recognition, regulation of transcription constitutes a kind of control tower that orchestrates plant response to pathogen. The following points illustrate this notion.

### 1.5.3 Transcriptional changes associated to the function of resistance proteins

Recent studies are starting to unravel the impact of NB-LRR proteins on transcriptional reprogramming. These data draw an emerging picture in which nuclear localized NB-LRRs mediate transcriptional reprogramming via their interaction with transcription factors. Two hybrid data generated by Muktar et al. largely support this idea (Muktar *et al.*, 2011). These authors tested interactions between *A. thaliana* NB-LRRs, and products of 8,000 immune-related genes including transcriptional regulators. A majority of NB-LRRs showing interactions, interacted with one or more transcriptional regulators. Transcriptional regulation occurs also through NLRs in animal cells : for instance, the NLR family members NLRC5 (IFN-γ–inducible nuclear protein) and CIITA (class II transactivator) can trans-activate some MHC genes involved in immunity by forming enhanceosomes through their interaction with promoter-assembled factors (Kobayashi e Van Den Elsen, 2012). In plants, interactions between transcriptional regulators and NLRs have also been demonstrated : for example, barley MLA10 interacts with two WRKY proteins, WRKY1 and 2 (Shen *et al.*, 2007). WRKY1 interacts directly with the Myb6 transcription factor and is able to suppress its DNA binding activity. The active form of MLA releases MYB6 from

WRKY1 repressor and stimulates its binding to DNA, which initiates the disease resistance signaling cascade (Figure C1-10) (Chang *et al.*, 2013). In *A. thaliana*, it was reported that a protein complex of Topless-related 1 (TPR1) (??) and a suppressor of npr1-1, constitutive 1 (SNC1) are implied in the regulation of defense responses. When plants are not challenged with a pathogen, plant immune responses are repressed by negative regulators. Upon pathogen attack, the protein complex of SNC1/TPR1 activates TPR1. The activated TPR1 represses the expression of negative regulators, which leads to activation of immune responses (Zhu *et al.*, 2010). Recent studies showed that the tobacco N immune receptor that provides immunity against Tobacco mosaic virus (TMV) infection is present in the nucleus and associates with the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 6 (SPL6) transcription factor. This association is detected only when the TMV effector, p50, is present in the cell. This suggests that N associates with SPL6 only during an active defense response. SPL6 function is required for defense against TMV. SPL6 from *A. thaliana* functions also in the resistance against the bacterial pathogen *P. syringae* expressing the AvrRps4 effector and positively modulates defense gene expression (Padmanabhan *et al.*, 2013).

### 1.5.4 Role of the Mediator and Elongator transcription complexes in plant immunity

Transcription regulation occurs at two levels, one involving the transcriptional apparatus and transcription factors, the other implicating chromatin structure and its regulators (Figure C1-11). These two processes are obviously interconnected. Concerning the first level, the key enzyme is RNA polymerase II (RNAPII). The recruitment of this enzyme during transcription initiation and its passage along the template during transcription elongation is regulated through the association and dissociation of several complexes. (i) The Mediator complex serves as a central scaffold within the pre-initiation complex and helps to regulate RNAPII activity. Mediator is also targeted by sequence-specific transcription factors and is essential to convert biological inputs to physiological responses *via* changes in gene expression. Depending on the organism, the Mediator complex consists of ~20 to 30 subunits. Several isoforms or alternative forms exist in cells, which may allow the complex to integrate a multitude of regulatory inputs (Casamassimi e Napoli, 2007). In

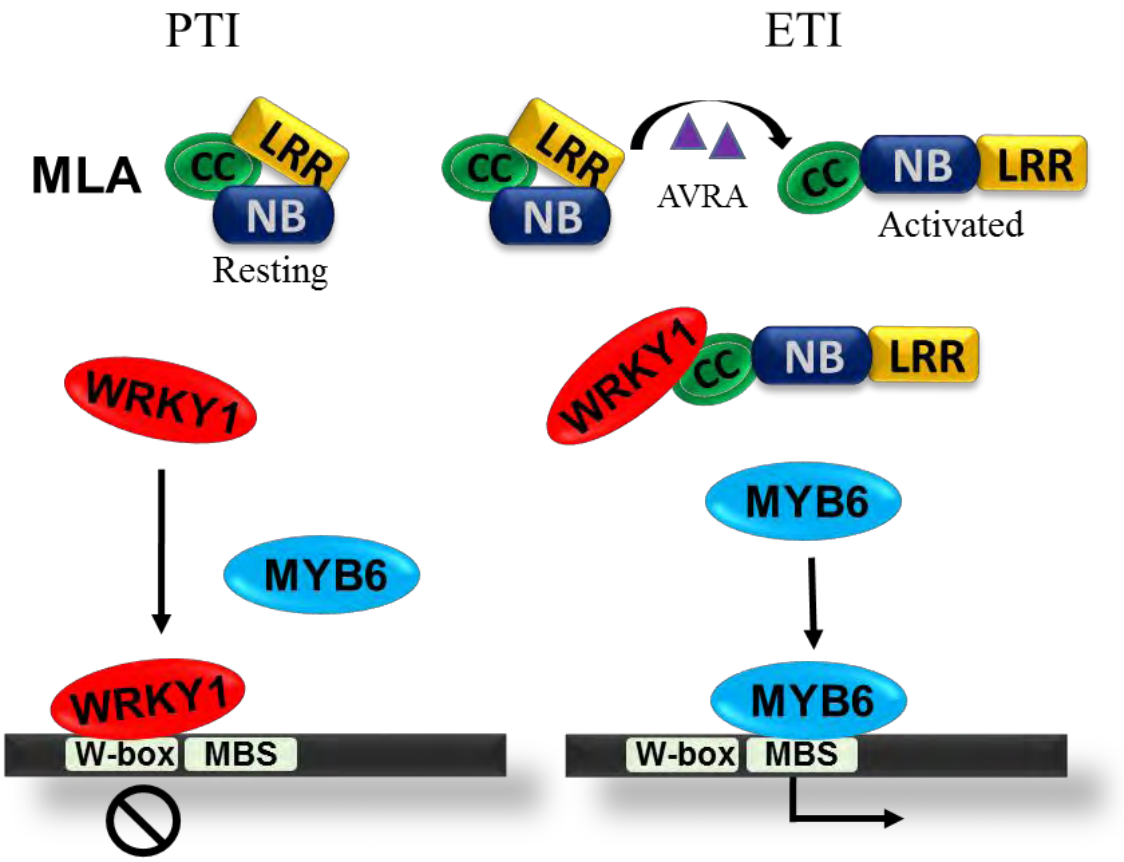



**Figure C1-10.** WRKY1 transcription factor interacts directly with the R protein (MLA). The barley Mildew A (MLA) gene encodes a CC-NLR receptor whereas MYB6 is a signaling component of the active form of this receptor. In PTI, in absence of an effector, MLA is in a resting state. The binding activity of MYB6 is restricted by the WRKY1 repressor. In ETI, after recognition of the AVRA effectors by MLA, MLA becomes activated. The activated form of MLA then releases MYB6 and activates defense gene expression. (Adapted from Chang *et al*, 2013)

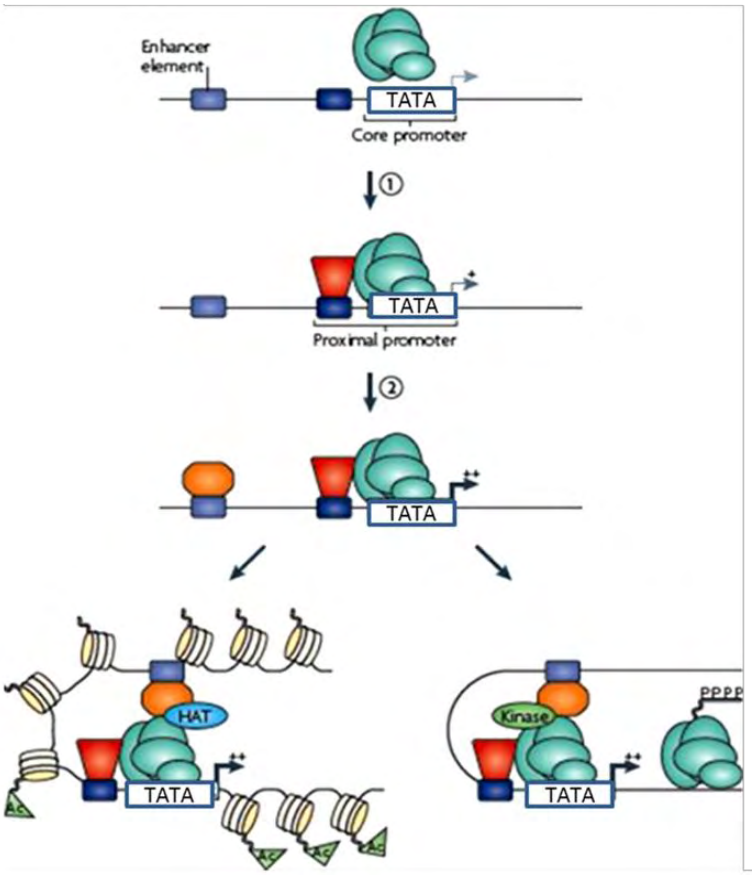



**Figure C1-11**. Transcriptional regulation by promoters and enhancers.

General transcription factors (light blue) bind to core promoter regions via the recognition of basal elements such as TATA boxes (TATA). However, these elements by themselves only provide very low levels of transcriptional activity due to the unstability of the interactions of the general factors with promoter region. Promoter activity can be increased by site-specific DNA binding factors (red) interacting with *cis*-elements in the proximal promoter region and stabilizing the recruitment of the transcriptional machinery through direct interaction of the site-specific factor and the general factors (step 1). Promoter activity can be further stimulated to higher levels by site-specific factors (orange) binding to enhancers (step 2). The enhancer factors can stimulate transcription by (A) recruiting a histone-modifying enzyme (for example a histone acetyltransferease, HAT) to create a more favorable chromatin environment for transcription (acetylated histones, Ac) or (B) recruiting a kinase that can phosphorylate the C terminal domain of RNA polymerase II and stimulate elongation. (Adapted from Farnham, 2009)

*A. thaliana*, 21 conserved and six species-specific Mediator subunits have been identified (Bäckström *et al.*, 2007). Several subunits (MED 8, 15, 16, 21, and MED 25) were shown to play a role in plant immunity, probably through the perception of signals activated by different hormones and the initiation of defense-associated transcriptional reprogramming. All of these Mediator subunits serve as positive regulators of defense against leaf pathogens: MED15 is involved in the activation of the SA-dependent signaling pathway (Canet, Dobón e Tornero, 2012). MED8 and MED25 mainly regulate JA-dependent signaling, but may also contribute to SA-dependent defense (Kidd *et al.*, 2009; Zhang *et al.*, 2012) whereas MED16 plays dual roles in mediating both SA and JA/ET signaling pathways (Zhang *et al.*, 2012). MED21 regulates resistance against fungal pathogens, likely by relaying signals from upstream regulators and factors modifying chromatin to RNAPII, then interconnecting the levels of the transcription apparatus with chromatin structure regulation (Dhawan *et al.*, 2009).

(2)The Elongator complex co-purifies with RNA polymerase II (RNAPII) during transcriptional elongation, and presumably renders DNA more accessible to the enzyme (Otero *et al.*, 1999; Hawkes *et al.*, 2002). The Elongator complex consists of six subunits (ELP1–ELP6), one of which displays an acetyl transferase (HAT) activity (Fichtner *et al.*, 2002). Acetylation is a well characterized histone modification and plays a role in the regulation of transcription (Gregory, Wagner e Hörz, 2001; Jenuwein e Allis, 2001).

In plants, mutations of the Elongator subunits result in pleiotropic effects including hypersensitivity to abscisic acid, resistance to oxidative stress, development of severely aberrant auxin phenotypes and disease susceptibility (Nelissen *et al.*, 2005; Chen *et al.*, 2006; Zhou *et al.*, 2009; Defraia, Zhang e Mou, 2010; Nelissen *et al.*, 2010). AtELP2 was shown to accelerate defense gene induction and allows rapid transcriptional changes required for plant immunity. Indeed, resistance mediated by two different R proteins RPS2 and RPS4, which involves such transcriptional changes, is compromised in *Atelp2* mutant plants (Defraia, Zhang e Mou, 2010). Recently AtELP2 was shown to maintain the histone acetylation level in several defense genes and to modulate the genomic DNA methylation status. It is therefore regarded as an epigenetic regulator of plant immune responses (Wang *et al.*, 2013).

### 1.5.5 Regulation of chromatin targeting of transcription regulators

In eukaryotic cells, transcription factors identify their DNA targets by scanning or hopping on the nuclear chromatin. (Hager, Mcnally e Misteli, 2009). It seems however (?) that transcription factors in plant immunity are actively kept away from chromatin at least by two different mechanisms, sequestration and destruction.

### 1.5.6 Sequestration of activity away from chromatin

The basic Leucine Zipper (bZIP) transcription factor AtbZIP10 is involved in pathogen-induced cell death, a mechanism used to restrict biotrophic pathogen propagation. In the absence of pathogen, the zinc finger Lesion Simulating Disease resistance 1, LSD1, a negative regulator of cell death interacts with bZIP10 and partially sequesters it in the cytoplasm. It has been hypothesized that the pathogen induce dissociation of bZIP10 from LSD1, allowing its translocation to the nucleus and the activation of immune-related gene expression (Kaminaka *et al.*, 2006).

This model is reminiscent of the mechanism by which the transcription activator, Nuclear Factor kB (NF-kB) is controlled in animal innate immunity. In resting cells, NF-kB is sequestered in the cytoplasm by the inhibitory protein Inhibitor of kB (IkB). Immune activation leads to the phosphorylation and subsequent degradation of IkB, releasing NF-kB to activate target genes in the nucleus (Hayden e Ghosh, 2004). Unlike NF-kB in animals, however, plant bZIP10 does not exhibit exclusive cytoplasmic localization in resting plant cells but was also found in the nucleus.

The immune regulator EDS1 is localized both in the nucleus and cytoplasm where it is a component of several complexes. EDS1 has been shown to interact with some transcription factors using yeast two-hybrid assays (Garc á *et al.*, 2010). Upon pathogen attack, part of the cytoplasmic EDS1 pool is redistributed to the nucleus. This event precedes an EDS1-dependent gene regulation. EDS1 controls PTI by forming complexes in the nucleus and the cytoplasm with PAD4 and SAG101, two other defense regulators (Aarts *et al.*, 1998; Feys *et al.*, 2005; Wiermer, Feys e Parker, 2005). These interactions restrict growth of virulent pathogens. EDS1 is also involved in the dowsnstream signaling of activated TIR-

NB-LRR receptors to control cell death and transcriptional regulation of defense pathways (Zhang *et al.*, 2003; Mestre e Baulcombe, 2006; Wirthmueller *et al.*, 2007).

These findings suggest that the nuclear import of immune activators constitutes a key process for the establishment of successful defense responses (Garc á e Parker, 2009; Heidrich *et al.*, 2011). Accordingly, mutation of the nucleoporin modifier of snc1,7, a subunit of the nuclear pore complex, compromises plant immunity due to the decreased nuclear accumulation of immune regulators, including EDS1 and NPR1 (Cheng *et al.*, 2009).

### 1.5.7 Transcriptional regulators may also be kept away from chromatin within the nucleus.

The transcriptional activator WRKY33 shows a pathogen-inducible association with defense genes, including *PAD3*. In unchallenged cells, WRKY33 forms a nuclear complex with the Mitogen-activated Protein Kinase 4 (MPK4). Upon pathogen infection, MPK4 dissociation allows WRKY33 to activate defense gene transcription (Andreasson *et al.*, 2005; Qiu, Fiil, *et al.*, 2008). Similarly, the ethylene-responsive transcription factor ERF 104, was also shown to be sequestered in the nucleus by MPK6 (Bethke *et al.*, 2009). Nuclear sequestration also concerns the JA-inducible transcription factor, MYC2, by members of the JAZ family of repressor proteins (Chini *et al.*, 2007; Thines *et al.*, 2007).

## 1.6 The *A. thaliana*/*R. solanacearum* pathosystem

### 1.6.1 *R. solanacearum*, a destructive bacterial plant pathogen

*R. solanacearum*, a *Beta*-proteobacterium, is pathogenic on more than 200 plant species. This pathogen affects *solanaceous* plants, such as tomato and potatoes and many others dicot and monocot families. This host range is expending and new hosts are frequently described. This soil borne bacteria is present all over the world in tropical and sub-tropical areas. Wilting symptoms are similar for many susceptible hosts but different disease names are used depending on the crop affected (Figure C1-12). Lethal wilts caused by the bacteria are among the most important bacterial diseases of plants.

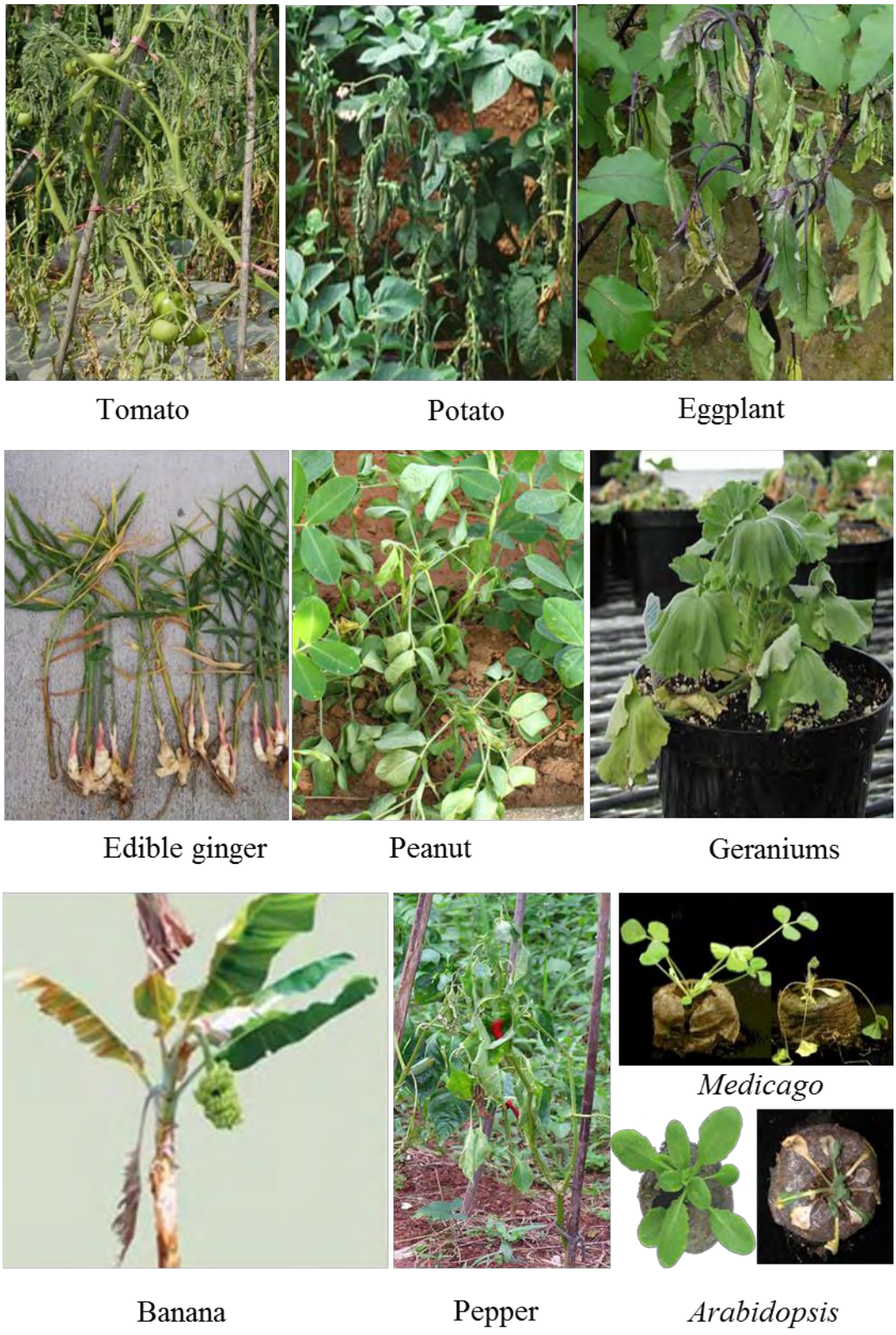



**Figure C1-12**. Bacterial wilt symptoms in plant caused by *R. solanacearum*.

**Biology**

The biology of *R. solanacearum* is adapted to environmental conditions. *R. solanacearum* can survive for years in most soils (Van Overbeek *et al.*, 2004; Alvarez, López e Biosca, 2008). When susceptible host are present, bacteria enter roots, invade xylem vessels and then spread rapidly to aerial parts of the plant through vasculature before causing plant death and returning to soil (Denny, 2006). Genomes of many strains have been sequenced. *R. solanacearum* genome is organized in two circular replicons called the chromosome and the megaplasmid. The chromosome sequence is rather well conserved between races, whereas the megaplasmid sequence is more variable. The megaplasmid carries most functions involved in adaptation to the environment or in pathogenicity. Among those, the type III and IV protein secretion systems, flagellar motility determinants, genes involved in chemotaxis and the extracellular polysaccharide (EPS) cluster (Genin e Denny, 2012).

## 1.6.2 Virulence determinants

*R. solanacearum* virulence factors enhance its ability to cause disease. Many gene products are required by *R. solanacearum* for successful infection of its hosts. Virulence factors include various plant cell-wall-degrading enzymes exported by the type II secretion pathway, and their expression is controlled by a complex virulence network (Schell, 2000; Genin e Boucher, 2002). Bacteria are able to overcome stressful conditions encountered within infected plants (reactive oxygen species, toxic compounds, low oxygen, iron depletion…) by expressing, in planta, several genes that promote stress tolerance during pathogenesis (Lavie *et al.*, 2002; Bhatt e Denny, 2004; Brown e Allen, 2004; Brown, Swanson e Allen, 2007; Colburn-Clifford e Allen, 2010; Flores-Cruz e Allen, 2011). Cytokinin, one of the phytohormones produced by *R. solanaceraum* has also been reported to participate to virulence (Delaspre *et al.*, 2007). However, two main determinants of virulence are EPS and the so-called hypersensitive response and pathogenicity (*hrp*) genes. EPS are produced both in culture and *in planta*. Accumulation of EPS is largely responsible for the vascular dysfunction that causes wilt symptoms on susceptible hosts. The *R. solanacearum hrp* genes, required to set up a functional type III Secretion System (T3SS) are necessary for disease development in susceptible plants and for elicitation of the plant

response in resistant plants. These genes have been grouped in 3 classes. The first class includes highly conserved genes among diverse animal and plant pathogenic bacteria and are named hrc (hrp-conserved). The second class contains transcriptional regulators of T3SS regulon genes, whereas the third one includes structural proteins and some secreted proteins like chaperones or other post-transcriptional regulatory proteins. T3SS allows delivery within plant cells of a battery of proteins called type III effector proteins known to collectively suppress plant defense and to favor bacterial multiplication and nutrition (Galán e Collmer, 1999; Tosi *et al.*, 2013). Studies performed in the sequenced strain GMI1000 have identified, to date, more than 40 T3E proteins transiting through this pathway and the number of potential substrates is estimated to be approximately 75 (Mukaihara e Tamura, 2009; Poueymiro e Genin, 2009). In addition, some T3E have been shown to trigger incompatibility on resistant hosts (AvrA on Tobacco, Pop P1 on petunia, and Pop P2 on *A. thaliana*) (Carney e Denny, 1990; Deslandes *et al.*, 2003; Poueymiro *et al.*, 2009).

### 1.6.3 Control of disease development

Means to control wilt disease caused by the soil-borne bacteria *R. solanacearum* are limited. Use of biological control agent (BCA) for protection has been evaluated with some promising success. In this context, *hrp* mutant strains able to colonize tomato plants without causing disease symptoms have been tested for their protective effect (Trigalet e Demery, 1986). The authors showed that root pre-inoculation with a *hrp* mutant leads to high protection rates against a subsequent inoculation with virulent strains (Trigalet e Trigaletdemery, 1990; Hanemian *et al.*, 2013). Furthermore, this strategy provided a durable protection by persisting several months within the plant without affecting fruit number and weight (Frey *et al.*, 1994). Protection was also achieved in the model plant *A. thaliana* using a similar approach (Feng *et al.*, 2012). Calcium levels were also shown to modulate disease severity: a decreased susceptibilty was associated to higher calcium concentrations (Jiang *et al.*, 2013). Some beneficial microorganisms have also potential to control bacterial wilt disease in tomato. The colonization of *P. syringae* fluorescence FPT9601-T5, a commercial plant-promoting rhizobacteria (PGPR), suppresses bacterial wilt disease. It was proposed recently that the activation of SA-dependent signaling

pathway and the suppression of JA-dependent signaling pathway seem to play key roles in *B. thuringiensis*-induced resistance to *R. solanacearum* in tomato plants (Takahashi *et al.*, 2013).

Specific resistance to several races of *R. solanacearum* was observed in some model plants as *A. thaliana* and *Medicago truncatula*, which are used to decipher the molecular mechanisms underlying resistance (Deslandes *et al.*, 2002; Vailleau *et al.*, 2007). The *A. thaliana* / *R. solanacearum* pathosystem is described in more details thereafter.

### 1.6.4 Identification of an *A. thaliana* ecotype resistant to *R. solanacearum* GMI1000

#### RRS1-R, an atypical TIR-NBS-LRR protein

The interaction between *R. solanacearum GMI1000*, a wide host range strain originally isolated from tomato in French Guyana, and the model plant A. thaliana has begun to be studied about 15 years ago. Resistant and susceptible ecotypes of *A. thaliana* to some specific strains of *R.solanacearum* were identified (Deslandes *et al.*, 1998; Yang e Ho, 1998). From the work of Deslandes and collaborators a resistance gene named RRS1-R was isolated from the resistant ecotype Niederzens (Nd-1), and the allelic gene (*RRS1-S*) found in the susceptible ecotype Colombia (Col-0) was also characterized. The nucleotide sequence indicates a high level of identify (98%) between the RRS1-R and RRS1-S proteins. Despite this overall conserved organization, the two genes differ in the position of a stop codon that leads in RRS1-S to a protein truncated by 90 amino acids. The *RRS1-R* gene encodes a protein whose structure combines the TIR-NBS-LRR domains found in several resistance proteins and a WRKY motif found in a family of transcription factors (Figure C1-13). Basically, the carboxy-terminal LRR domain is involved in protein–protein interaction and typically confers effector recognition specificity (Farnham e Baulcombe, 2006; Sela *et al.*, 2012). NLR activation after effector recognition requires nucleotide exchange at the NBS domain (Lukasik e Takken, 2009). In the “off” state, the NBS domain adopts a “closed” structure where ADP is preferentially bound and coordinates intermolecular interactions to stabilize this structure. Activation is thought to require release of the ADP to be replaced by ATP and adoption of an “open” structure.

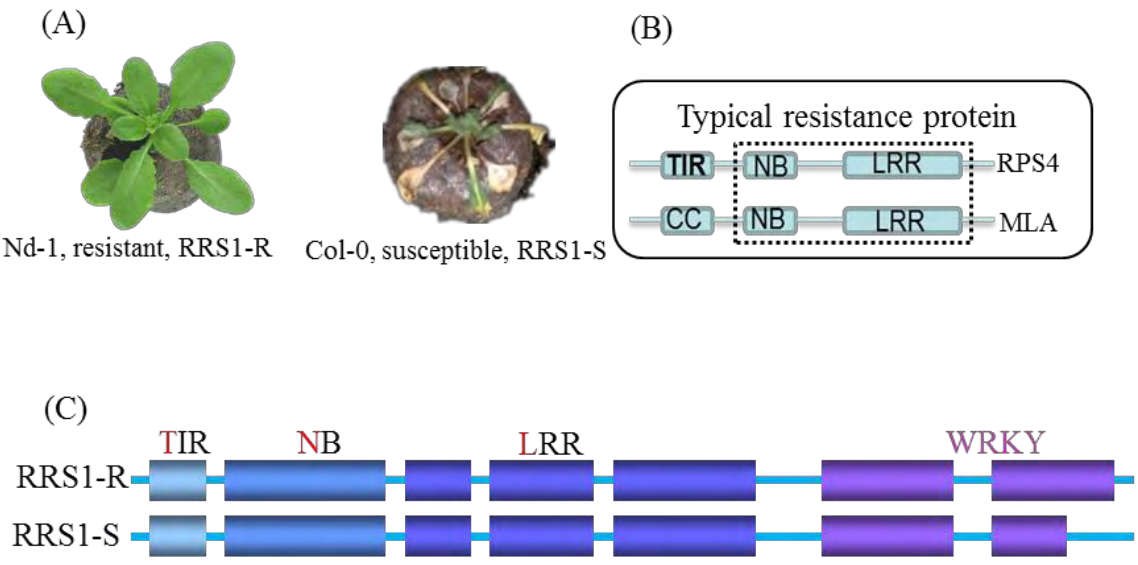



**Figure C1-13.** Schematic representation of the RRS1-R and RRS1-S genes in accessions Col-0 and Nd-1.

(A) The response to strain GMI1000 of both ecotypes. (B) Schematic structures of typical NB-LRR resistance protein without WRKY transcription factor (TFs) domain. (C) RRS1-R is an atypical resistance protein including a TIR (The Toll/interleukin-1 receptor), NB (nucleotide binding), LRR (Leucine-rich repeat), nuclear localization signal (NLS), and a WRKY domain.

This structural change is then thought to promote homo-oligomerization via the NBS domain, which in turn enables the N-terminal domains to engage in downstream signaling (Qi and Innes, 2013). More recently, the identification of *RRS1* and *RPS4* as dual genes involved in the resistance of several pathogens among which *R. solanacearum* (Narusaka *et al.*, 2009), underlines the importance of the *RRS1* genes in resistance to pythopathogens and potentially links molecular data described on RRS1- and RPS4-mediated resistances to *R. solanacearum* and *P. syringae* respectively. Recently, heterodimerization of the TIR domains of RRS1 and RPS4 was demonstrated that could plays a role in receptor activation after perception of the effector. (Williams et al., XV Congress on Molecular Plant-Microbe Interactions, Kyoto, 2011, Japan). Following this activation step the WRKY domain of RRS1 could then take part in gene reprogramming. Although genetically defined as a recessive allele, RRS1-R behaves as a dominant resistance gene in transgenic plants. Additionally, RRS1-mediated resistance is salicylic-, NDR1- and EDS1 dependent (Deslandes *et al.*, 2002).

### 1.6.5 Pop P2 an avirulence protein of *R. solanacearum* GMI1000 strain involved in the resistance mediated by RRS1-R.

Pop P2 is an *R. solanacearum GMI000* strain T3E that belongs to the *YopJ/Avrxv* family (Figure C1-14) (Staskawicz *et al.*, 2001; Orth, 2002). It was recently shown to have an acetyl tranferase activity (Tasset *et al.*, 2010) and to interact with multiple plant targets (Bernoux, Deslandes and collaborators, unpublished results). It interacts directly with the resistance protein RRS1-R as well as with the RRS1-S protein (Deslandes *et al.*, 2003). This ability to interact with thr R protein interaction is with Pop P1, another *YopJ/Avrxv* protein that confers avirulence in petunia (Lavie *et al.*, 2002). It was further demonstrated that both PopP2 and RRS1 proteins colocalize and directly interact in the plant cell nucleus (Deslandes *et al.*, 2003). Furthermore it was proposed that Pop P2 is required for bacterial fitness on host plants such as eggplant or bean (Macho *et al.*, 2010).

### 1.6.6 Transcriptional reprogramming in response to *R. solanacearum*.

Transcriptomic analyses from tomato stems after *R. solanacearum* inoculation of strain *8107S* (race 1, biovar 4, phylotype 1) have been performed. Gene expression profiles at

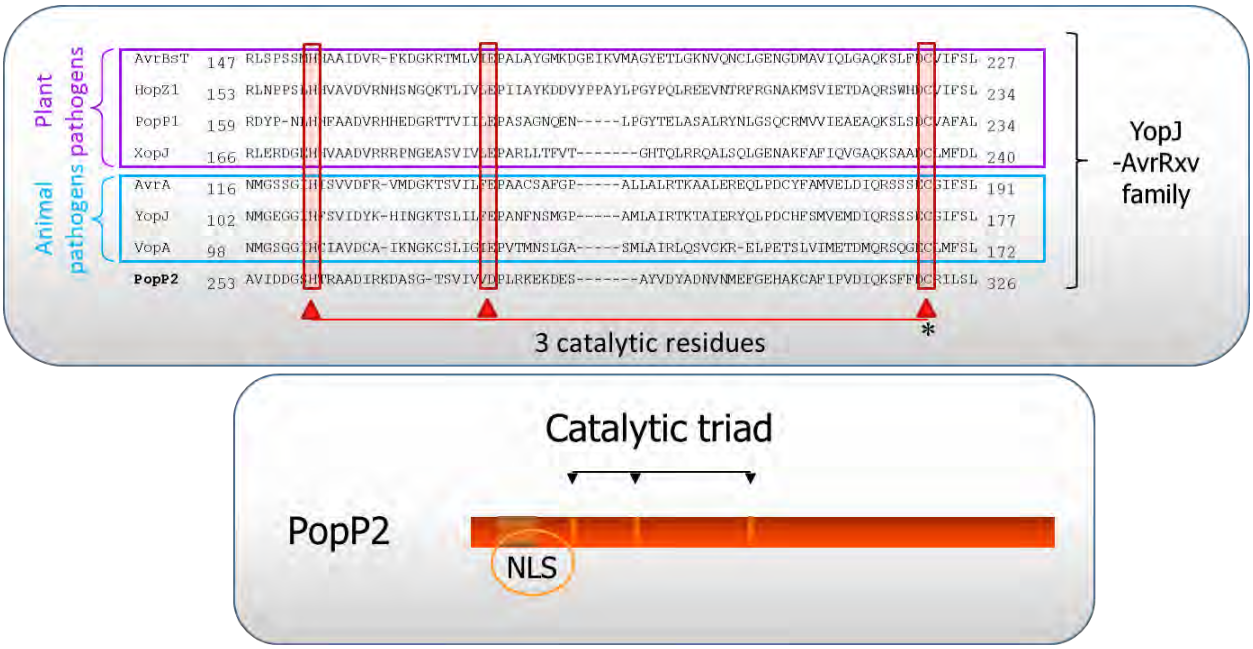



**Figure C1-14.** Sequence alignment of different members of the YopJ/AvrRxv effector family from plant and animal bacterial pathogens. The conserved residues in the catalytic core (H, D/E, and C) are highlighted (red boxes). The star indicates the position of the main cysteine catalytic residue. The accession numbers for the proteins are: *Xanthomonas campestris* pv. *vesicatoria* AvrBsT (AAD39255); *Pseudomonas syringae* pv. *syringae* HopZ2 (ABK13722); *R. solanacearum* PopP1 (CAF32331) and PopP2 (CAD14570); *Xanthomonas campestris* pv. *vesicatoria* XopJ (YP_363887); *Salmonella enterica* AvrA (AAB83970); *Yersinia pestis* YopJ (NP_395205); and *Vibrio parahaemolyticus* VopA (AAT08443). PopP2 autoacetylation is essential for RRS1-R mediated immunity in *Arabidopsis*.

1dpi were analyzed using an Affymetrix Tomato Genome ArrayGeneChip representing over 9,200 tomato genes. Results for resistant cultivar and susceptible cultivar 1 day after stem inoculation showed no change in gene expression was, but expression levels of over 140 genes, including pathogenesis-related, genes involved in hormone signaling and in lignin biosynthesis, increased in a resistant cultivar. (Ishihara *et al.*, 2012).

ATH1 Microarray were used in order to measure transcriptional regulations in susceptible or resistant ecotypes of *A. thaliana* in response to strain GMI1000 of *R. solanacearum*. Gene expression was marginally affected in leaves during the early stages of infection. Major changes in transcript levels occured between 4 and 5 days after pathogen inoculation, at the onset of appearance of wilt symptoms. Up-regulated genes in diseased plants included abscisic acid (ABA)-, senescence- and basal resistance-associated genes (Hu *et al.*, 2008). Comparative transcriptomic analyses between mutant plants showing an increased resistance to virulent bateria compared to susceptible wild type plants were also performed and allowed the identification of a set of up-regulated genes, including a number of ABA–responsive, defense related genes encoding antibiotic peptides and enzymes involved in the synthesis and activation of antimicrobial secondary metabolites (Hernandez-Blanco *et al.*, 2007). These data as well as the increased susceptibility of some ABA mutants (*abi1-1*, *abi2-1*, and *aba1-6*) to *R. solanacearum* support a direct role of ABA in resistance to this pathogen. Using CATMA arrays, another study considering roots and leaves separately was performed by Denance et al (Denance *et al.*, 2013). By combining transcriptomic and metabolomic data, they demonstrated a general repression of indole metabolism in the roots of a cell wall mutant, *wat1-1*, that was correlated with a decreased susceptibility to a virulent strain of *R. solanacearum*.

Transcriptomic analyses were also performed following induced resistance in susceptible plants, through the inoculation of *hrp* mutant bacteria, prior to inoculation with virulent bacteria (Feng *et al.*, 2012). A high proportion of genes differentially regulated in plants that were protected against virulent bacteria were related to abscisic acid-associated pathways.

Altogether, all these analyses revealed that a high number of plant genes (1352 genes called thereafter "genes responsive to *R. solanacearum"*) are found to be significatively deregulated in several transcriptomic analyses following plant interaction with the pathogenic bacteria. . It also appeared that phytohormones involved in plant development such as ABA and auxin play a major role in the establishment of the plant response to *R. solanacearum*. Genetic data confirmed these observations. ,

In the context of ETI driven by PopP2/RRS1-R partners, it is hypothesized that part of this transcriptional regulation results from the functionality of the WRKY domain of RRS1.

## 1.7 Objectives of the Thesis

The major objective of this thesis concerns the identification of RRS1-R and RRS1-S primary targets. These genes should constitute important clues for the establishment of the appropriate plant response during the interaction with pathogenic *R. solanacearum* bacteria. The involvement of these genes in plant resistance or susceptibility can then be tested by genetic approaches. This study is aimed at a better understanding of the initial signaling steps following pathogen perception, that trigger specific resistance.

A highly sensitive approach, namely DNA adenine methyltransferase identification (DamID), was therefore developed to identify *in vivo* binding sites of RRS1-R and RRS1-S proteins. This approach is based on the covalent linking of a "fingerprint" in the vicinity of the DNA-binding sites of the protein of interest. The fingerprints can be further mapped by simple molecular approaches. First developed in *Drosophila melanogaster* (Van Steensel e Henikoff, 2000), DamID was successfully adapted to *A. thaliana,* and its feasibility demonstrated by using the well-known yeast GAL4 transcription factor (Germann *et al.*, 2006). The method was further used to establish a genome-wide map of the target sites of LHP1, a regulatory chromatin protein in *A. thaliana* (Zhang, Xiaoyu *et al.*, 2007). This approach constitutes an alternative to chromatin immunoprecipitation (ChIP) with a higher sensitivity and presents the main advantage of detecting transitory associations to DNA.

# Chapter II

## RRS1 expression profiles and binding to W box *cis* elements

## 2.1 Introduction

In order to define optimal conditions for target gene identification, it was important to focus on plant tissues supporting *RRS1-R* and *RRS1-S* gene expression. Expression profiles of these genes were unknown, since most of the experiments used to decipher the complex mechanisms underlying resistance or susceptibility were conducted in transitory expression systems (*Nicotiana benthamiana* or *A. thaliana* leaves). More generally, regulation of NB-LRR resistance gene expression is not well documented at the transcriptional level, which is however a preliminary step in the elucidation of the regulation of protein function. Information on the pattern of expression of resistance genes should provide clues about their roles in plants and during bacterial infection.

Similarly to WRKY transcription factors, RRS1-R and RRS1-S proteins fulfill potential functions in the regulation of gene expression. The WRKY transcription factor family is among the ten largest families of transcription factors in higher plants (Ulker e Somssich, 2004). WRKY transcription factor family consists of 74 members in *A. thaliana* (Eulgem *et al.*, 2000). The family has expanded during the evolution of plants. This expansion is likely to be associated to defense mechanisms co-evolving in land plants together with their adapted pathogens. Recent studies suggest that these transcription factors have evolutionary links with transposons such as mutator elements and could have originated from an atypical boundary element domain found in BEAF and DREF proteins, that play a role in gene regulation and are potentially linked to nuclear organization, and in transposases from animals (Pandey e Somssich, 2009). The first two reports on WRKY proteins defined DNA binding proteins that played potential roles in the regulation of gene expression by sucrose (SPF1) (Ishiguro e Nakamura, 1994) or during germination (ABF1 and ABF2) (Rushton *et al.*, 1995). A third report identified WRKY1, WRKY2 and WRKY3 from parsley (*Petroselinum crispum*) and gave the name WRKY to this family (Rushton *et al.*, 1996). This work also provided the first evidence that WRKY proteins play roles in regulating plant responses to pathogens, and many reports have confirmed this observation (Eulgem e Somssich, 2007). WRKY proteins are important regulators of plant disease resistance toward biotrophic pathogens. For example, disruptions of WRKY40 or WRKY60 show enhanced resistance against *P. syringae* and *Golovinomyces orontii* (Xu

*et al.*, 2006; Shen *et al.*, 2007). Likewise, WRKY11 and WRKY17 also function as negative regulators of plant resistance against *P. syringae* (Journot-Catalino *et al.*, 2006). A recent study suggests that WRKY51 may, on the contrary, function as a positive regulator of basal defense against *P. syringae* (Gao *et al.*, 2011). Moreover, Hwang et al. showed that heterologous expression of OsWRKY6 gene in *A. thaliana* enhanced disease resistance to *X. campestris pv. Campestris* (Hwang, Yie e Hwang, 2011). Recent studies also showed that AtWRKY46, AtWRKY70 and AtWRKY53 positively regulate basal resistance to *P. syringae*, and they play overlapping and synergistic roles in basal defense (Hu, Dong e Yu, 2012). In addition, WRKY25 and WRKY72 were also shown as regulators in the response to *P. syringae* pv. *maculicola* strain ES4326 and *Hyaloperonospora arabidopsidis* (Zheng *et al.*, 2007; Bhattarai *et al.*, 2010).

Taking together, current results suggest that WRKY TFs in plants act in a complex defense response network both as positive and negative regulators (Eulgem e Somssich, 2007). Furthermore recent data indicate that a single WRKY transcription factor regulates commonly transcriptional reprogramming associated with multiple plant programs. (Rushton *et al.*, 2010).

The WRKY domain (about 60 residues in length), corresponding to the conserved DNA binding domain of this protein family (Figure C2-1) (Rushton *et al.*, 1996; Eulgem *et al.*, 2000), contains the WRKY signature and also has an atypical zinc-finger structure at the C-terminus. Phylogenetic data show that the WRKY family in higher plants is divided into several Groups (I, IIa + IIb, IIc, IId + IIe, and III) (Figure C2-2) (Zhang e Wang, 2005). These transcription factors bind to the DNA element termed W box (T/CTGACT/C). Indeed, gel shift experiments, random binding site selection, yeast one-hybrid screens and co-transfection assays performed with many different WRKY proteins have shown that the W box is the minimal consensus sequence required for specific DNA binding (Rushton *et al.*, 1996; Ciolkowski *et al.*, 2008).

Transcriptional regulation of WRKY genes is well documented and many of them are regulated in response to abiotic or biotic stresses. Data concerning WRKY *A. thaliana* gene expression are summarized in the Table C2-1. However, due to its very specific structure,

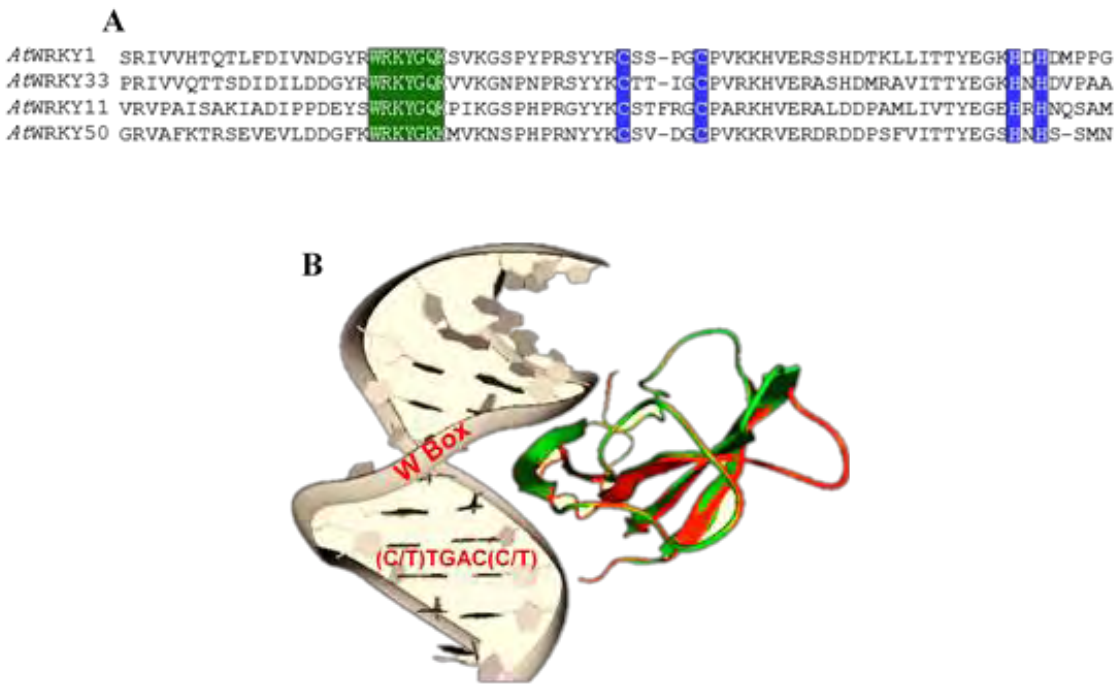



**Figure C2-1**. Homology models of *At*WRKY DNA-binding domain. (A) The WRKY domain consensus for each WRKY subfamily in higher plants. Each consensus sequence using WRKY domains comes from Arabidopsis thaliana. The WRKY motif is highlighted in green and the cysteines and histidines that form the zinc finger are shown in blue. (B) The overlay of the protein-DNA models of AtWRKY33 DNA-binding domain (green) is displayed. W Boxes are defined as elicitor-responsive elements (C/T) TGAC (C/T).

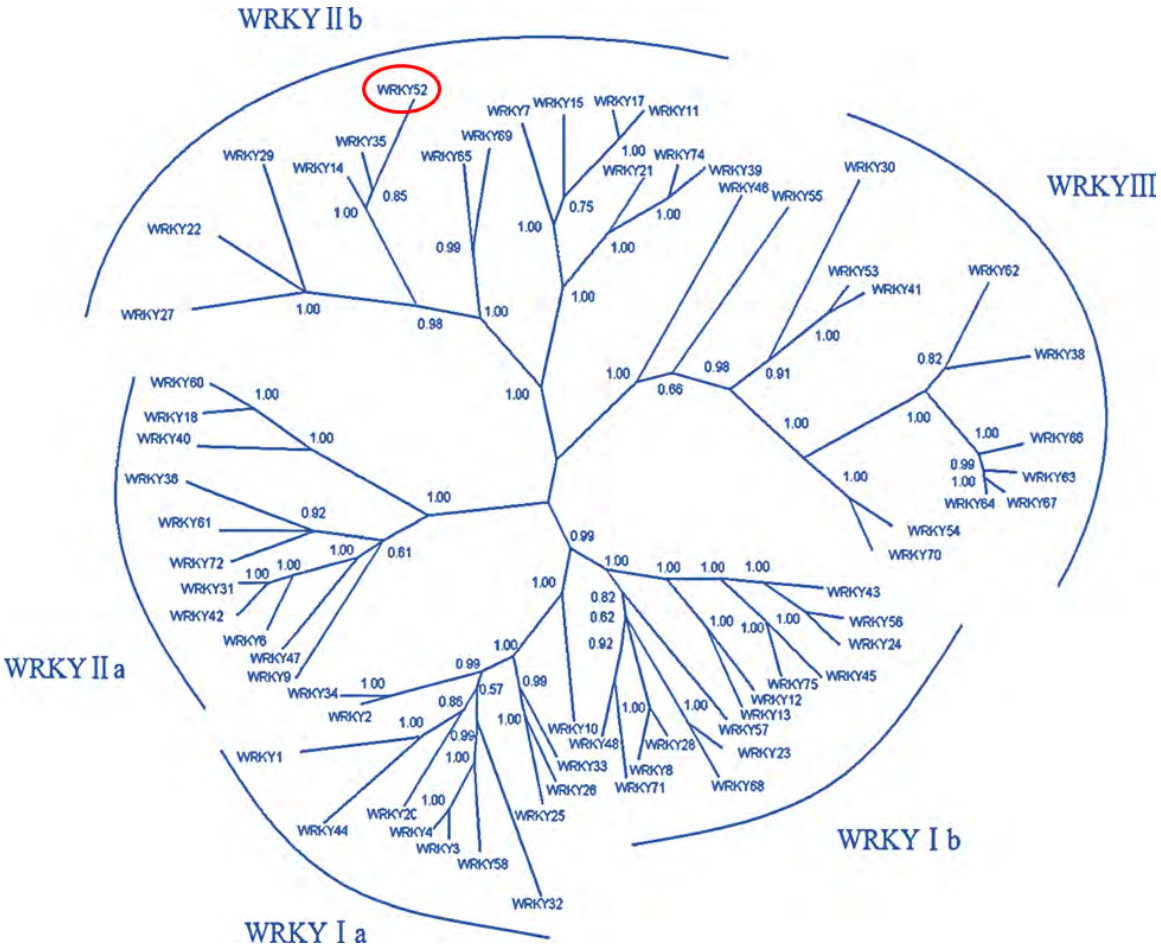



**Figure C2-2**. Phylogenetic tree of the *At*WRKY family in *A. thaliana*. Phylogenetic tree based on the nucleotide sequence data. The numbers indicate the Bayesian probabilities for each phylogenetic clade. The gene corresponding to *RRS1* (WRKY52) is circled. (Adapted from Wang *et al*, 2011).

**Table C2-1.** WRKY gene expression in *Arabidopsis thaliana*.

| Gene | Locus | Spatial Expression | Repressed by | Induced by |
|---|---|---|---|---|
| *AtWRKY1; ZAP1* | At2g04880 | Root, flower, leaf, stem, siliques | | SA |
| *AtWRKY3* | At2g03340 | | | SA, pathogen (incompatible P. syringae) |
| *AtWRKY4* | At1g13960 | | | JA, SA, sucrose, senescence, cold, salinity, pathogen (incompatible P. syringae, B. cinerea) |
| *AtWRKY6* | At1g62300 | | AtWRKY53 knockout | Senescence, pathogen (bacteria, oomycetes, fungi, virus), herbivory (B. brassicae), H2O2, methyl viologen, high light in CATdeficient mutants |
| *AtWRKY7* | At4g24240 | | | Senescence |
| *AtWRKY8* | At5g46350 | | | Pathogen (harpin, P. syringae) |
| *AtWRKY9* | At1g68150 | | | Pathogen (LPS, harpin) |
| *AtWRKY10; MINISEED3* | At1g55600 | Floral buds, pollen grains, pollen tubes, ovules, developing seeds | Pathogen (Pseudomonas fluorescens WCS417r) | |
| *AtWRKY11* | At4g31550 | | | β-aminobutyric acid in Col-0, pathogen (chitin), methyl viologe |
| *AtWRKY15* | At2g23320 | | | NO, pathogen (chitin), herbivory (B. brassicae), β-minobutyric acid in Col-0, AtWRKY53 knockout |
| *AtWRKY17* | At2g24570 | | | Pathogen (chitin) |
| *AtWRKY18* | At4g31800 | | AtWRKY53 knockout | SA, pathogen (chitin), herbivory (B. brassicae), β-aminobutyric acid in Col-0 |
| *AtWRKY22* | At4g01250 | | AtWRKY53 knockout | SA, wounding, pathogen (P. syringae, chitin, flagellin, harpin, elf18), methyl viologen |
| *AtWRKY23* | At2g47260 | | | Auxin, nematode infection |
| *AtWRKY25* | At2g30250 | Roots | ABA (24h), JA, cold (6h) | SA, ethylene, NO, NaCl, mannitol, cold (24h), heat stress, pathogen (harpin, P. syringae, herbivory (B. brassicae), β-aminobutyric acid in Col-0 |
| *AtWRKY26* | At5g07100 | | | Herbivory (B. brassicae) |
| *AtWRKY28* | At4g18170 | | | Methyl viologen |
| *AtWRKY29* | At4g23550 | | AtWRKY53 knockout | Pathogen (P. syringae, chitin, flagellin, harpin, elf18) |
| *AtWRKY30* | At5g24110 | | | Herbivory (B. brassicae), β-aminobutyric acid in Col-0 & ABA1/NPQ2 mutant, methyl viologen, H2O2 |
| *AtWRKY31* | At4g22070 | | | |

| | | | | |
|---|---|---|---|---|
| *AtWRKY32; PcWRKY1-similar* | At4g30935 | | | |
| *AtWRKY33* | At2g38470 | Mature leaves, roots, shoots, inflorescences | Heat | SA, INA, BTH, NaCl, mannitol, cold, H2O2, ozone oxidative stress, UV, cycloheximide, wounding, pathogen (P. syringae, chitin, flagellin, harpin), herbivory (B. brassicae), MBF1coverexpression (enhanced thermotolerance), β-aminobutyric acid in Col-0, methyl viologen |
| *AtWRKY34* | At4g26440 | | | Sucrose, β-aminobutyric acid in Col-0 |
| *AtWRKY38* | At5g22570 | | | SA, herbivory (B. brassicae), pathogen (compatible P. syringae), β-aminobutyric acid in Col-0 |
| *AtWRKY40* | At1g80840 | | | SA, NO, wounding, pathogen (P. syringae, Pseudomonas fluorescens WCS417r, chitin, harpin), herbivory (B. brassicae), MBF1c overexpression (enhanced thermotolerance), β-aminobutyric acid in Col-0 & ABA1/NPQ2 mutant, methyl viologen |
| *AtWRKY41* | At4g11070 | | Pathogen (compatible P. syringae) | Pathogen (incompatible P. syringae, flagellin), β-minobutyric acid in Col-0 |
| *AtWRKY42* | At4g04450 | | AtWRKY53 knockout | |
| *AtWRKY44; AtTTG2* | At2g37260 | Young leaves, trichomes, seed coats, root hairless cells | | |
| *AtWRKY46* | At2g46400 | | | BTH, osmotic stress, potassium starvation, NaCl, herbivory (B. brassicae), MBF1c overexpression (enhanced thermotolerance), β-aminobutyric acid in Col-0 & ABA1/NPQ2 mutant, methyl viologen |
| *AtWRKY47; ABF2-similar* | At4g01720 | | | Senescence, pathogen (bacteria, virus) |
| *AtWRKY48* | At5g49520 | | | Osmotic/mechanical stress, pathogen (P. syringae, chitin, LPS), methyl viologen |
| *AtWRKY50* | At5g26170 | | | Herbivory (B. brassicae), β-aminobutyric acid in Col-0 |

| | | | | |
|---|---|---|---|---|
| *AtWRKY52; AtRRS1-R* | At5g45260 | | | SA, NDR1, pathogen |
| *AtWRKY53* | At4g23810 | | | Senescence, SA, NO, H2O2, wounding, pathogen (P. syringae, chitin, flagellin, harpin), herbivory (B. brassicae), β-aminobutyric acid in Col-0, overexpression of GATA4 |
| *AtWRKY54* | At2g40750 | | β-minobutyric acid in Col-0 | Herbivory (B. brassicae), β-aminobutyric acid in ABA1/NPQ2 mutant |
| *AtWRKY55* | At2g40740 | | | SA, pathogen (P. syringae, flagellin, harpin), β-aminobutyric acid in Col-0 |
| *AtWRKY58* | At3g01080 | | | β-aminobutyric acid in Col-0 |
| *AtWRKY60* | At2g25000 | | | Senescence, wounding, pathogen (virus, oomycetes) |
| *AtWRKY61* | At1g18860 | | Pathogen (Pseudomonas Fluorescens WCS417r), β-aminobutyric acid in ABA1/NPQ2 mutant | |
| *AtWRKY62* | At5g01900 | | *AtWRKY53* knockout | MeJA, SA, pathogen (compatible P. syringae), β-aminobutyric acid in Col-0 |
| *AtWRKY70* | At3g56400 | Leaves, floral abscission zones, flower sepals | heat, darkness, UV-B | Senescence, SA, cycloheximide, pathogen (chitin), herbivory (B. brassicae), β- aminobutyric acid in Col-0, AtWRKY53 knockout |
| *AtWRKY72* | At5g15130 | | | Pathogen (chitin), β-aminobutyric acid in Col-0 & ABA1/NPQ2 mutant |
| *AtWRKY75* | At5g13080 | | Pathogen (Pseudomonas Fluorescens WCS417r) | Pi deprivation, pathogen (harpin), herbivory (B. brassicae); β-aminobutyric acid in Col-0 |

Adapted from Rushton et al, 2010

RRS1, referenced in the WRKY family as WRKY52, a member of Group III, was not, in most studies, included in analyses of whole WRKY gene family.

In this chapter, results concerning "prerequisites", for the *in vivo* target identification, will be presented:

*RRS1-R* and *RRS1-S* transcription profiles were studied in plants and the effect of biotic or abiotic stresses on these profiles was evaluated. It was also necessary to confirm the binding properties of both RRS1-S and RRS1-R proteins to DNA, and this is the second point addressed in this chapter.

## 2.2 Results

### 2.2.1 *RRS1-S* and *RRS1-R* gene expression profiles

#### 2.2.1.1 The two *RRS1* genes have similar expression profiles in plantlets: they are mainly expressed in the stele at the transition zone between hypocotyl and roots

*RRS1-R* and *RRS1-S* promoter sequences were defined as the intergenic region existing between the *RPS4* and *RRS1* neighboring genes, which is a short region of about 250 base pairs. *RRS1-R* and *RRS1-S* promoter sequences showed a high level of similarity but also some interesting differences (Figure C2-3) residing especially in a putative TATA box sequence and a MYB transcription factor binding site. These unusually short length promoters (named P250S and P250R for RRS1-S and RRS1-R respectively) were used to drive expression of a GFP::GUS reporter gene in *A. thaliana*. A longer sized DNA fragment was also chosen for comparison (3kb, named P3000S and P3000R). The promoter activity of the *RRS1* genes can be deduced from a GFP::GUS reporter gene profiling (Karimi *et al.*, 2002). These promoters were first tested in transient expression in *Nicotiana benthamiana* (*N. benthamiana*) leaves. GFP and GUS expression was detected for all tested contructs (not shown). Expression of the reporter gene was then analyzed in Col-0 (susceptible ecotype), Nd-1 (resistant ecotype) or in a resistant transgenic line containing *RRS1*-R in a Col-0 genetic background (CH1-2). Altogether, about 80 transgenic lines were generated and 10 to 15 days old, *in vitro* grown, T2 plantlets analyzed. In these plants,

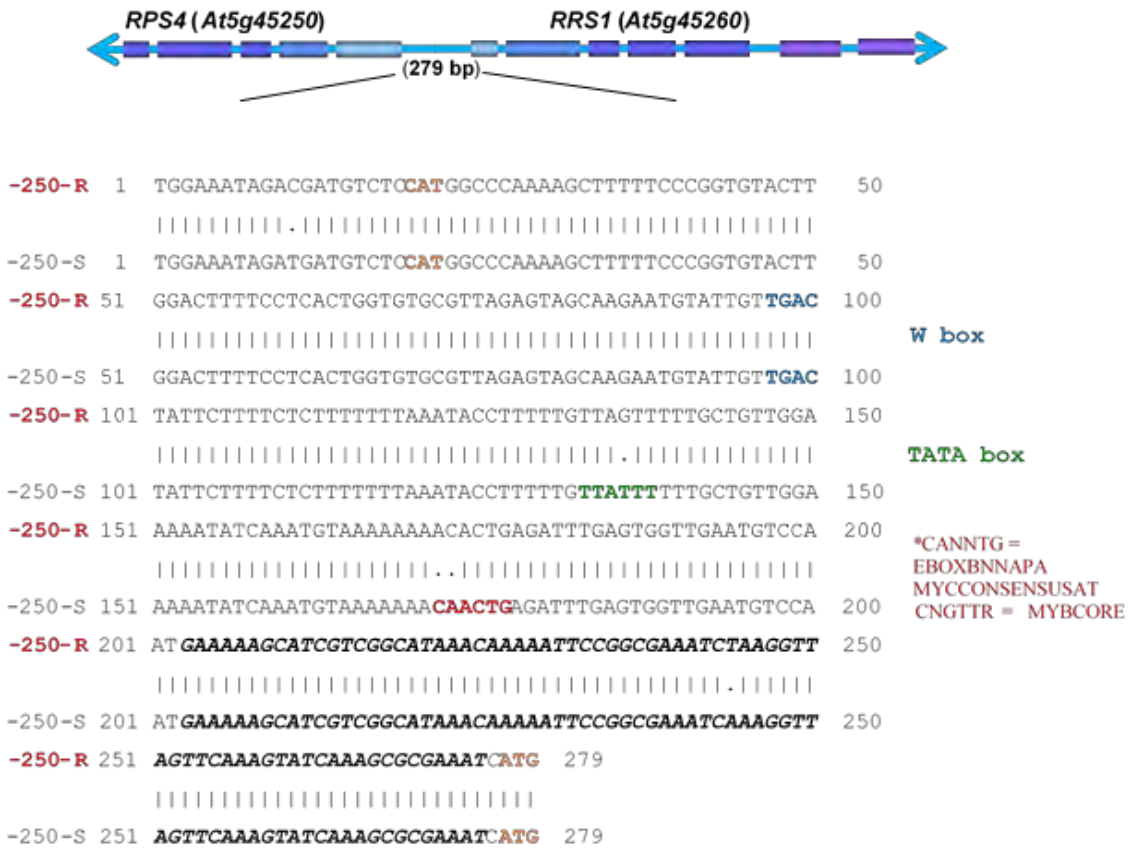



**Figure C2-3**. Promoter structure of *RPS4* and *RRS1* R-gene pairs. The orientation of the genes is indicated by arrows. The distance between start codons is indicated in brackets. A few differences observed in the two sequences from Nd-1 (upper line) and Col-0 (lower line) are marked with a dot. ATG codon are in brown letters. Some *cis* elements are highlighted with colors: W-box in blue, TATA box and MYB core binding sequences are in green and red respectively.

expression of the GFP gene was not detectable, suggesting that these promoters conducted a low level of transcription. Expression of the GUS reporter gene was systematically detected in vasculature, in the older part of roots, in collar and in basal hypocotyl region (Figure C2-4). RRS1 expression was sometime detected outside the vascular system, in all root cell layers, but to a lower level. Expression was also sporadically (sometimes) detected in veins of cotyledons and young leaves as well as in hydatodes. Patterns were similar for all promoters tested in the three genetic backgrounds. In parallel, the level of RRS1 messenger was quantified in Col 0 and Nd-1 plantlets by performing qRT-PCR on aerial parts or roots (Figure C2-5). Results support a higher level of RRS1 gene expression in roots than in aerial part of plantlets. We decided to focus our study on roots, since *R. solanacearum* is a soil borne pathogen. Basal region of hypocotyls were also included in our analysis. Transcriptional activity was observed in pericycle cells and in two endodermal cells located at the protoxylem poles on transversal sections performed in hypocotyls and roots. These results are illustrated in Figure C2-6, Figure C2-7 and Figure C2-8.

Reporter gene expression was followed in roots from adult plants, grown in soil. Expression was detected in many locations, in older parts of roots, very often at lateral root branching points. However this expression was not homogenous in all the root system and no obvious pattern of expression could be drawn from our observations. Transversal sections indicate that, as in plantlets, the reporter gene was mainly expressed in pericycle cells. These observations were similar with all promoters in all genetic backgrounds (Figure C2-9).

#### 2.2.1.2 *RRS1* gene expression pattern may depend on environmental growth conditions

GUS expression was monitored in adult plants subjected to water deprivation for several days. Plants did not exhibit wilting symptoms, although the soil was dried out. Under such conditions, reporter gene expression was completely repressed in roots, in all the lines we tested (Figure C2-10).

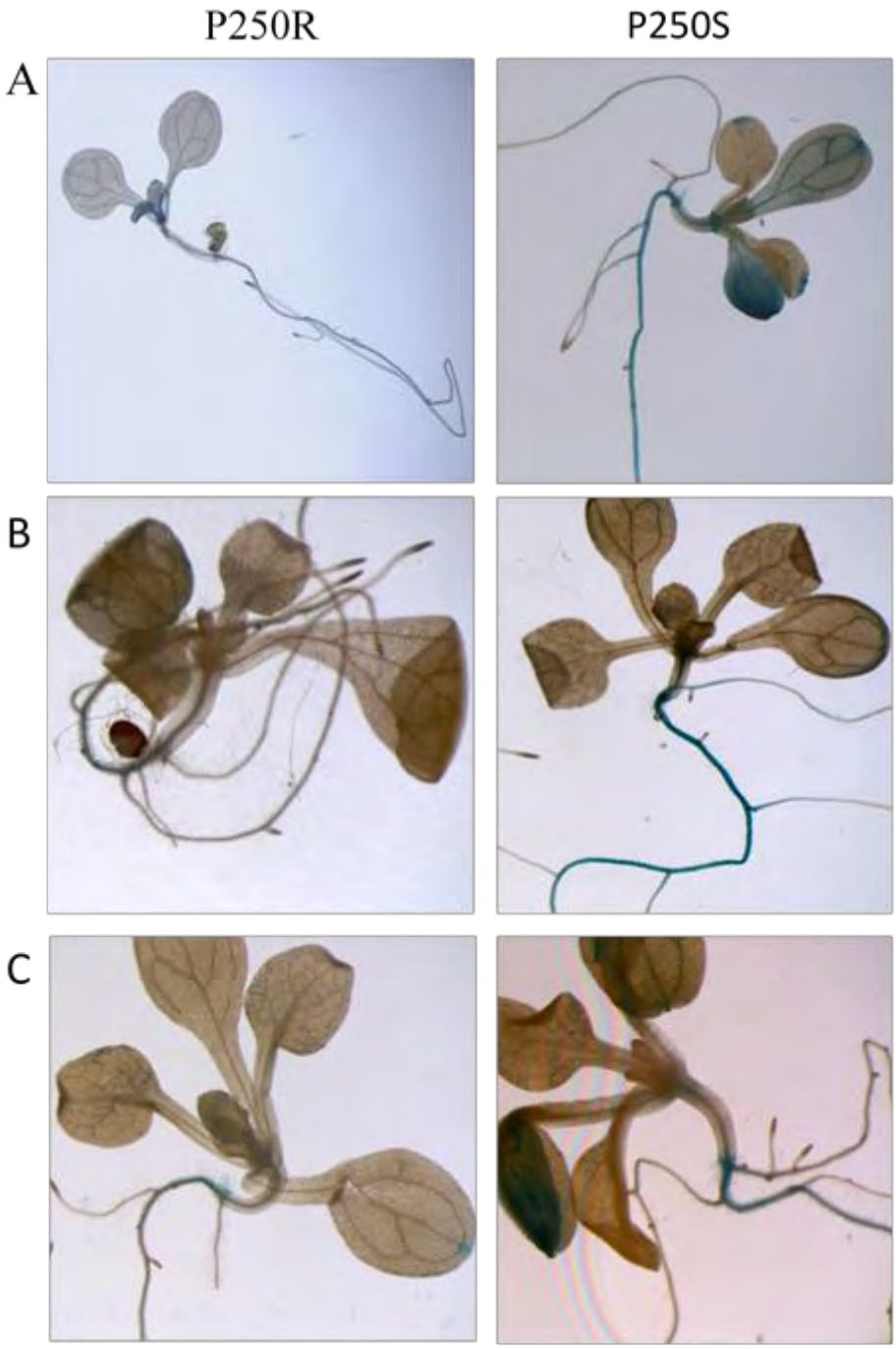



**Figure C2-4.** GUS reporter gene expression in *A.thaliana* plantlets. Promoter used to drive GUS transcription are indicated above each column: (P250 S): RRS1-S gene promoter. (P250 R): RRS1-R gene promoter. Genetic background correspond to: (A) Nd1, Niderzenz-1 (B) Col 0 Colombia-0, (C) CH1-2 transgenic Col 0 containing the RPS4-RRS1 locus from Nd1. Plants were grown 10 to 15 days in vitro.

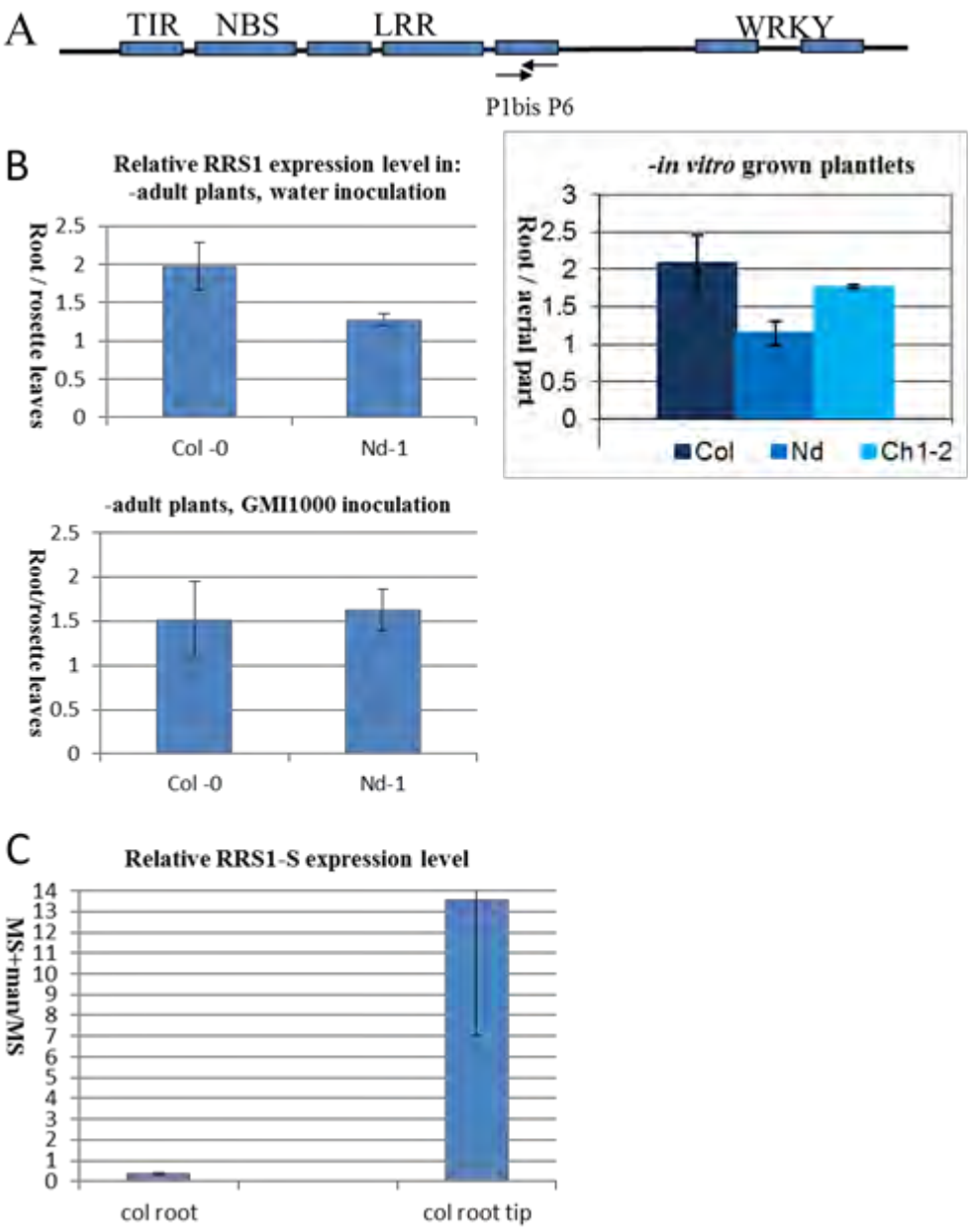



**Figure C2-5.** Relative RRS1 expression level in plants. A: Position of primers used in QRT-PCR experiments on the *RRS1* gene. B: Results were obtained from two independant experiments by using P1bis/P6 primer pair for PCR; Material from adults plants was harvested 3 days post inoculation. Plantlets were grown 10 days on Ms medium. C: 7 days old Col-o plantlets grown on MS medium were transfered on MS+ 200mM mannitol for 24H before RNA extraction from roots without tips or root tips (about 100 root tips from the differentiation zone).

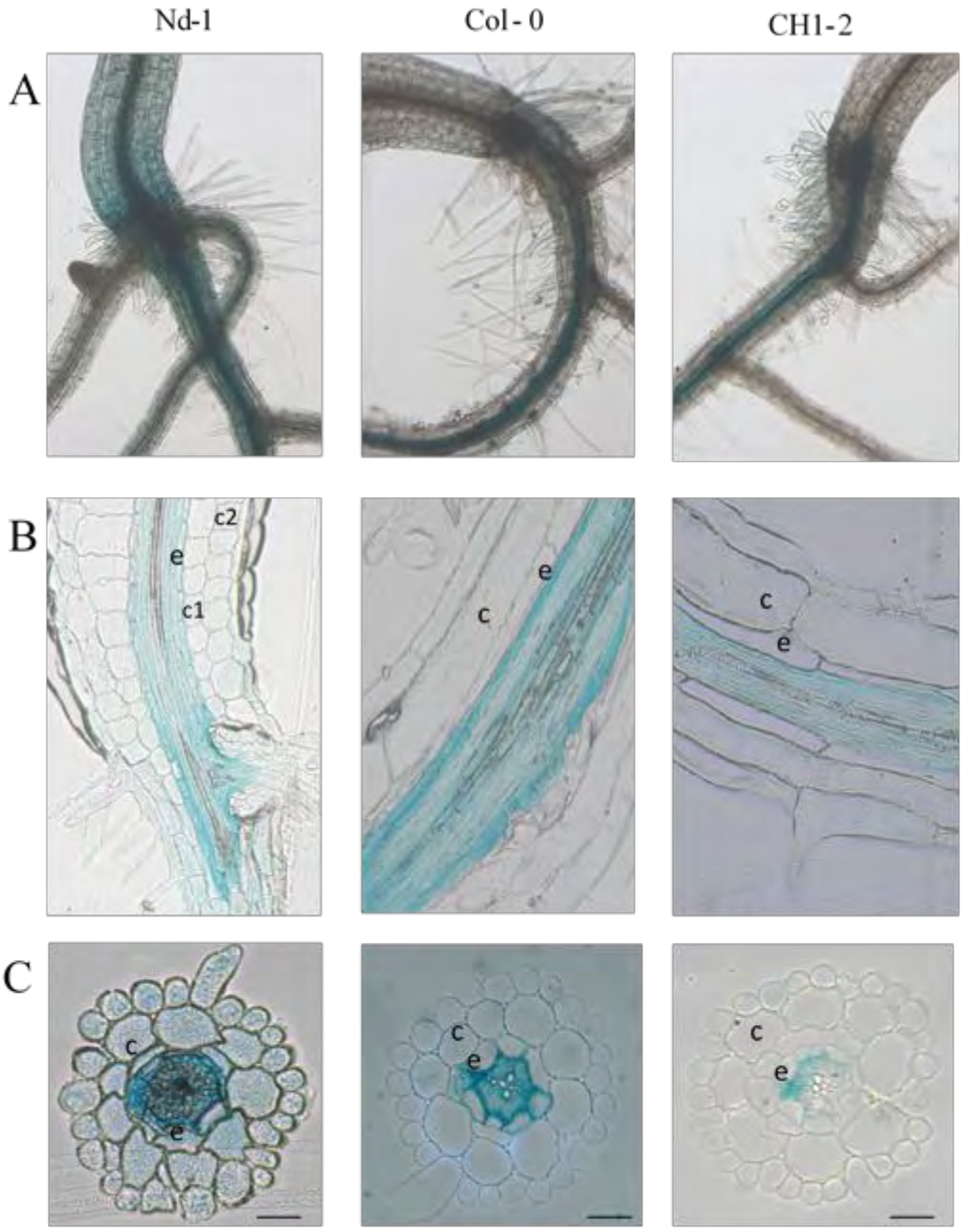



**Figure C2-6.** *P250R:GUS* expression in the transition zone from basal hypocotyl to roots. 1st column: Nd-1 background, 2nd column Col-0 background, 3rd column CH1-2 background. A: whole plantlets. B: longitudinal section. C: transversal section. e: endodermis. c: cortex. Size bars (C) 20µM.

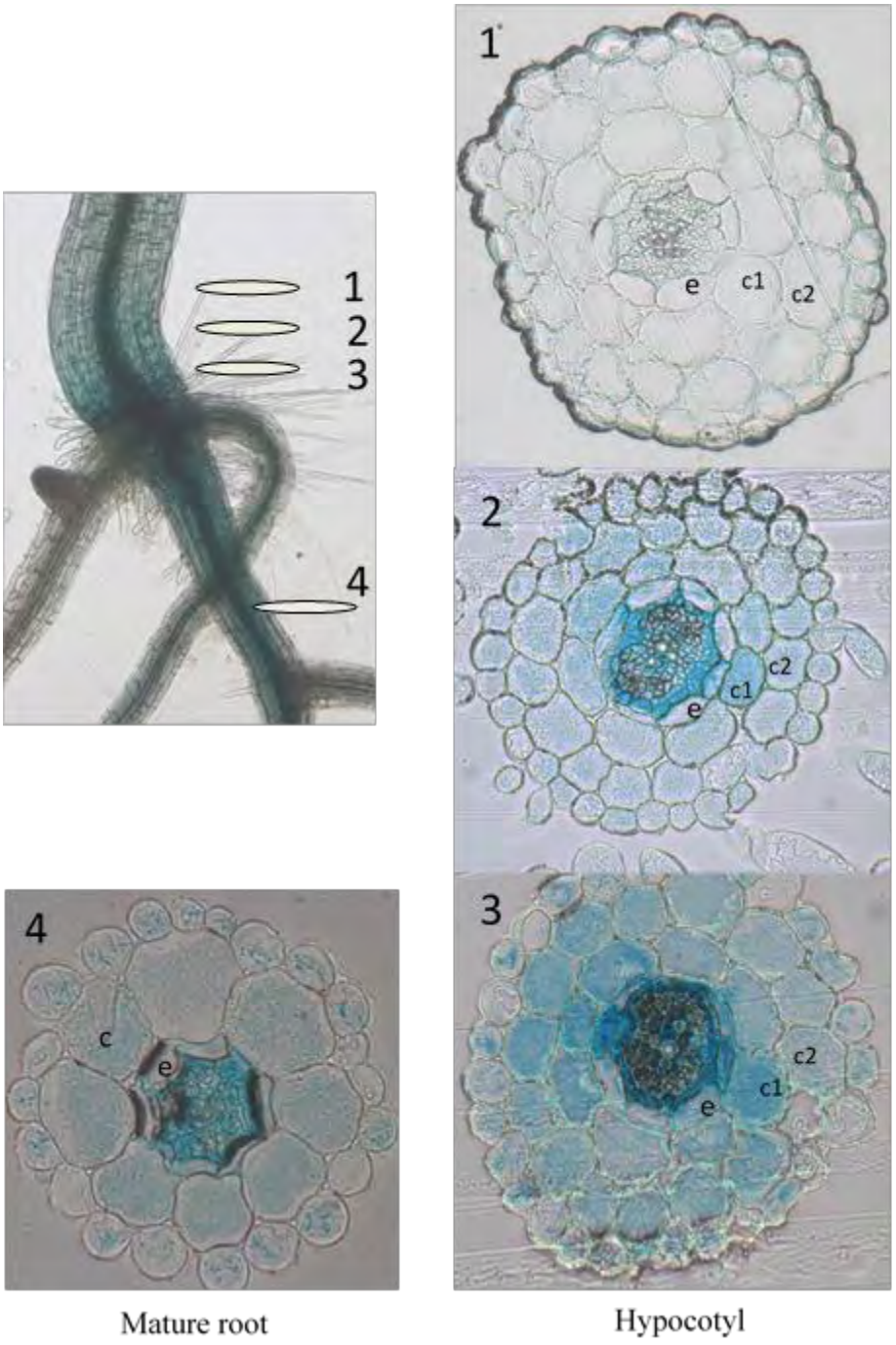



**Figure C2-7**: *P250R:GUS* expression in transversal sections of Nd-1 plants. (1-3). Sections in hypocotyl. (4). Sections in mature root. e : endodermis. c: cortex.

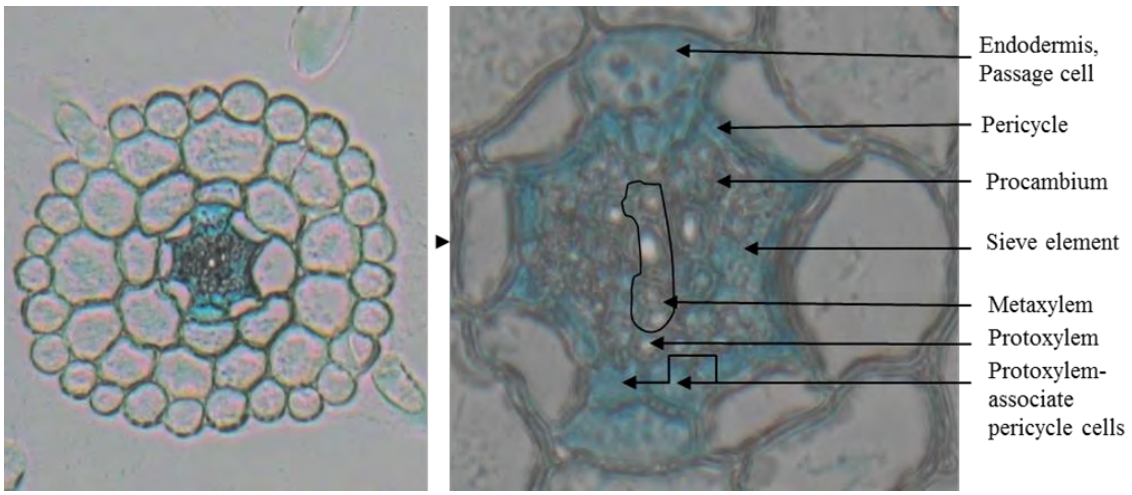



**Figure C2-8.** Details of *P250R:GUS* expression in vascular tissues observed from hypocotyl tranversal section (Nd-1 genetic background).

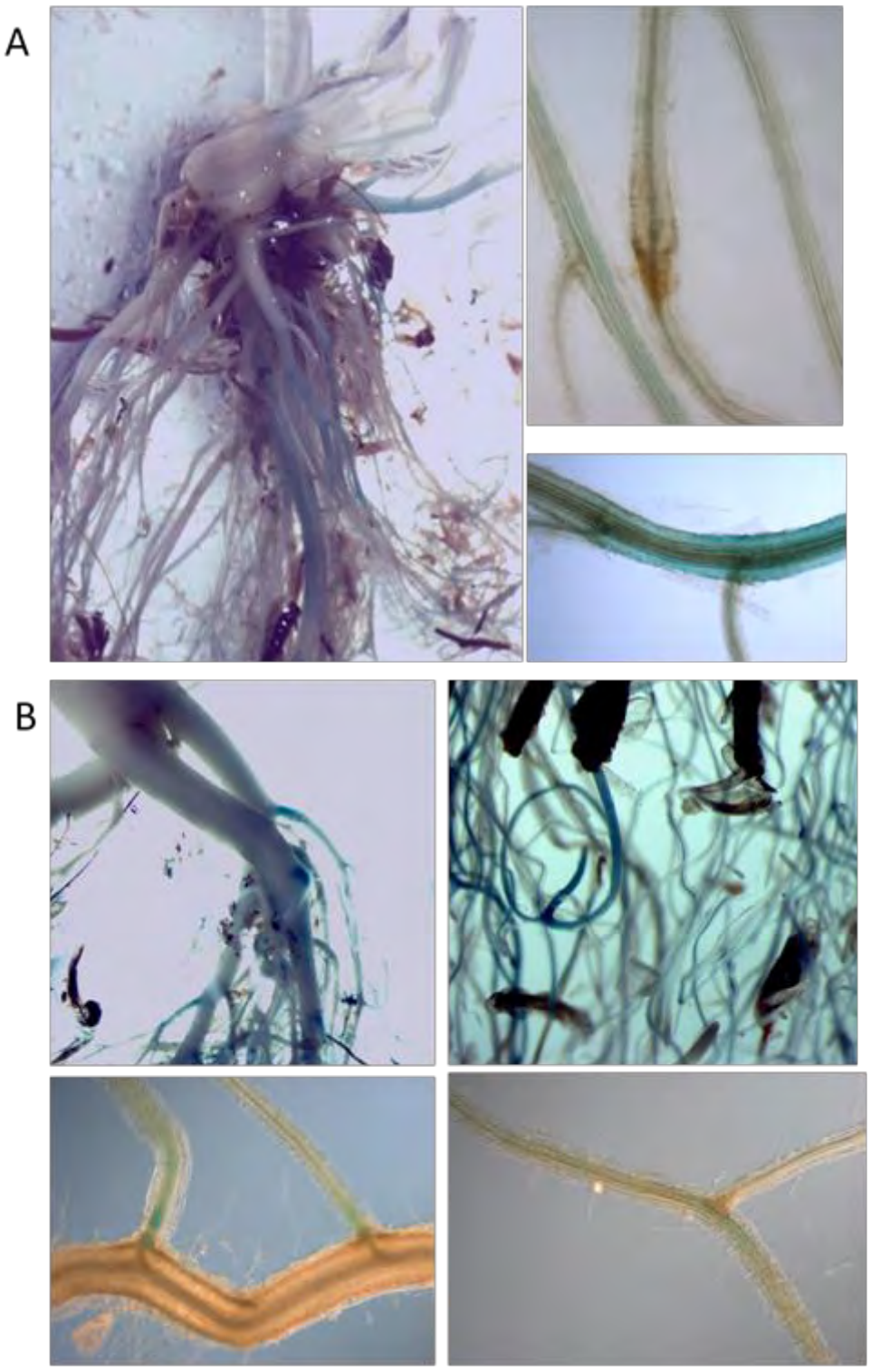



**Figure C2-9.** *RRS1-R* gene promoter:GUS reporter gene expression in roots from adult plants grown in soil. (A) P250 R in Col-0, (B) P250 R in Nd-1.

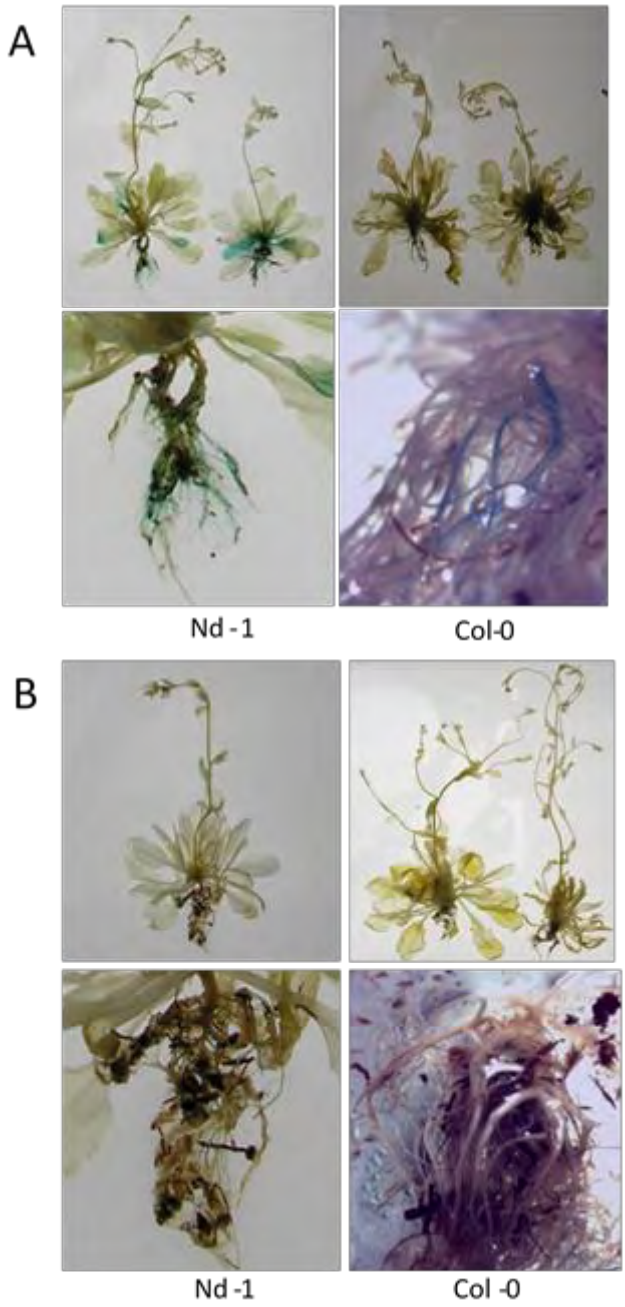



**Figure C2-10.** Down regulation of *P250R:GUS* expression in plants grown in reduced watering conditions: A: Plants were normally watered. B: Plants were water deprivation.

Plantlets grown *in vitro* were challenged by adding sodium chloride (100mM), or mannitol (200mM). In response to these compounds (Figure C2-11), GUS gene expression was modified: it slightly decreased in the transition zone from roots to hypocotyls and highly increased in root tips in comparison to untreated plants. QRT-PCR was performed on the same material to follow directly RRS1 gene expression after transfer on mannitol containing culture medium. Results showed for the Col-0 plants an increase of the level of expression in root tips (Figure C2-5c).

#### 2.2.1.3 No detectable change in *RRS1* root expression observed following *R. solanacearum* inoculation

Adults plants were inoculated by dipping roots in a bacterial suspension as previously described (Deslandes *et al.,* 1998). GMI1000 bacteria expressing a *LacZ* reporter gene were used in these experiments, to allow both detection of bacteria and RRS1 gene expression. Gus expression was observed at different times after inoculation and compared to expression in water-soaked roots. Based on the observation of roots from 10 inoculated plants, in Col0 and Nd1 background, it appeared that the presence of bacteria had no detectable impact on the *GUS* expression profile. However, root tips were often damaged before observation during the soil extraction step. According to the results obtained *in vitro*, it was interesting to follow *Gus* expression in root tips. To this end, Jijjys containing the growing plants were "scalped" to recover as much as possible inoculated root tips. A high variation of expression was observed within roots of a single plant, probably reflecting a variation in growing conditions, and no conclusion could be drawn. Observations are illustrated in Figure C2-12a and b.

#### 2.2.1.4 Pop P2 effector impacts *RRS1* gene expression in *A. thaliana* plantlets.

An estradiol-inducible promoter was fused to the coding sequence of Pop P2 in order to induce Pop P2 within the plant cell. A Flag Tag was also added for its detection (Neil Ledger and Laurent Deslandes, unpublished work). This construct was introduced in the RRS1-GUS reporter lines. Several generated lines were shown to express Pop P2 following estradiol addition (Figure C2-13). Plants were grown directly on a medium containing the inducer, or were transferred onto this medium after one week of growth on MS medium.

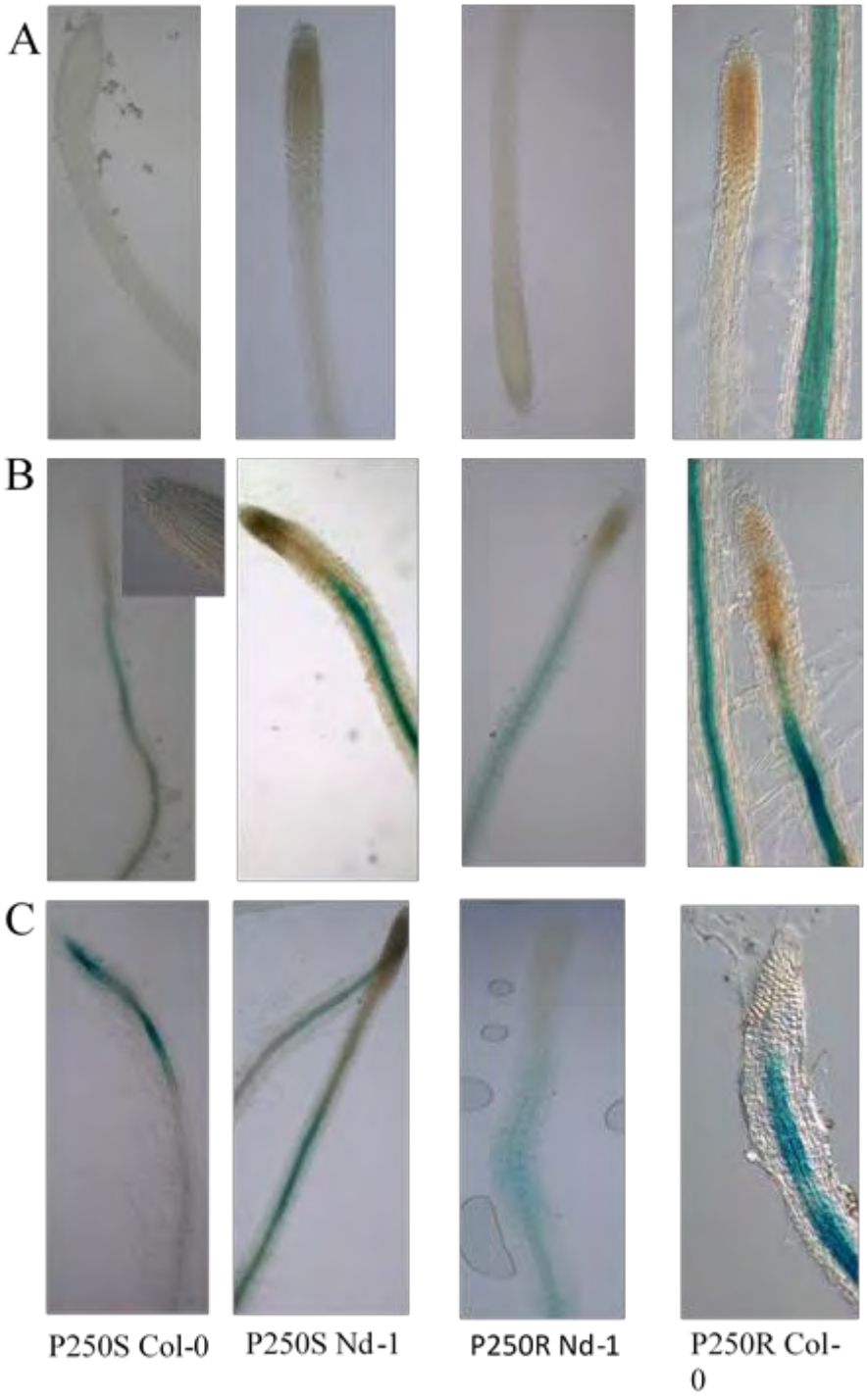



**Figure C2-11.** Induction of *GUS* expression in root tips in response to abiotic stresses. Plant were grown on MS medium and tranferred after 10days for 24 hours on a new medium as indicated below: A:MS, B: MS + mannitol 200 mM, C:Ms +NaCl 100mM (iso osmotic stresses).

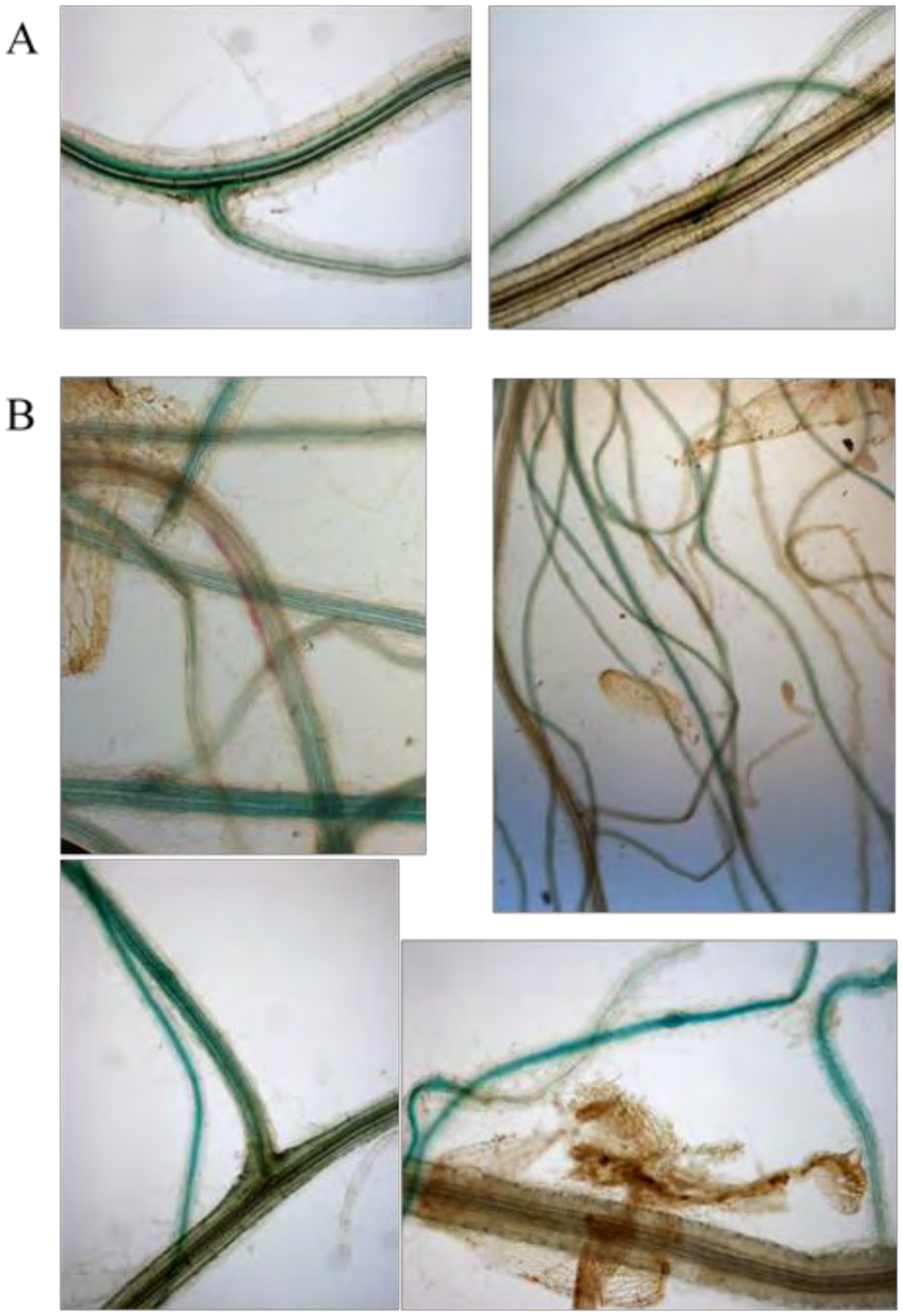



**Figure C2-12a.** P250R: *GUS* expression in adults Nd-1 plants grown in soil and root inoculated. A: mock inoculation with water. B: GMI1000 inoculation. Activity of the bacterial reporter gene is revealed with magenta Gal.

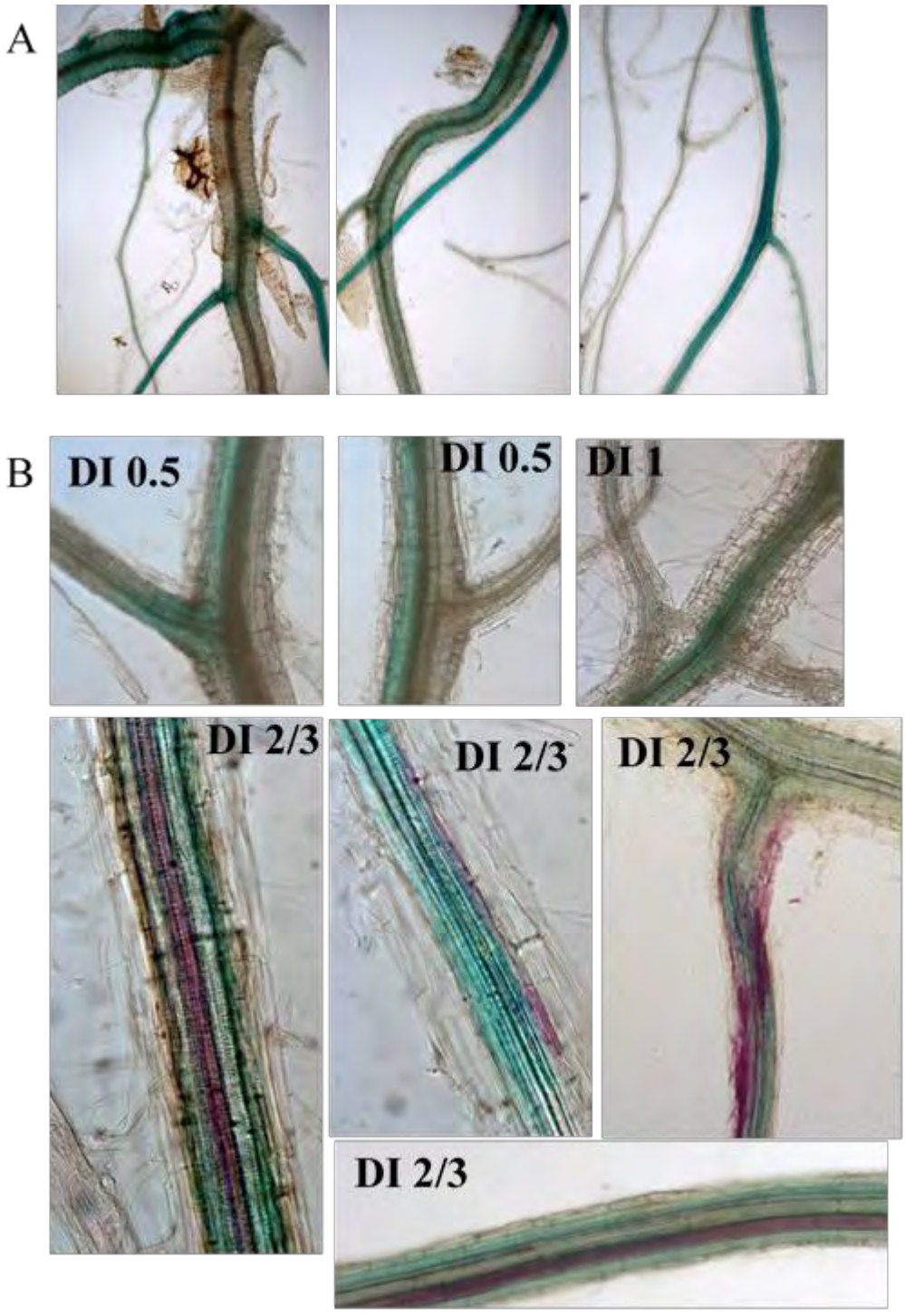



**Figure C2-12b.** P250R: *GUS* expression in adult Col-0 plants grown in soil and root innoculated: A: mock inoculation with water. B:GMI1000 inoculation. Disease index is indicated on each view. Activity of the bacterial reporter gene is revealed with magenta Gal.

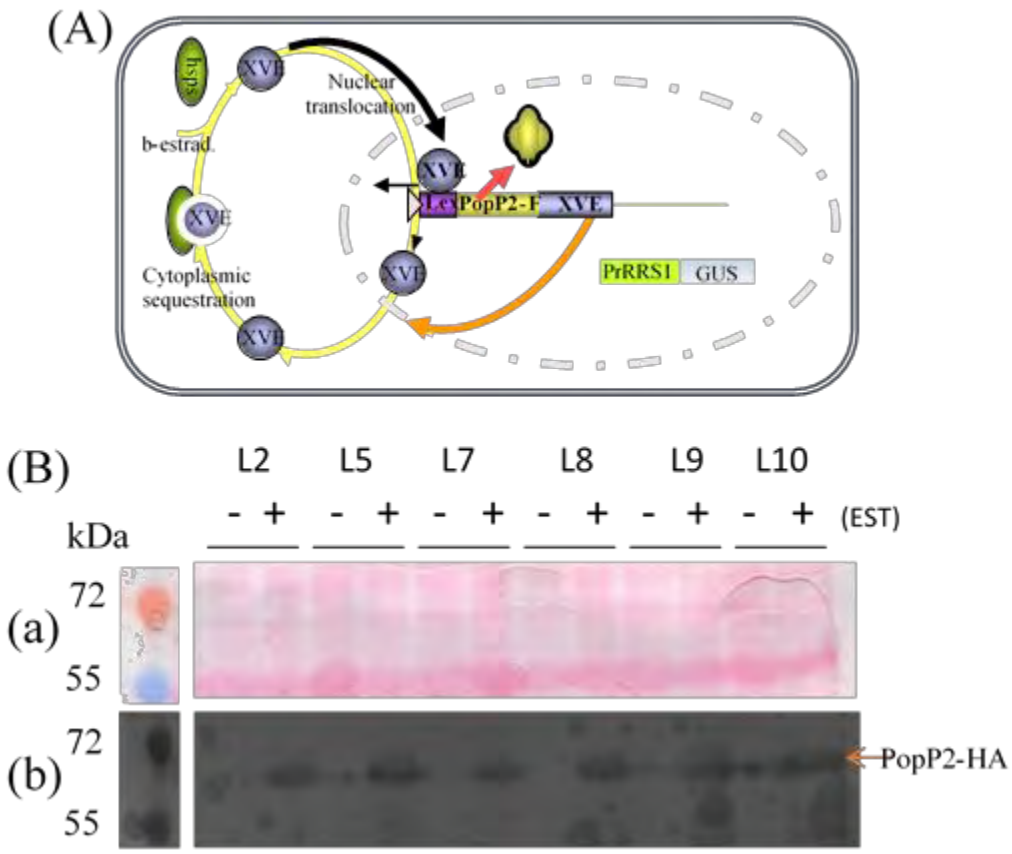



**Figure C2-13.** Estradiol induction of PopP2 in plantlets expressing the reporter genes. (A) *LexA* promoter activity is regulated by estradiol. Estradiol is directly applied on MS medium for 30 minutes and expression is checked 12 hours later. (B) Control of Pop P2 induction (a) Ponceau staining was performed to ensure equal loading. (b) Westernblot was performed with HA antibody. Each line correspond to a transgenic plant containing different reporter genes as indicated bellow (promoter/genetic background). (L2)P250R /Col-0, (L5) P250 R/Nd-1, (L7) - (L8)- (L9) P250S/Col-0, (L10) P250S /Nd-1.

Analysis of the Gus expression pattern of 10 days old plantlets expressing Pop P2 showed, for most of the lines, an expression profile similar to the one already observed without Pop P2. Nevertheless, GUS expression driven by P250S was modified in a Col-0 genetic background. In 3 lines containing this reporter gene, expression was lower in older parts of roots and at the transition between hypocotyls and root and was also observed in the elongation and differentiation zone of the root (Figure C2-14).

### 2.2.2 Specific binding of RRS1-R and RRS1-S WRKY domains to W boxes

In an attempt to identify RRS1 target sequences *in vivo*, a preliminary step was to demonstrate the binding activity of these proteins to W boxes in vitro. This binding activity had been previously tested for a protein similar to RRS1-R, SLH cloned by Noutoshi and collaborators from the *Arabidopsis Nossen* ecotype (Noutoshi *et al.*, 2005). These authors used gel shift experiments in order to demonstrate that the WRKY domain of the SLH protein was able to recognize W box sequences. Because of their similarities with SLH, RRS1-R WRKY domain (WRKY-R) should be able to bind the W box. However, since RRS1-R and RRS1-S differ in their C-terminal region, containing the WRKY domain, differences in the binding to DNA could be expected. In order to check that both RRS1-S and RRS1-R WRKY domains were able to bind to W box DNA sequences *in vitro*, WRKY domains were expressed in E. coli and purified as described in material and methods. Western blot were performed to control the protein production (Figure C2-15b). A biotin-labeled oligonucleotide containing a W box sequence, or a mutated version of this sequence that do not allow WRKY protein binding (Noutoshi *et al.*, 2005), was used in gel shift experiments. Results indicate that both WRKY-R and WRKY-S bind to W box containing sequences. Specificity of binding was demonstrated using unlabeled oligonucleotides in competition experiments. Super shift using GST antibody confirmed the nature of retarded complexes (Figure C2-15c).

## 2.3 Discussion

We report expression profiles driven by the RRS1-S and RRS1-R promoters in resistant and susceptible ecotypes. Under our experimental conditions, promoters of the two genes drive similar expression profiles in the two resistant and susceptible genetic backgrounds.

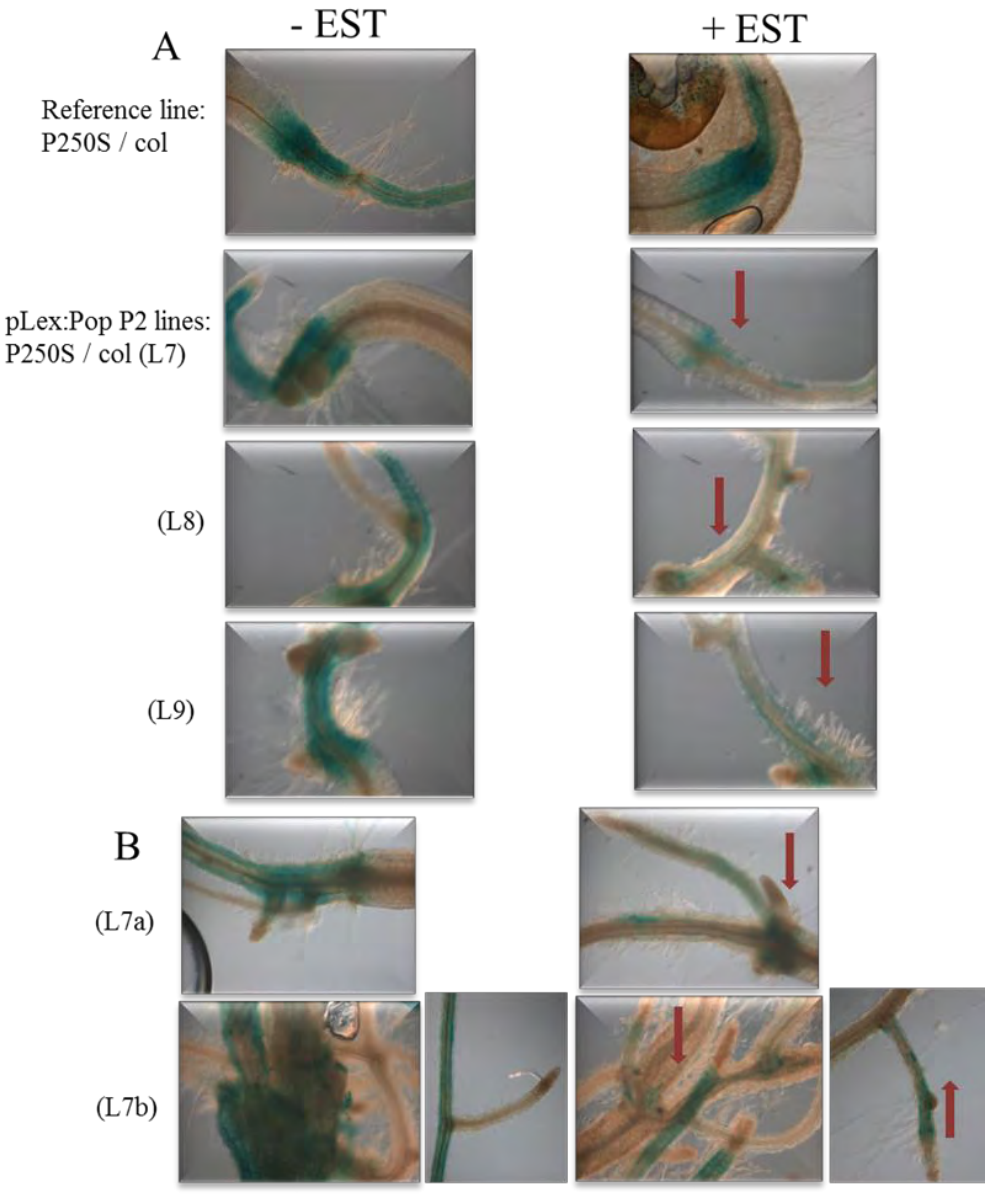



**Figure C2-14.** Impact of Pop P2 induction in GUS expression driven by a P250S promoter in Col-0 genetic background. A: GUS expression in 10 days old plantlets grown on MS medium with (+EST 10mM, first column) or without estradiol (-EST second column). Red arrow highlight the down regulation of GUS expression observed with estradiol when Pop P2 is induced in tree lines (L7, L8, L9) containing the inducible *Pop P2* gene. B: plants from line L7 were first grown on MS medium and then tranferred for 6 days on MS +EST. GUS expression was tested on 13 days and 21 days old plantlets (L7a) and (L7b) respectively. Red arrow highlight the enhanced regulation of GUS expression observed with estradiol.

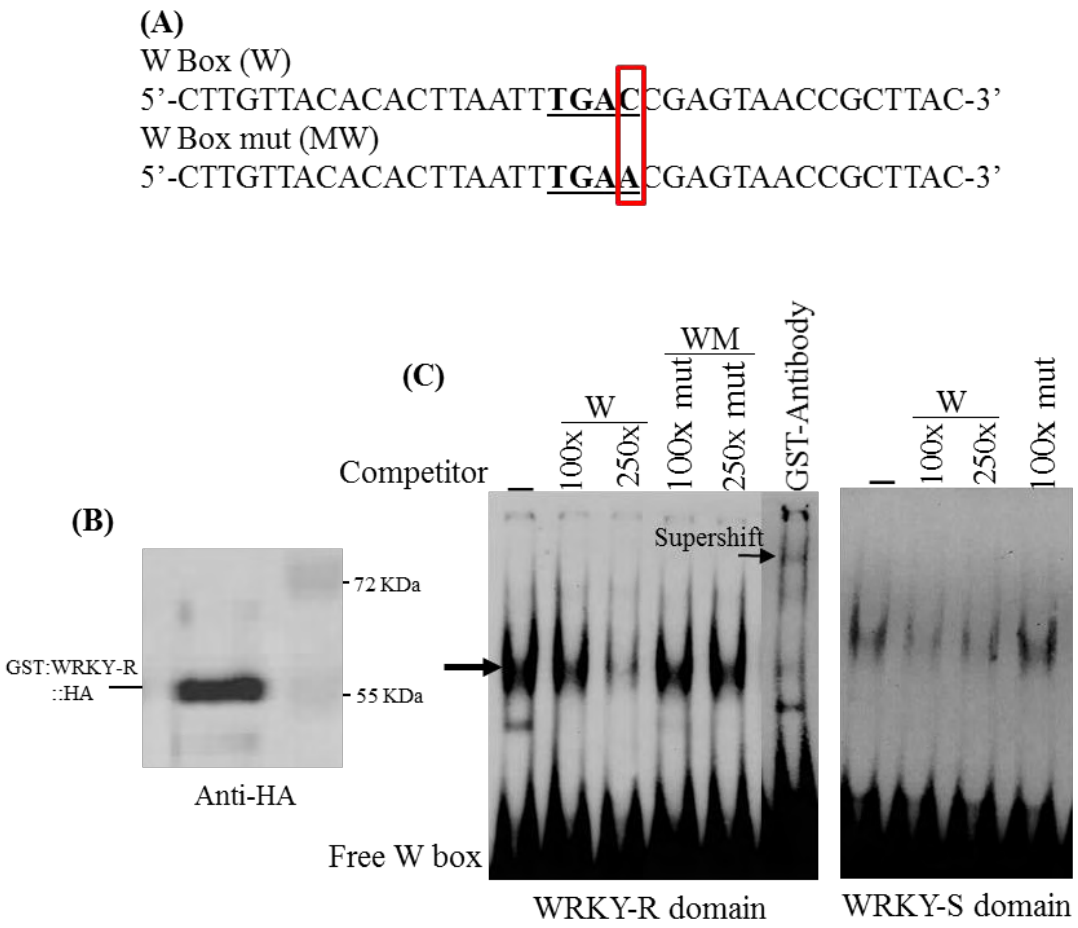



**Figure C2-15.** DNA-binding activity of the RRS1 protein WRKY domains.

(A) The sequences of W Box (W) and W Box mut (WM). Core sequences of the W-box are underlined. A box surrounds the position of the substituted base in the mutant W-box. (B) The GST-fused proteins of the WRKY domain were express in *E.coli* and purified. (C) Gel shift assay of the RRS1-R protein WRKY domains. The WRKY domains were incubated with labeled W Box. The dash (-) indicate the absence of competitor. The competitor W and WM were added in 100- and 250-fold molar excess as indicated. GST-Antibody were used for supershift.

A short promoter, corresponding to the intergenic region, drives a stable and reproducible expression in comparison to the expression driven by a longer promoter, which was more variable. In order to deepen our analysis on roots, transversal sections were performed and allowed to visualize RRS1 expression in pericycle cells and some in specific endodermal cells located at the protoxylem poles. The significance of this pattern and its relevance to *R. solanacearum* infection is interesting. Pericycle and endodermal cells adjacent to the protoxylem poles have features that distinguish them from neighboring cells. For example, lateral roots initiate from the pericycle cells immediately adjacent to the two protoxylem poles (Laskowski *et al.*, 1995). Furthermore, the formation of the Casparian band in the anticlinal walls of the endodermal cells and the deposition of suberin lamellae into the entire walls begin opposite the phloem strands and spread toward the protoxylem, resulting in the presence of thin-walled endodermal cells, called passage cells, opposite to the protoxylem poles. Passage cells are thought to offer a lower resistance pathway for water flow into the stele (Peterson e Enstone, 1996). As published recently, *R. solanacearum* bacteria progress through the root by pericycle cells located at the xylem poles when inoculated *in vitro* on Col-0 *A. thaliana* plantlets (Digonnet *et al.*, 2012). Vessel invasion by bacteria in tomato roots also starts with bacterial multiplication in protoxylem cells (Vasse, Frey e Trigalet, 1995). It can be hypothesized that root-invading bacteria are directly in contact with cells in which RRS1 genes are expressed, leading possibly to the elaboration of a rapid plant response. Expression of a resistance gene in response to pathogens we already described following nematode infection. The HS1 pro-1 promoter fused to a GUS reporter gene indicates that expression increases after nematode infection specifically in the nematode feeding site (Thurau *et al.*, 2003). We could imagine that genes involved in resistance to root pathogens are possibly directly expressed in the site of infection and studies concerning such genes should be developed in the future to gain insight into interactions with pathogens invading roots. According to our observations in plantlets, the expression profile of RRS1 genes appears to resemble the expression of the *PHO1* gene. This gene encodes an inorganic phosphate transporter induced upon phosphate starvation and is important for inorganic phosphate loading into xylem vessels (Hamburger *et al.*, 2002). In the context of *R. solanacearum* infection, no effect of bacterial inoculation on RRS1 gene expression could be detected. Additional experiments, using hydroponic

cultures of *A. thaliana* plants and labelled bacteria will be performed to strengthen these results. Nevertheless, RRS1 expression is obviously modified in response to abiotic stress and is possibly affected by the induction of Pop P2 in the plant cells. The reproducibility of experiments apparently strongly affected by the growth/environmental conditions suggests that environmental conditions influence the promoter activity but constitutes actually a main obstacle to draw final conclusions on some points of this analysis. Immunolocalization of the protein was also considered but, due to the poor quality of antibodies raised against the RRS proteins, this approach could not be developed

However, the ability of RRS1 WRKY domains to recognize W box sequences *in vitro*, open the possibility of undertaking the *in vivo* target identification of both RRS1-R and RRS1-S genes.

## 2.4 Conclusion and perspectives

The work presented in this chapter allows the visualization of RRS1-R and RRS1-S promoter activity in plants and ensures the DNA binding properties in vitro using gel retardation assays of the both RRS1-R and RRS1-S WRKY domains.

The main prospects open up by these data are following:

- Concerning the RRS1 gene expression profiles:

- (i) It will be interesting to identify *cis*-elements, within the promoters, involved in expression patterning. Birker and co-authors already proposed the importance of several known *cis*-elements define by interspecies sequence comparison as putatively important regulatory sequences (Birker *et al.*, 2009). Mutated promoters can be used to drive GUS expression and to complement resistance in order to establish a structure-function relationship.

- (ii) In order to avoid heterogeneity in expression level and pattern, it will be necessary to grow plants in more controlled conditions. A phenotyping platform available in the laboratory in a next future will be used. In order to test the effect of R. solanacearum infection on expression pattern, hydroponic cultures, for root visualization, and

luminescent bacteria (Monteiro *et al.*, 2012), to focus expression analysis at sites of infection, will be used.

-(iii) Comparison of RRS1 and RPS4 gene expression profiles should give new clues to the functioning of this dual resistance gene system as the two genes are head to head and share inverted promoters.

-Concerning the binding properties of RRS1-R and RRS1-S WRKY domains:

Even if the main objective of these experiments was to ensure binding properties of both domains to W boxes, in order to undertake in vivo the characterization of binding sites, they open new possibilities to identify parameters that will modify DNA binding. Indeed transcription factors will have their affinity modified by post-translational regulation such as, phosphorylation or acetylation events. Due to the Pop P2 acetylase activity, we will check the impact of Pop P2 on gel retardation experiments by co-expressing the WRKY domains of RRS1 proteins in *E.coli*. It will be also necessary to check whether RPS4 can interfere with the binding properties of RRS1.

Altogether, that will allow us to add some new data on the mechanisms of action of RRS1-S and R proteins.

# Chapter III

## *In vivo* identification of DNA binding sites of RRS1-R proteins

## 3.1 Introduction

In order to locate binding sites of a DNA-binding protein in the genome, chromatin immunoprecipitation is the most popular method (Kuo e Allis, 1999). This method uses the possibility to crosslink *in vivo* proteins to their DNA targets. Specific protein-antibody complexes are then purified by immunoprecipitation using a specific antibody against the protein of interest or against a tag fused to the protein (De Folter *et al.*, 2007). In order to generate genome-wide binding profile of a protein, whole genome tilling array (ChIP-CHIP) or high throughput sequencing technologies (ChIP-SEQ) can be employed. (Kim e Ren, 2006). The signal to noise ratio of the ChIP experiment which depends on the expression level of the protein as well as the efficiency of the antibody, constitutes a major obstacle.

Dynamics of the Protein-DNA interaction in chromatin environment constitutes a critical point to access the *in vivo* binding sites of a protein. Detection of binding for transient protein/DNA interactions is very poor and constitutes a major limitation of this approach.

An alternative method is the "DNA adenine methyltransferase identification" (DamID) (Van Steensel e Henikoff, 2000). DNA adenine methyltransferase (Dam) from *E. coli*, which specifically methylates the adenine residue in a GATC recognition sequence, is a small sized protein, which can be easily fused to the DNA-binding protein of interest. When expressed in cells, the fusion proteins bind to genomic DNA and introduce N-6-adenine methylation to nearby GATC sequences. Locations of methylation can then be identified with methylation-sensitive restriction enzymes Dpn I and Dpn II. Because adenine methylation in GATC sequence does not occur endogenously in eukaryotes, this method has been successfully applied to several model systems, e.g. budding yeasts, cultured mammalian cells, fruit flies, and plant cells (Orian *et al.*, 2003; Bianchi-Frias *et al.*, 2004; Weber *et al.*, 2005; Venkatasubrahmanyam *et al.*, 2007; Zhang, X. *et al.*, 2007).

The main advantage of DamID is the high sensitivity of detection due to the high methylation activity by the Dam enzyme, which allows fingerprints of binding sites following transitory association to DNA.

By using such a method, the restricted expression patterns and low activity of *RRS1-S* and *RRS1-R* genes should not be any more a limitation for the detection of protein/DNA interaction. In addition, because transcription dynamics is a main feature of transcriptional reprogramming in ETI, the possibility to map transitory associations to DNA with the DamID method is definitively an advantage. We therefore decided to use this approach to get a genome-wide fingerprint of the RRS1-R or RRS1-S DNA binding sites *in vivo*.

## 3.2 Results

### 3.2.1 Set up of the Dam ID approach

#### 3.2.1.1 Engineering a Gateway destiny vector enabling the generation of the appropriate Dam fusions

The Dam sequence was amplified from *E. coli* DNA and N-term tagged with the triple HA tag. The terminator sequence from the *RRS1-R* gene was added at the 3' end of the chimaera construct. This DNA fragment was introduced into a Gateway vector allowing the cloning of protein fusion for protein production in *E. coli*, for transitory expression experiments in *N. benthamiana* or for generation of transgenic plants.

#### 3.2.1.2 Fusion to Dam does not inhibit binding to W box of WRKY domain of RRS1-R and RRS1-S

Dam fusions corresponding to the WRKY-R or WRKY-S domains were expressed in *E. coli* as described in the "Materials and Methods" section. After protein purification, gel retardation assays were performed using similar conditions than those used to test the binding activity of the WRKY domain *in vitro*. Results shown in Figure C3-1 demonstrated a specific binding of the tested fusion proteins to the W box containing oligonucleotides.

#### 3.2.1.3 The RRS1-R and RRS1-S fusion proteins do possess a Dam activity

In order to check whether the RRS1-R::Dam fusion protein had a methyl transferase activity, we transiently over-expressed it in N. benthamiana. The expression of the fusion protein was detected by Western blot as shown in Figure C3-1a. Total DNA was then purified from the same leaf material and digested with methylation sensitive enzymes DpnI

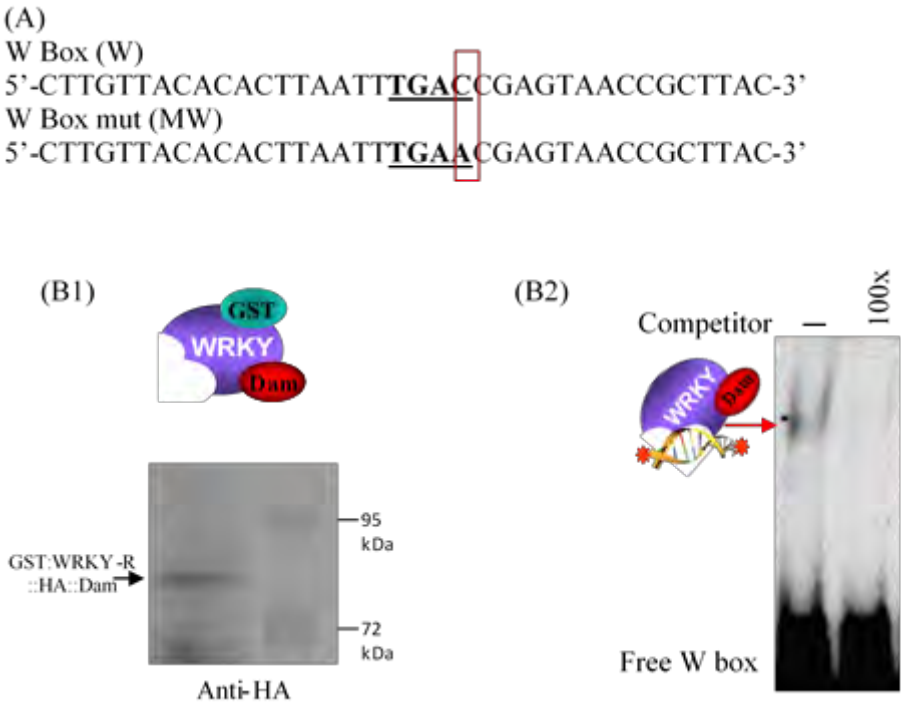



**Figure C3-1.** DNA-binding activity of the WRKY-R::Dam fusion protein.

(A) Sequences of W Box (W) and W Box mut (WM). Core sequences of the W-box are underlined. A box surrounds the position of the substituted base in the mutant W-box. (B): Western blotting detection of the GST-purified WRKY-R::Dam recombinant proteins (B1). Gel shift assay with WRKY-R::Dam protein incubated with labeled W box C (-) or W box and 100 fold molar excess unlabeled specific competitors (B2).

or DpnII which cut either only methylated GATC sites or only non-methylated GATC sites, respectively. Analysis of DpnI digestion products by agarose gel elctrophoresis revealed that no methylated GATC sequence ($GA^{m}TC$) was detected when the fusion protein was not expressed and reversely, that a smear of digested DNA was obtained when the fusion protein was expressed, indicating digestion of methylated GATC sites. DpnII digestion profiles confirmed these results, which demonstrates that the RRS1-R :: Dam fusion protein does possess a methyl transferase activity on plant DNA (Figure C3-1b).

#### 3.2.1.4 Generation of transgenic *A. thaliana* plants expressing the RRS1::Dam fusions

A. thaliana plants were transformed with plasmids expressing either the RRS1-S::Dam or the RRS1-R::dam fusion proteins under the control of the native RRS1-S or RRS1-R promoters, respectively, in order to obtain a specific and low level activity of Dam. Control lines were generated to evaluate the methylation background associated to the Dam protein expressed under the same promoter. Plants in each genetic background and for each construct were generated.

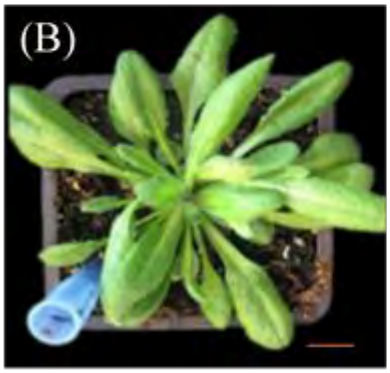
Table C3-1 recapitulates the number of normal plants selected in each case. At that point, we focused our study on transgenic plants obtained in the resistant Nd-1 genetic background. Levels of Dam and Dam fusion transgene expression were analyzed by quantitative RT-PCR in some selected lines. Normalization was performed using the EF1-$\alpha$ elongation factor gene, in order to select lines with a reduced methylation background (Van Blokland *et al.*, 1998; Germann e Gaudin, 2011). qRT-PCR results are presented in Figure C3-3. Two lines for each construct and background were selected for further analyses. This expression level was also checked on the next generations of plants, prior to any further experiment.

#### 3.2.1.5 Expression of the RRS1-R :: Dam fusion does not compromise plant resistance to R. solanacearum

Functionality of the RRS1-R fusion protein for resistance to R. solanacearum was addressed. Knock-out (KO) plants for RRS1-S in the Col 0 susceptible ecotype were

**Table C3-1.** Summary of transgenic lines and genetic background used for DamID

| Construct | Genetic background | T1 transgenic lines |
|---|---|---|
| p250R:RRS1-R::Dam | Nd-1 | 2 |
| | Nd-1/Inducible Pop P2 | 2 |
| p250R::Dam | Nd-1 | 13 |
| | Nd-1/Inducible Pop P2 | 10 |
| p250S:RRS1-S::Dam | Col-0 | 0 |
| | Col-0/Inducible Pop P2 | 2 |
| p250S::Dam | Col-0 | 9 |
| | Col-0/Inducible Pop P2 | 8 |


Transgenic plant containing the p250R:RRS1-R::Dam transgene in Nd-1. (A) Developmental problems of most of the obtained transgenic lines are exemplified (B) Normal phenotypes. All plants are 5 weeks old. In the table the number of normal T1 transgenic line obtained is indicated in the last column. Size bars: 1cm.

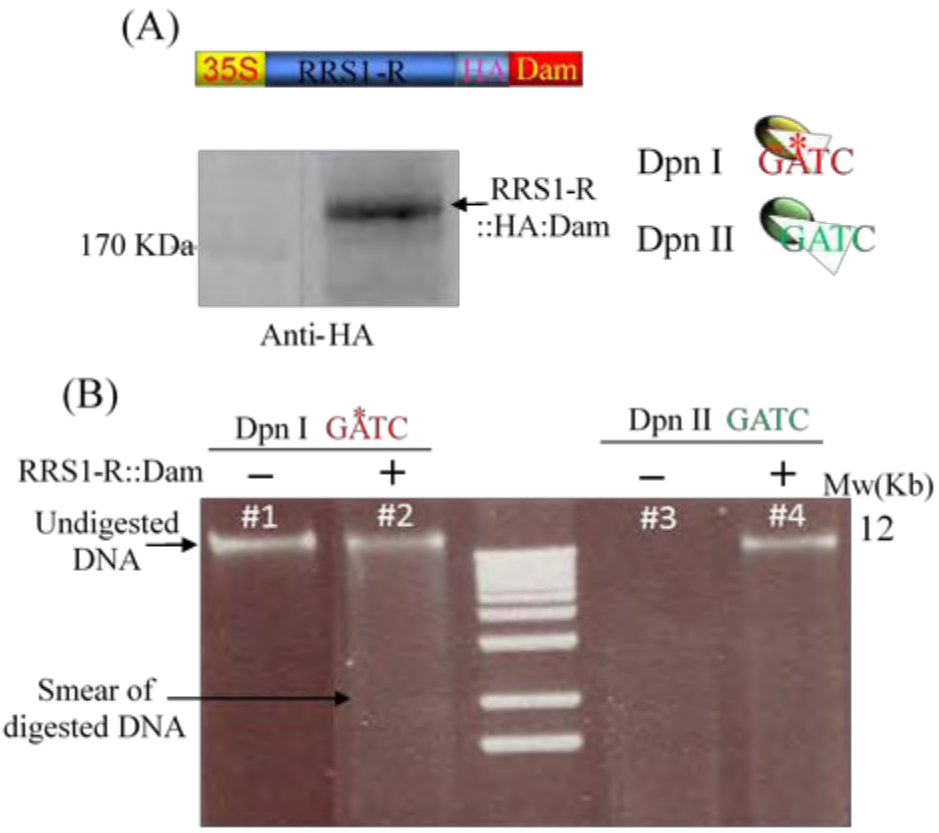



**Figure C3-2.** RRS1-R::Dam methylates plant DNA. (A) Anti-HA antibodies were used in Westernblot experiment to control the transient expression of *p35S:RRS1-R::HA::Dam* in *N.benthamiana*. (B) 1 mg of DNA was digested by Dpn I and Dpn II separately. Dpn I cut methylated GATC sites, reversely, DpnII only digest non-methylated GATC sites. The digested DNA was electrophoresed on 1% agarose gel. In lane #1 and #3 the Dam fusion protein was not expressed. In lane #2 and #4 the fusion protein has been transiently expressed.

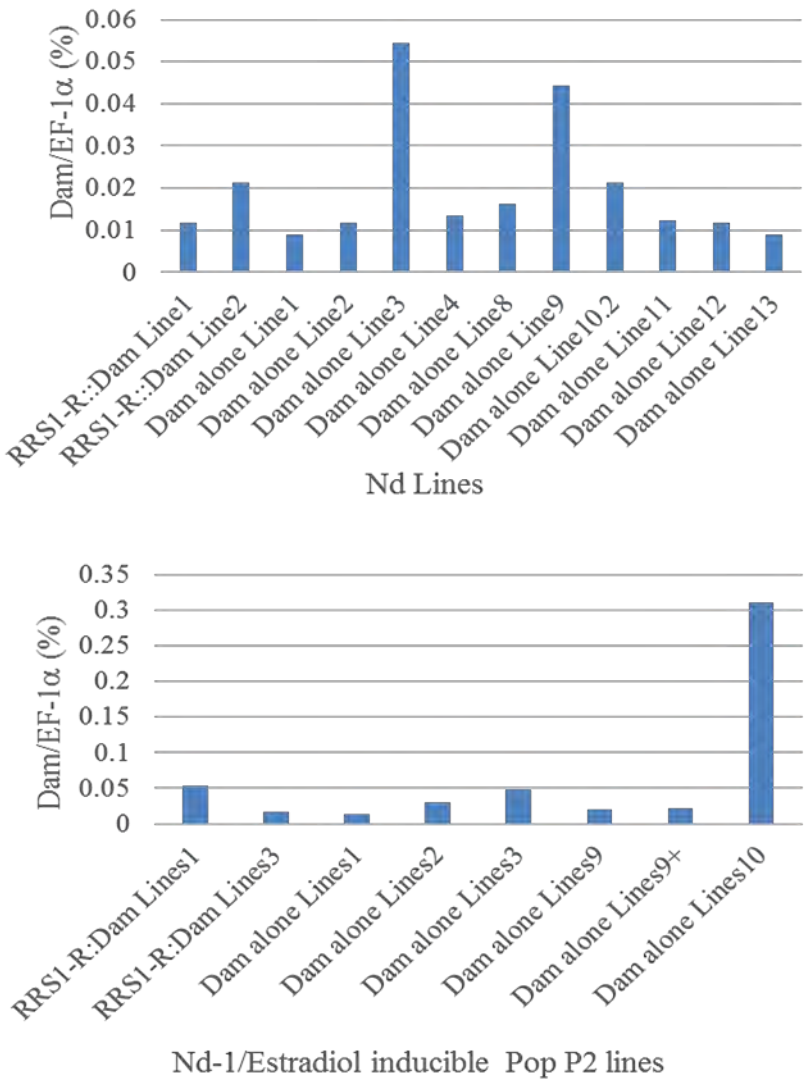



**Figure C3-3**. Expression level of Dam/EF-1a in Nd 1 and Nd-1/Estradiol inducible PopP2 lines. The expression level of Dam gene and elongation factor 1-alpha gene (*EF-1α*, At5g60390) were checked by qRT-PCR in plantlets used FARM identification.

transformed with the RRS1-R protein expressed under the control of its own promoter (genomic sequence) or with the construct used for DNA target identification. Unfortunately, as the RPS4 sequence from the resistant ecotype, which is also necessary for complementation was not included in the complementation experiment, it was not possible to complement the resistance even with the wild type RRS1-R sequence. This point is still under investigation.

Nd-1 plants expressing RRS1-R:dam and RPS1-R wild type genes were resistant, indicating that the addition of the Dam protein to RRS1-R did not disturb the resistance function of the wild type protein.

## 3.2.2 In vivo identification of Fragments Associated to the RRS1-R driven Methylation (FARMs)

### 3.2.2.1 FARMs characterization from leaves of T1 adult plants

Plant material, corresponding to Nd-1 mature rosette leaves, expressing the protein fusion or only the dam protein, was harvested under greenhouse conditions. DNA was purified and PCR products were obtained according to the flowchart presented in Figure C3-4 (methods, this chapter). Amplification products obtained from plants expressing the fusion protein were cloned and one hundred clones randomly chosen for sequencing. We then performed a small scale test to evaluate by Q-PCR if the corresponding fragments were also amplified in a control line expressing Dam alone.

Internal primers were designed for 16 FARMs Results are presented in Figure C3-5. Most of the corresponding fragment were largely enriched in samples corresponding to DNA amplified from plants expressing the RRS1-R::Dam fusion protein.

Through this first survey of methylated targets, 85 positive blastn hit on tair10 were detected. 70 sequences matched to upstream -3000 promoter sequences. Some of the corresponding genes (29), were nuclear-located, others corresponded to pseudogenes, transposons, chloroplastic or mitochondrial sequences. Specific features of these sequences are presented in Table C3-2. One clone corresponding to the promoter sequence

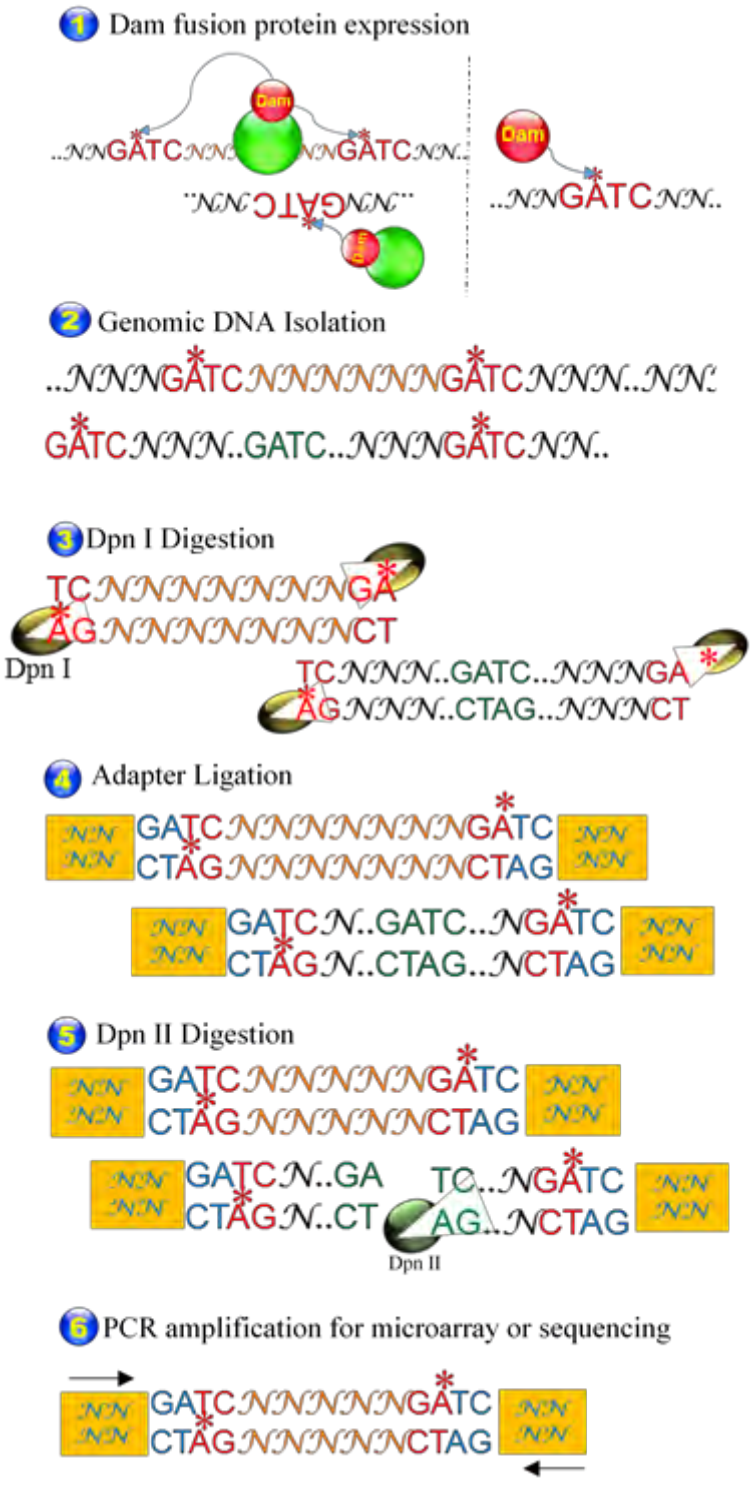



**Figure C3-4.** Protocol of DNA adenine methyltransferase identification (DamID).

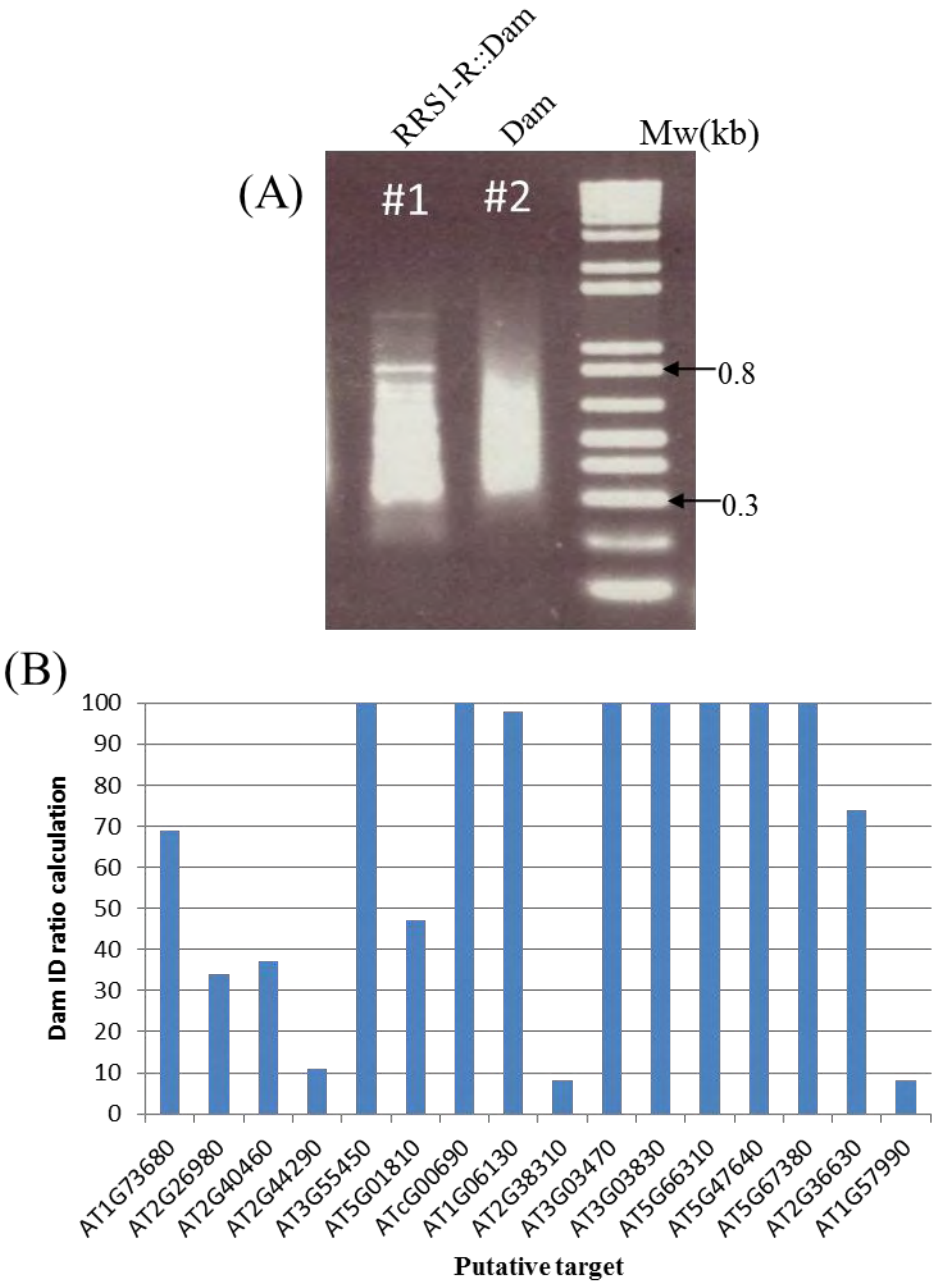



**Figure C3-5.** (A): FARMs amplified from Nd-1 mature leaves expressing RRS1-R::Dam (#1), or Dam alone (#2). (B): Ratio of expression deduced from qPCR experiments on DNA from plantlets expressing RRS1-R::Dam fusion or Dam. They corresponds to efficiency$^{(CT\ RRS1\text{-}R::\ DAM/CT\ DAM)target}$/ to efficiency$^{(CT\ RRS1\text{-}R::DAM/CT\ DAM)reference}$. CT for the reference were obtained from average of CT for two fragments not present in the identified targets.

**Table C3-2.** FARMs hits identified from leaves

| | |
|---|---|
| **Hits on nuclear gene promoter** | |
| AT1G06130 | glyoxalase 2-4 (GLX2-4) |
| AT1G49160 | WNK protein kinase |
| AT1G64710 | GroES-like zinc-binding dehydrogenase family protein |
| AT1G73680 | alpha dioxygenase |
| AT1G76090 | S-adenosyl-methionine-sterol-C-methyltransferase |
| AT2G07711 | pseudogene, similar to NADH dehydrogenase subunit 5 |
| AT2G14610 | PR1 salicylic-acid responsive. |
| AT2G14620 | xyloglucan endotransglucosylase/hydrolase 10 (XTH10) |
| AT2G26980 | CBL-INTERACTING PROTEIN KINASE 3 (CIPK3) |
| AT2G29560 | CYTOSOLIC ENOLASE (ENOC) |
| AT2G31370 | Basic-leucine zipper (bZIP) transcription factor family protein(WNK7) |
| AT2G36240 | pentatricopeptide (PPR) repeat-containing protein |
| AT2G38310 | Regulatory components of ABA receptor 10 (RCAR10); PYR1-like 4 (PYL4) |
| AT3G03470 | Cytochrome P450, Family 87, Subfamily A, Polypeptide (CYP89A9) |
| AT3G03830 | SMALL AUXIN UP RNA 26 (SAUR26) |
| AT3G03840 | SMALL AUXIN UP RNA 27 (SAUR27) |
| AT3G03850 | SMALL AUXIN UP RNA 28 (SAUR28) |
| AT3G10960 | AZA-GUANINE RESISTANT1 (AZG1) |
| AT3G46170 | NAD(P)-binding Rossmann-fold superfamily protein |
| AT3G55450 | PBS1-LIKE 1 (PBL1) |
| AT4G16141 | GATA type zinc finger transcription factor family protein |
| AT4G30935 | WRKY DNA-BINDING PROTEIN 32 (WRKY32); (ATWRKY32) |
| AT4G30940 | BTB/POZ domain with WD40/YVTN repeat-like protein |
| AT5G01810 | CBL-INTERACTING PROTEIN KINASE 15 (CIPK15) |
| AT5G47635 | Pollen Ole e 1 allergen and extensin family protein |
| AT5G47640 | NUCLEAR FACTOR Y, SUBUNIT B2 (NF-YB2) |
| AT5G66310 | ATP binding microtubule motor family protein |
| AT5G67380 | Casein kinase II (CK2) catalytic subunit (alpha 1) |
| AT5G67385 | Phototropic-responsive NPH3 family protein |
| **Hits on coding/ intron sequences** | |
| AT2G28550 | RELATED TO AP2.7 (RAP2.7) |
| AT2G40460 | Major facilitator superfamily protein |
| AT3G03260 | HOMEODOMAIN GLABROUS 8 (HDG8) |
| AT3G03680 | C2 calcium/lipid-binding plant phosphoribosyltransferase family protein |
| AT1G65320 | CBS DOMAIN CONTAINING PROTEIN 6 (CBSX6) |
| AT5G28640 | ANGUSTIFOLIA 3 (AN3) |
| **Hits on downstream gene sequences** | |
| AT1G08910 | EMBRYO DEFECTIVE 3001 (EMB3001) |
| AT1G28670 | Arabidopsis thaliana lipase |
| AT1G28685 | Potential natural antisense gene, locus overlaps with AT1G28680 |
| AT1G29650 | transposable element gene; non-LTR retrotransposon family (LINE) |
| AT2G44490 | PENETRATION 2 (PEN2) |
| AT3G10970 | Haloacid dehalogenase-like hydrolase (HAD) superfamily protein |
| AT3G62620 | sucrose-phosphatase-related |
| AT3G62630 | Protein of unknown function (DUF1645) |

| | |
|---|---|
| AT4G33960 | Leucine-rich repeat (LRR) family protein |
| AT4G33970 | unknown protein |
| AT5G25260 | SPFH/Band 7/PHB domain-containing membrane-associated protein family |
| AT5G65720 | NITROGEN FIXATION S (NIFS)-LIKE 1 (NFS1) |
| AT5G65740 | zinc ion binding |
| **Hits on mitochondria and chloroplast** | |
| ATCG00170 | RNA polymerase beta' subunit-2 |
| ATCG00480 | ATP SYNTHASE SUBUNIT BETA (PB) |
| ATCG00560 | PHOTOSYSTEM II REACTION CENTER PROTEIN L (PSBL) |
| ATCG00570 | PHOTOSYSTEM II REACTION CENTER PROTEIN F (PSBF) |
| ATCG00580 | PHOTOSYSTEM II REACTION CENTER PROTEIN E (PSBE) |
| ATCG00610 | tRNA-Trp |
| ATCG00630 | subunit J of photosystem I. |
| ATCG00640 | RIBOSOMAL PROTEIN L33 (RPL33) |
| ATCG00650 | RIBOSOMAL PROTEIN S18 (RPS18) |
| ATCG00690 | PHOTOSYSTEM II REACTION CENTER PROTEIN T (PSBT) |
| ATCG00700 | PHOTOSYSTEM II REACTION CENTER PROTEIN N (PSBN) |
| ATCG00730 | PHOTOSYNTHETIC ELECTRON TRANSFER D (PETD) |
| ATCG00750 | RIBOSOMAL PROTEIN S11 (RPS11) |
| ATCG00760 | RIBOSOMAL PROTEIN L36 (RPL36) |
| ATCG00770 | RIBOSOMAL PROTEIN S8 (RPS8) |
| ATCG00780 | RIBOSOMAL PROTEIN L14 (RPL14) |
| ATCG00870 | hypothetical protein |
| ATCG00900 | CHLOROPLAST RIBOSOMAL PROTEIN S7 (RPS7); (RPS7.1) |
| ATCG00920 | RIBOSOMAL RNA16S (RRN16S.1) |
| ATCG00930 | RIBOSOMAL RNA23S (RRN23S.1) |
| ATCG00950 | RIBOSOMAL RNA4.5S (RRN4.5S.1) |
| ATCG00960 | RIBOSOMAL RNA5S (RRN5S) |
| ATCG00970 | NADH dehydrogenase unit. |
| ATCG01010 | RIBOSOMAL RNA23S (RRN23S.2) |
| ATCG01180 | tRNA-Ile |
| ATCG01200 | RIBOSOMAL RNA 16S (RRN16S.2) |
| ATCG01210 | RIBOSOMAL PROTEIN S12B (RPS12B) |
| ATCG01230 | RIBOSOMAL PROTEIN S7 (RPS7.2) |
| ATCG01240 | 30S chloroplast ribosomal protein S7 |
| ATCG01250 | NADH dehydrogenase ND2 |
| ATCG01260 | tRNA-Leu |
| ATMG00090 | ribosomal protein S3 |
| ATMG00110 | Cytochrome c biogenesis protein. |
| ATMG00640 | b subunit of mitochondrial ATP synthase |
| ATMG00650 | NADH DEHYDROGENASE SUBUNIT 4L (NAD4L) |
| ATMG00660 | hypothetical protein |
| ATMG00690 | hypothetical protein |

of the gene encoding a receptor-like cytoplasmic kinase, PBL1 (for, PBS1-like 1) was selected for further analysis.

### 3.2.2.2 Functional analysis of PBL1, a candidate target gene

The PBL1 gene was defined as an interesting candidate for the following reasons:

- Two clones were amplified on methylated DNA from two independent experiments with Nd-1 mature leaves.

- FARM was well positioned in a gene promoter; sequence perfectly matched to our expectations: GATC sites on both sides of the FARM, 6 W boxes in the promoter. The sequence of the *pBL1* promoter and localization of the Farm is shown in Figure C3-6.

- This gene plays an important role in the context of plant immunity:

PBL1 is a protein closely related to the receptor-like cytoplasmic kinase BIK1 (Botrytis-induced kinase 1) that directly interacts with PAMP-responsive receptors (PRR). Activation of these PRRs results in the rapid phospholylation of PBL1 and BIK1 which then dissociate from the receptors to activate downstream signaling (Lu *et al.*, 2010; Zhang, J. *et al.*, 2010). PBL1 was also shown to trigger PTI by mediating defense induced by Pep-1, a damage-associated molecular pattern (Yamaguchi, Pearce e Ryan, 2006; Liu *et al.*, 2013). As previously reported in the introduction (chapter xxx), PBL1 is also targeted by the AvrPphB effector which leads to the disruption of the PTI signaling.

It was therefore interesting to ask whether such a gene was really targeted by RRS1-R and if this targeting modified its expression.

Several experiments were performed in order to try to identify a relationship between RRS1-R and PBL1: (i) at the level of gene expression regulation. (ii) at a functional level.

(i-a) A GUS reporter gene was fused to the *PBL1* promoter and co-expressed in *N. benthamiana* with RRS1-R as described in methods. Pop P2 which stabilizes RRS1 in the plant nucleus was also co-expressed in some samples. A repression of Gus activity driven by the PBL1 promoter was observed in the presence of RRS1-R. The repression was

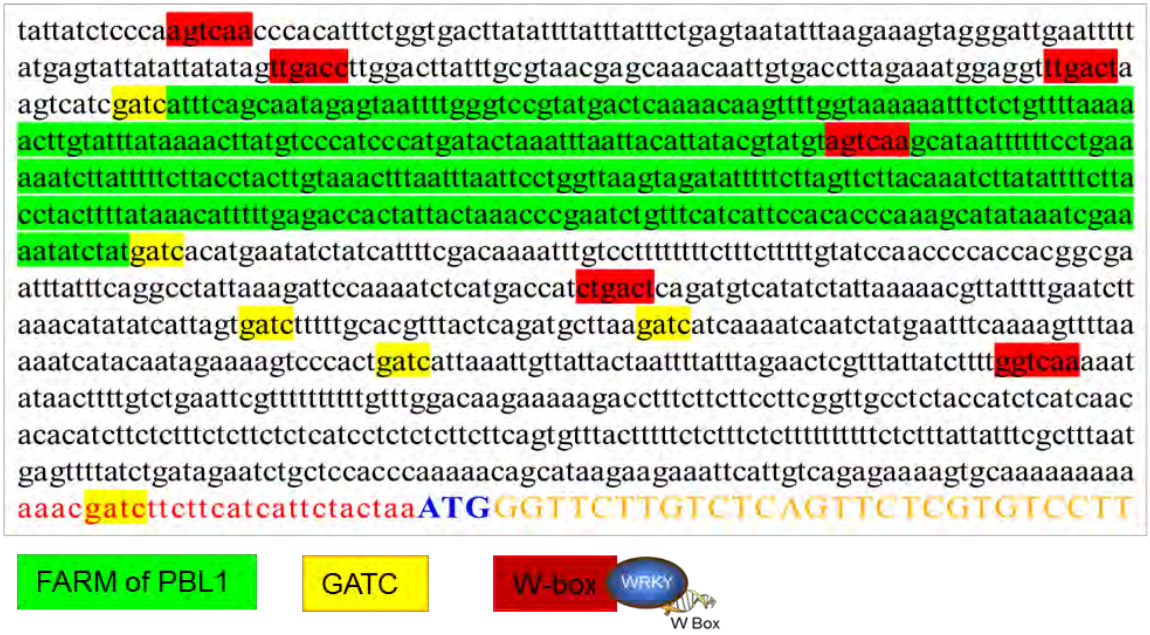



**Figure C3-6.** *PBL1* promoter sequence (Upstream -1000bp). The Fragment Associated to RRS1-R driven Methylation (FARM, green highlight) is positioned in the *PBL1* promoter. Six W-boxes (Red highlight) and GATC (yellow highlight) sites are highlighted.

stronger when RRS1 was stabilized by PopP2. PopP2 alone also repressed the PBL1 activity. This effect could result from the stabilization by PopP2 of some *N. benthamiana* RRS1-related protein. These preliminary results have nevertheless to be confirmed (FigureC3-7a).

(i-b) Transgenic Nd-1 and Col-0 *A. thaliana* plants expressing this reporter gene were obtained. They were challenged with *R. solanacearum* GMI1000 bacteria and stained for GUS expression. The *GUS* gene expression was observed in plantlets grown in vitro inoculated or not with bacteria. Bacteria or water-treated adult plants grown in soil were also stained at different time points following inoculation. The first results did not allowed us to notice any modification of expression in response to the bacteria. The expression driven by the *PBL1* promoter was from restricted to some part of the roots: elongation zone of the root apex in many cases, some cells at the base of lateral root emergence in Col-O. In Nd-1, expression was more localized to root vasculature (not shown). However tissue section should be done to conclude definitively.

(i-c) QRT-PCR experiments were performed on cDNA prepared from plant leaves at several time points following inoculation with a virulent strain or an avirulent strain showed an increased expression as soon as 6H post inoculation in all the tested interactions (Figure C3-7b).

(ii) An *A. thaliana* mutant was obtained from the NASC stock center (SAIL_1236_D07). This mutant line which was in a susceptible Col-0 background, and showed no expression of the PBL1 gene, was tested for its response to *R. solanacearum*. No significant difference was obtained in wilting symptoms between mutant and wild type plants. The mutation was then introduced in an Nd-1 background but also appeared to have no effect on the plant resistance to virulent bacteria (Figure C3-7c).

### 3.2.2.3 FARMs identification from in vitro grown Nd-1 plantlets containing or not the inducible PopP2 gene

Before setting up a whole genome analysis performed in a similar way that in leaf DNA, a preliminary identification of several FARMs obtained from the T2 plantlets was performed.

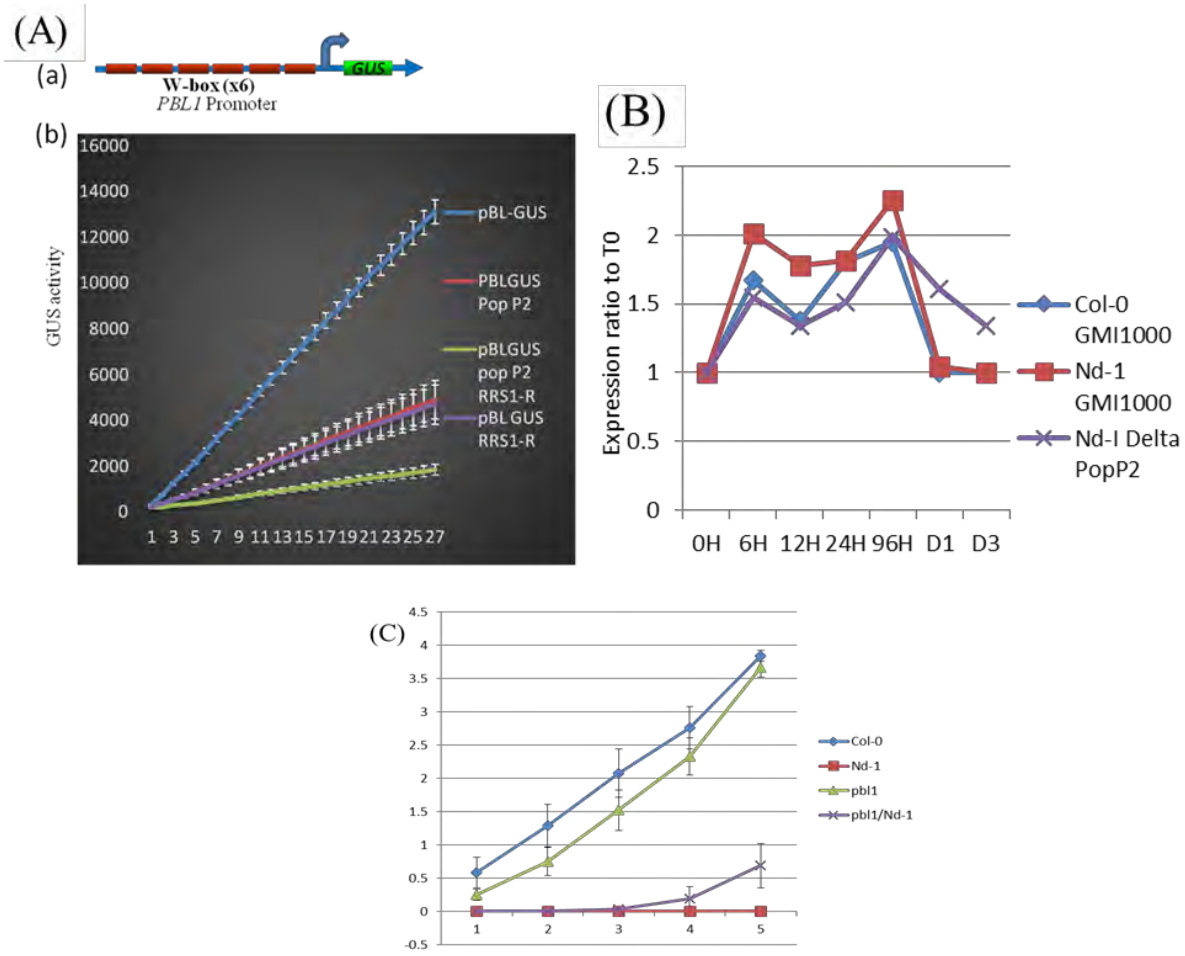



**Figure C3-7.** Expression and function of *PBL1*. (A)Evaluation of the effect of RSS1-R on the regulation of *PBL1* gene expression in *N. benthamiana*. (a) Scheme of the pPBL1: GUS reporter gene used in transcient assays. (b) Fluorometric analysis of the GUS activity: The reporter gene was expressed alone or co-expressed with PopP2, RRS1-R or both PopP2 and RRS1-R proteins. Error bars were calculated from two technical replicates (B)*PBL1* gene expression level was measured by PCR following inoculation with virulent strains (GMI1000 on Col-0, GMI1000 ΔPopP2 on Nd-1) and with an avirulent strain (GMI1000) on Nd-1. (C) 16 plants Col-0 wild type or pbl1 mutant plants, Nd-1 wild type or pbl1 mutant plants were inoculated in two independent experiments with GMI1000 bacteria. Disease index was noticed as describe in methods.

DNA from Nd-1 plantlets grown for 10 days *in vitro* on MS plates was purified from two independent experiments. Amplification products were also cloned from a control line expressing only Dam. Sequences from this background control were eliminated. Two experiments were conducted in parallel on DNA from plants expressing Pop P2. The efficiency of PopP2induction was controlled by western blot and by glucuronidase activity test, as the *uid A* gene was also present as an estradiol inducible gene in the transgenic plant (Figure C3-8). We retrieved 61 and 91 hits on tair 10 respectively without Pop P2 and with Pop P2 (Figure C3-8, Table C3-3, Table C3-4, and Figure C3-9). In both set of genes, several interesting candidates were obtained: genes responsive to *R. solanacearum* (defined in transcriptome analyses), genes responsive to PopP2 induction in the plant (transcriptome analysis, unpublished results) and genes coding for interacting partners of Pop P2 (in a two-hybrid screening performed by Laurent Deslandes). In addition, it appeared that the RPS4 and EDS1 coding sequences were putative targets of RRS1-R in Nd-1 plants (see Figure C3-10a and Figure C3-10b for FARM characteristics). As previously explained, RPS4 is a NBS-LRR protein partner of RRS1 in the recognition of effectors (Narusaka *et al.*, 2009), and interaction between TIR domains of these proteins has been demonstrated. It has been also shown that EDS1, a central regulatory hub in plant immunity, is involved in RPS4/RRS1 driven ETI in response to *P. syringae pv tomato* strain *DC3000* expressing the AvrRps4 effector (Bhattacharjee *et al.*, 2011; Heidrich *et al.*, 2011; Heidrich *et al.*, 2013). EDS1 is also necessary for full resistance to *R. solanaceraum* expressing the effector PopP2 (Laurent Deslandes, personal communication). Another interesting gene *WAT1* (walls are thin) retained our attention among RRS1-R targets in Nd-1 when PopP2 was induced. WAT1, a gene required for secondary cell-wall deposition was shown to conferred resistance to *R. solanaceraum* (Denance *et al.*, 2013). WAT1 was recently demonstrated to be a vacuolar auxin transport facilitator required for auxin homoeostasis (Ranocha *et al.*, 2013). The identification of all these FARMs, well related to our study strengthens the value of our preliminary analysis.

Unfortunately, we do not yet have access to the RRS1-R binding sites at a whole genome level and it is therefore too early to have a global view of the results. However, some conclusions (to be validated in the near future) can be drawn from the data obtained so far:

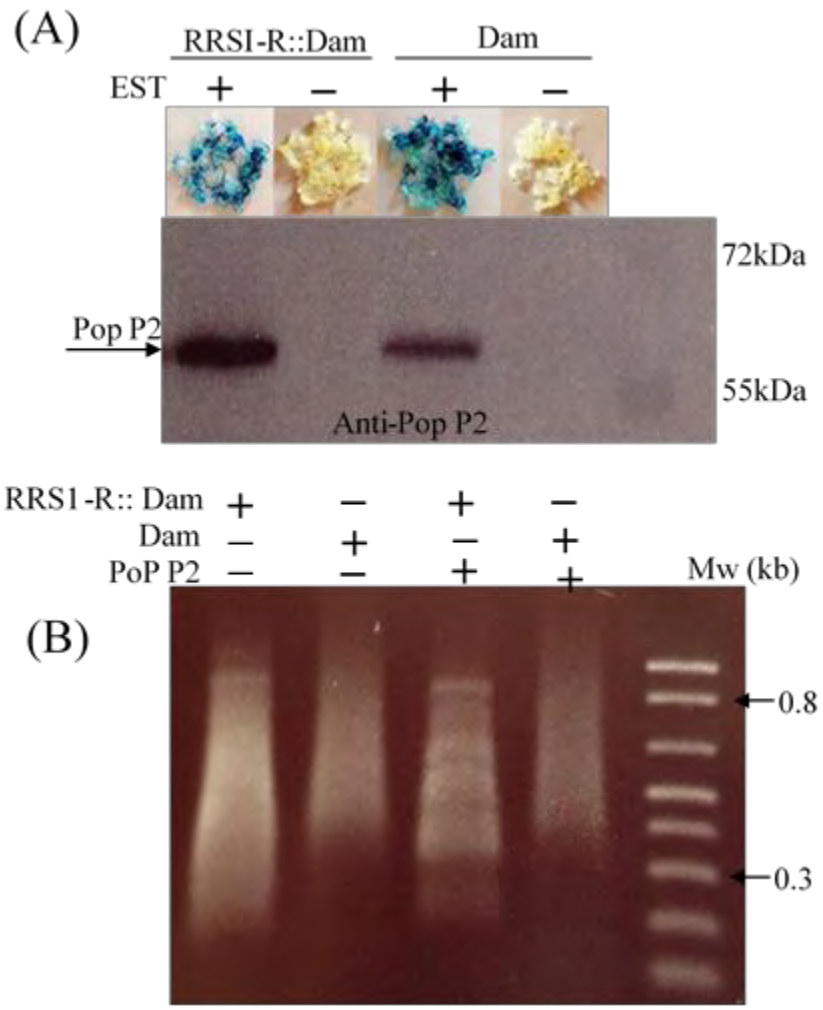



**Figure C3-8.** FARM amplification in plantlets. (A): GUS staining and Pop P2 Western blot detection in plantlets expressing an inducible GUS gene and an inducible PopP2 gene after induction by Estradiol (EST). (B): FARMs were amplified from Nd-1 (-PopP2) and Nd-1/inducible PopP2 line (+PopP2), expressing RRS1-R:: Dam or Dam alone.

| | TAIR10 | AFFY1 | Ralsto R | RRS1 | POP | Function |
|---|---|---|---|---|---|---|
| **FARMs in Nd-1** | 61 | 37 | 1 | 4 | 6 | 3 |
| **FARMs in ND/iPop** | 91 | 72 | 11 | 8 | 19 | 1 |

**Figure C3-9.** Quantitative overview of FARMS hits in comparison to several data sets. TAIR10: number of Hits on TAIR 10. AFFY1: number of Hits present on AFFY1 microarray. Ralsto R: number of Hits identified as *Ralstonia* responsive genes. RRS1: number of Hits in RRS1 responsive genes. POP: number of Hits in PopP2 related transcriptomes or in PopP2 interacting partners. Function: number of Hits with known function in response to *Ralstonia*.

**Table C3-3.** FARMs hits identified from Nd-1 plantlets without inducible Pop P2.

| AGI | annotation |
|---|---|
| AT3G44590 | 60S acidic ribosomal protein family |
| AT3G17010 | AP2/B3-like transcriptional factor family protein |
| AT5G37490 | ARM repeat superfamily protein |
| AT3G01770 | ATBET10_BET10__bromodomain and extraterminal domain protein 10 |
| AT3G48090 | ATEDS1_EDS1__alpha/beta-Hydrolases superfamily protein |
| AT1G73640 | AtRABA6a_RABA6a__RAB GTPase homolog A6A |
| AT1G70290 | ATTPS8_ATTPSC_TPS8__trehalose-6-phosphatase synthase S8 |
| AT3G58510 | DEA(D/H)-box RNA helicase family protein |
| AT5G63050 | EMB2759__embryo defective 2759 |
| AT4G30030 | Eukaryotic aspartyl protease family protein |
| AT2G17020 | F-box/RNI-like superfamily protein |
| AT4G30450 | glycine-rich protein |
| AT1G06230 | GTE4__global transcription factor group E4 |
| AT3G24450 | Heavy metal transport/detoxification superfamily protein |
| AT4G37280 | MRG family protein |
| AT5G42965 | Polynucleotidyl transferase, ribonuclease H-like superfamily protein |
| AT5G07020 | proline-rich family protein |
| AT5G38210 | Protein kinase family protein |
| AT2G07719 | Putative membrane lipoprotein |
| AT3G12915 | Ribosomal protein S5/Elongation factor G/III/V family protein |
| AT5G45250 | RPS4_Disease resistance protein (TIR-NBS-LRR class) family |
| AT3G24460 | Serinc-domain containing serine and sphingolipid biosynthesis protein |
| AT1G30240 | unknown protein |
| AT2G07713 | unknown protein |
| AT2G07776 | unknown protein |
| AT3G19274 | unknown protein |
| AT4G32020 | unknown protein |
| AT5G41774 | unknown protein |
| AT4G37800 | XTH7__xyloglucan endotransglucosylase/hydrolase 7 |
| AT2G05460 | pre-tRNA; tRNA-Cys (anticodon: GCA) |
| AT2G07683 | pseudogene of Ulp1 protease family protein |
| AT2G07703 | transposable element gene; copia-like retrotransposon family |
| AT2G07709 | transposable element gene; copia-like retrotransposon family |
| AT2G07763 | pseudogene, similar to NADH dehydrogenase |
| AT2G07809 | Pseudogene of ATMG00600 |
| AT2G07812 | Pseudogene of ATMG01100 |
| AT2G12110 | transposable element gene |
| AT2G16170 | transposable element gene |
| AT2G26220 | pseudogene, similar to phosphoenolpyruvate/phosphate translocator precursor |

| | |
|---|---|
| AT3G47330 | transposable element gene |
| AT5G37390 | transposable element gene |
| | |
| **Hits on chloroplastic or mitochondrial DNA** | |
| ATCG00190 | |
| ATCG00905 | |
| ATCG00960 | |
| ATCG00970 | |
| ATCG00980 | |
| ATCG01130 | |
| ATCG01150 | |
| ATCG01160 | |
| ATCG01170 | |
| ATMG00020 | |
| ATMG00110 | |
| ATMG00510 | |
| ATMG00530 | |
| ATMG00540 | |
| ATMG00600 | |
| ATMG00630 | |
| ATMG00810 | |
| ATMG01340 | |
| ATMG01370 | |
| ATMG01390 | |

**Table C3-4.** FARMs hits identified from Nd-1 plantlets / iPopP2.

| AGI | annotation |
|---|---|
| AT5G05600 | 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein |
| AT5G06530 | ABCG22_AtABCG22__ABC-2 type transporter family protein |
| AT4G26200 | ACS7_ATACS7__1-amino-cyclopropane-1-carboxylate synthase 7 |
| AT5G08370 | AGAL2_AtAGAL2__alpha-galactosidase 2 |
| AT5G46750 | AGD9__ARF-GAP domain 9 |
| AT4G29680 | Alkaline-phosphatase-like family protein |
| AT1G32190 | alpha/beta-Hydrolases superfamily protein |
| AT1G73480 | alpha/beta-Hydrolases superfamily protein |
| AT3G15500 | ANAC055_ATNAC3_NAC055_NAC3_NAC domain containing protein 3 |
| AT3G57040 | ARR9_ATRR4__response regulator 9 |
| AT4G29740 | ATCKX4_CKX4__cytokinin oxidase 4 |
| AT3G17310 | AtDRM3_DRM3_S-adenosyl-L-methionine-dependent methyltransferases superfamily protein |
| AT2G27050 | AtEIL1_EIL1__ETHYLENE-INSENSITIVE3-like 1 |
| AT1G47990 | ATGA2OX4_GA2OX4__gibberellin 2-oxidase 4 |
| AT1G68460 | ATIPT1_IPT1__isopentenyltransferase 1 |
| AT5G66460 | AtMAN7_MAN7__Glycosyl hydrolase superfamily protein |
| AT1G74650 | ATMYB31_ATY13_MYB31__myb domain protein 31 |
| AT5G60890 | ATMYB34_ATR1_MYB34__myb domain protein 34 |
| AT4G22753 | ATSMO1-3_SMO1-3__sterol 4-alpha methyl oxidase 1-3 |
| AT1G03780 | AtTPX2_TPX2__targeting protein for XKLP2 |
| AT1G80730 | ATZFP1_ZFP1__zinc-finger protein 1 |
| AT3G54810 | BME3_BME3-ZF_GATA8_Plant-specific GATA-type zinc finger transcription factor family protein |
| AT5G59140 | BTB/POZ domain-containing protein |
| AT3G59440 | Calcium-binding EF-hand family protein |
| AT2G41430 | CID1_ERD15_LSR1__dehydration-induced protein (ERD15) |
| AT5G11540 | D-arabinono-1,4-lactone oxidase family protein |
| AT5G23940 | DCR_EMB3009_PEL3__HXXXD-type acyl-transferase family protein |
| AT2G07727 | Di-haem cytochrome, transmembrane;Cytochrome b/b6, C-terminal |
| AT5G20250 | DIN10_RS6__Raffinose synthase family protein |
| AT5G05598 | Encodes a Defensin-like (DEFL) family protein |
| AT5G56452 | FBD-like domain family protein |
| AT3G17320 | F-box and associated interaction domains-containing protein |
| AT4G08980 | FBW2__F-BOX WITH WD-40 2 |
| AT1G33811 | GDSL-like Lipase/Acylhydrolase superfamily protein |
| AT3G14550 | GGPS3__geranylgeranyl pyrophosphate synthase 3 |
| AT1G79840 | GL2_HD-ZIP IV family of homeobox-leucine zipper protein with lipid-binding START domain |
| AT5G04290 | KTF1_SPT5L__kow domain-containing transcription factor 1 |

| | |
|---|---|
| AT5G10290 | leucine-rich repeat transmembrane protein kinase family protein |
| AT1G74660 | MIF1__mini zinc finger 1 |
| AT1G78610 | MSL6__mechanosensitive channel of small conductance-like 6 |
| AT5G46760 | MYC3__Basic helix-loop-helix (bHLH) DNA-binding family protein |
| AT4G30020 | PA-domain containing subtilase family protein |
| AT5G05590 | PAI2__phosphoribosylanthranilate isomerase 2 |
| AT1G68450 | PDE337__VQ motif-containing protein |
| AT2G43880 | Pectin lyase-like superfamily protein |
| AT3G27400 | Pectin lyase-like superfamily protein |
| AT5G04310 | Pectin lyase-like superfamily protein |
| AT5G06540 | Pentatricopeptide repeat (PPR) superfamily protein |
| AT2G27770 | Plant protein of unknown function (DUF868) |
| AT5G56450 | PM-ANT__Mitochondrial substrate carrier family protein |
| AT2G39360 | Protein kinase superfamily protein |
| AT1G69610 | Protein of unknown function (DUF1666) |
| AT1G78080 | RAP2.4_WIND1__related to AP2 4 |
| AT5G48540 | receptor-like protein kinase-related family protein |
| AT5G13250 | RING finger protein |
| AT5G13730 | SIG4_SIGD__sigma factor 4 |
| AT1G61550 | S-locus lectin protein kinase family protein |
| AT3G13445 | TBP1_TFIID-1__TATA binding protein 1 |
| AT1G80130 | Tetratricopeptide repeat (TPR)-like superfamily protein |
| AT5G67180 | TOE3__target of early activation tagged (EAT) 3 |
| AT1G15750 | TPL_WSIP1__Transducin family protein / WD-40 repeat family protein |
| AT4G17020 | transcription factor-related |
| AT4G31620 | Transcriptional factor B3 family protein |
| AT5G66690 | UGT72E2__UDP-Glycosyltransferase superfamily protein |
| AT5G61490 | Uncharacterised conserved protein (UCP012943) |
| AT1G02391 | unknown protein |
| AT1G80133 | unknown protein |
| AT2G07738 | unknown protein; |
| AT2G07795 | unknown protein; |
| AT2G30032 | unknown protein; |
| AT2G36030 | unknown protein; |
| AT2G39370 | unknown protein; |
| AT3G14560 | unknown protein; |
| AT3G54802 | unknown protein; |
| AT3G54804 | unknown protein; |
| AT4G17010 | unknown protein; |
| AT4G29735 | unknown protein; |
| AT4G36170 | unknown protein; |

| | |
|---|---|
| AT5G13260 | unknown protein; |
| AT5G44574 | unknown protein; |
| AT5G44575 | unknown protein; |
| AT1G75500 | WAT1__Walls Are Thin 1 |
| AT2G07739 | Ycf1 protein |
| AT5G13740 | ZIF1__zinc induced facilitator 1 |
| AT2G01010 | rRNA; 18SrRNA |
| AT2G07759 | pre-tRNA; tRNA-Ser (anticodon: GCT) |
| AT4G17005 | transposable element gene; copia-like retrotransposon family |
| | |
| **Hits on chloroplastic or mitochondrial DNA** | |
| ATMG00320 | |
| ATMG00330 | |
| ATMG00516 | |
| ATMG00520 | |

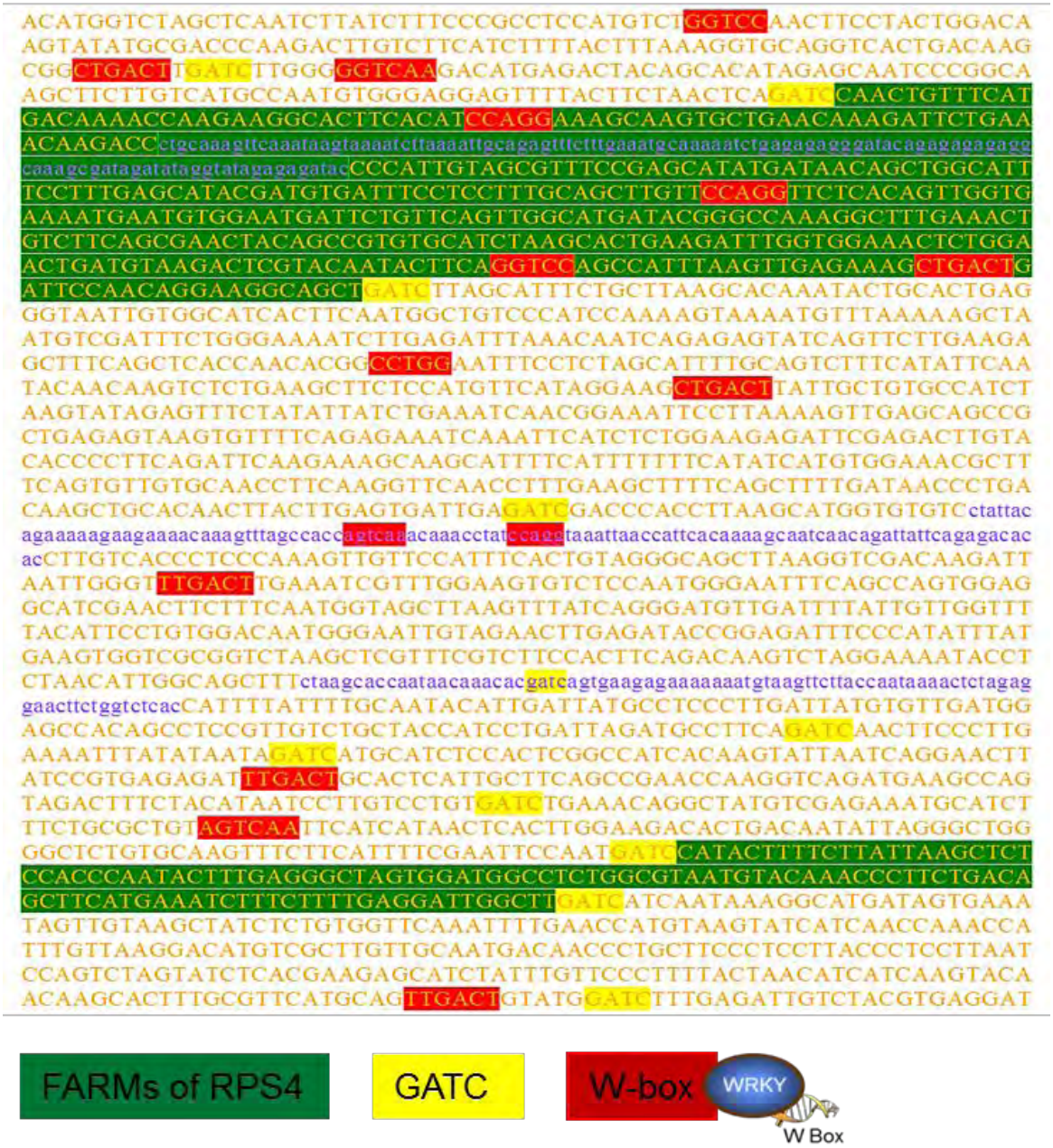



**Figure C3-10a**. FARMs within RPS4 and EDS1 coding sequence.

Fragments Associated to RRS1-R driven Methylation (FARM, green highlight) are localized in the coding sequence. Several W-boxes (Red highlight) and GATC (yellow highlight) sites are highlighted.

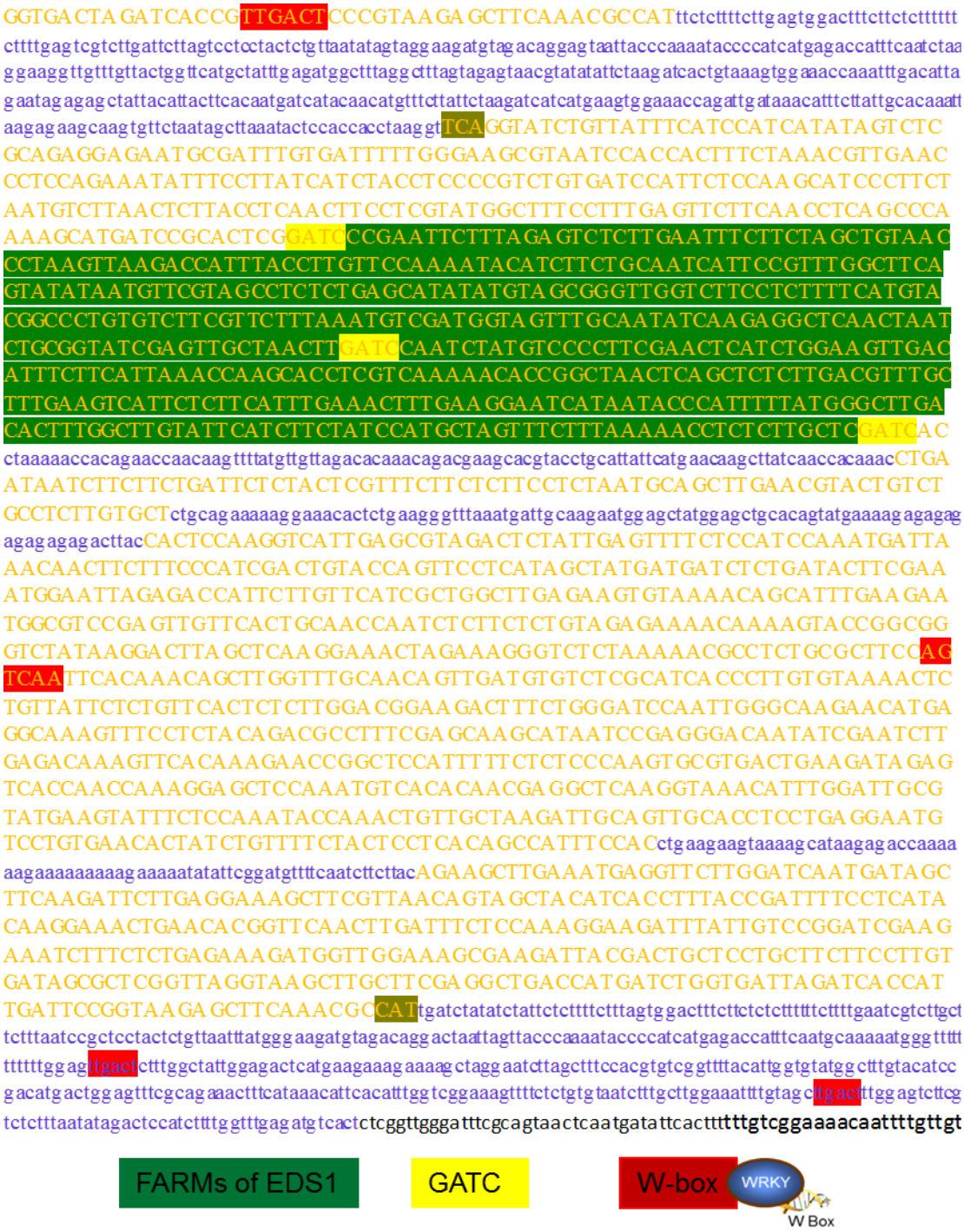



**Figure C3-10b**. FARMs within RPS4 and EDS1 coding sequence.

Fragments Associated to RRS1-R driven Methylation (FARM, green highlight) are localized in the coding sequence. Several W-boxes (Red highlight) and GATC (yellow highlight) sites are highlighted.

-RRS1-R is located both on promoter and coding sequences in the nucleus.

- RSS1-R is also able to bind to some chloroplastic and mitochondrial DNA sequences

-RRS1-R binding sites localization is probably different in Nd-1 plantlets and in Nd-1 expressing an inducible form of PopP2: we observed much more binding to promoter sequences in the presence of the effector protein.

## 3.3 Discussion

In order to identify the *in vivo* binding sites of RRS1-R, a DamID approach was developed. A pilot experiment was performed using *A. thaliana* Nd-1 leaves from plants grown in a greenhouse. Most sequences identified corresponded to gene promoter regions in this experiment, suggesting that RRS1-R might participate to the regulation of the expression of the corresponding genes under these conditions. We focused our interest on a gene encoding PBL1, a kinase mainly involved in PTI. Preliminary results obtained in transient expression experiments in *N. benthamiana* suggest that RRS1-R negatively regulates gene expression. The existence and implication of such a regulation in *A. thaliana* remains unknown. It can be proposed that this regulation allows to finely adjust the levels of PBL1 involved in PTI. It would be interesting to check whether under pathogen attack (or under less favorable conditions for the plant than the greenhouse), RRS1-R is released from PBL1 promoter, allowing an increase expression of this gene.

Several other genes such as *PYL4, NFY-B2, SAUR27, and PR1* were also identified (Table C3-2). These genes were indeed already detected by our transcriptomic analyses. The validation of these genes is underway and will allow to check whether RRS1-R is really directly involved in their regulation.

This first experiment suggested that the Dam ID approach is appropriate to identify *in vivo RSS-1* DNA targets. We therefore initiated a study aimed at the identification of RRS1-R DNA binding sites in the presence or not of the cognate avirulence protein PopP2. This experiment was performed using plantlets, grown under aseptic and under rich nutrient medium conditions. The inducible induction of PopP2 in Nd-1 plants leads to a specific effector-triggered immunity in response to a *R. solanacearum* mutant strain that does not

express PopP2 (Neil Ledger unpublished, not shown). Previous studies in the group also showed that gene expression in plants expressing an inducible *PopP2* gene, overlaps, to some extent, to that of plants inoculated with *R. solanacearum* (unpublished results). We therefore assume that Pop P2 induction leads to the activation of similar molecular mechanisms than the ones associated to the responses to *R. solanacearum.*

An overview of the candidate RRS1 target genes identified using the DamID approach in unchallenged Nd-1 plants, leads us to propose that the RRS1-R protein is associated to the coding regions of genes or to transposons or pseudogenes. Transposons and pseudogenes are usually located in repressed chromatin regions. Repression of transcription arises from different types of mechanisms. Passive repression relies on mechanisms of steric hindrance to counteract the function of transcriptional activators. Active repression is rather due to an intrinsic repressive capacity, conferred by repression domains, or/and by recruitment of regulators, including chromatin remodeling factors that can promote the formation of a repressive chromatin state (Krogan e Long, 2009). Transcription factors binding to coding regions are well documented in animals as paused complexes of transcription, mainly at the 5' end of the coding sequence of a gene. Genes exhibiting engaged and paused transcription complexes are supposed to present a permissive chromatin status that allow genes that are transcribed at low basal levels to be constantly accessible and primed for bursts of transcription activation in response to specific signals (Adelman e Lis, 2012). It is therefore assumed that the genes targeted in their coding sequences by RRS1-R are "silenced" and also "primed" for subsequent activation by various stimuli. Interestingly, the coding regions of *RPS4* and *EDS1*, encoding major partners of RRS1 proteins for the establishment of resistance, were identified as targets of RRS1-R in Nd-1 plants. This observation suggests that the expression of these genes may be subject to such a control by RRS1-R in unchallenged plants. Following PopP2 induction, RRS1-R is bound to regions corresponding to promoters of genes where it could, in cooperation with other regulatory proteins, modulate gene transcription. However, our FARM cloning approach, coding sequence and promoter sequence of the same gene was not characterized without and with PopP2 and more data will be necessary to conclude.

This study is still underway and our hypotheses will have to be reevaluated after the completion of the analysis at a whole genome scale. Nevertheless, we propose a model presented in Figure C3-11. We hypothesize that RRS1-R is associated to "silencing" complexes to inhibit autoimmunity in "naïve" (uninfected) plants which are not submitted to stress. This mechanism allows a rapid induction of gene activity in response to various stimuli. The relocalization of RRS1-R to promoter sequences in presence of PopP2 would then affect this silencing status. Interestingly, PopP2 has been shown to autoacetylate and to acetylate several of its targets, among which bromodomain proteins known to interact with histones (Tasset *et al.*, 2010). In addition, the expression of six histone deacetylase encoding genes (out of a total of sixteen genes in *A. thaliana*) is modified in response to PopP2 induction in plants (unpublished results). Acetylation of histones is an important epigenetic modulator, regulating DNA accessibility by controlling chromatin structure (Figure C3-12). Mechanisms of transcriptional regulation crosstalk with structure/ epigenetic regulation of gene expression are already well documented as a strategy developed by pathogens to manipulate their hosts (Bierne, Hamon e Cossart, 2012). More generally, these epigenetic mechanisms play a major role in the plant responses to environmental stresses (Berr *et al.*, 2012; Gutzat e Scheid, 2012).

## 3.4 Conclusion and perspectives

Identification of in vivo targets from the pilot experiment, performed on plantlets expressing or not PopP2, allowed identification of genes associated to cloned FARMS that could represent RRS1-R putative targets. The following experiments will be now a priority:

- Farms DNA sequences will be analyzed to identify W boxes. Then interaction of RRS1-R to selected boxes will be controlled by ChIP. This validation step will constitute a key point. To this end a tagged version of RRS1-R, in a Pop P2 inducible background to ensure stabilization of the RRS1 protein will be used.

- The impact of binding on gene expression will be monitored. To this end, the biological material prepared for the global approach will be used for gene expression evaluation by qRT-PCR, which is going on. A correlation between binding of RRS1-R on a FARM with

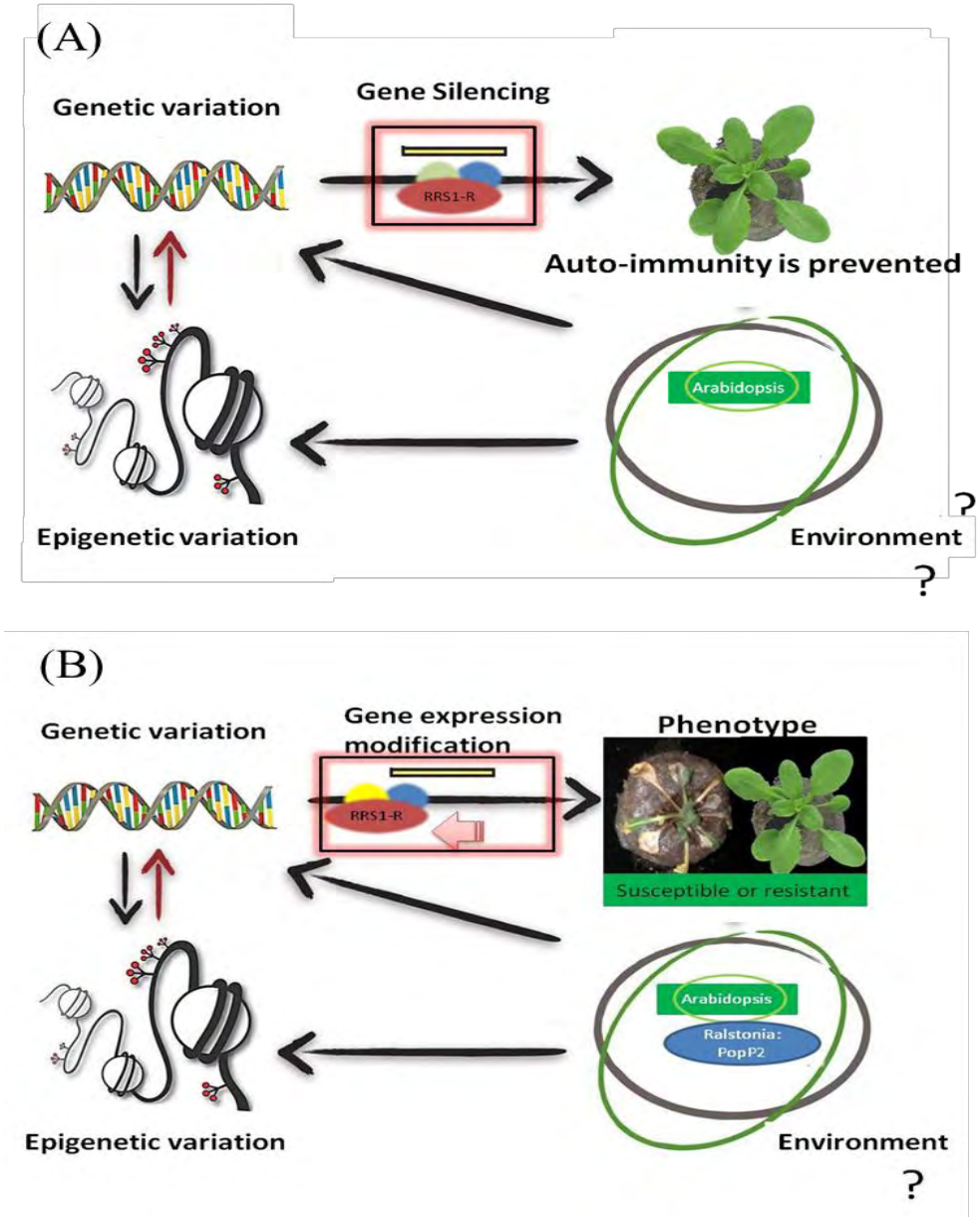



**Figure C3-11.** Model for RSS1-R mode of action. RRS1-R represses expression of genes related to defense, which leads to inhibition of autoimmunity. In the presence of PopP2 the repression is lifted. The pink arrow depicts the delocalization of regulatory complexes containing RRS1-R allowing the alteration of expression of defense associated gens.

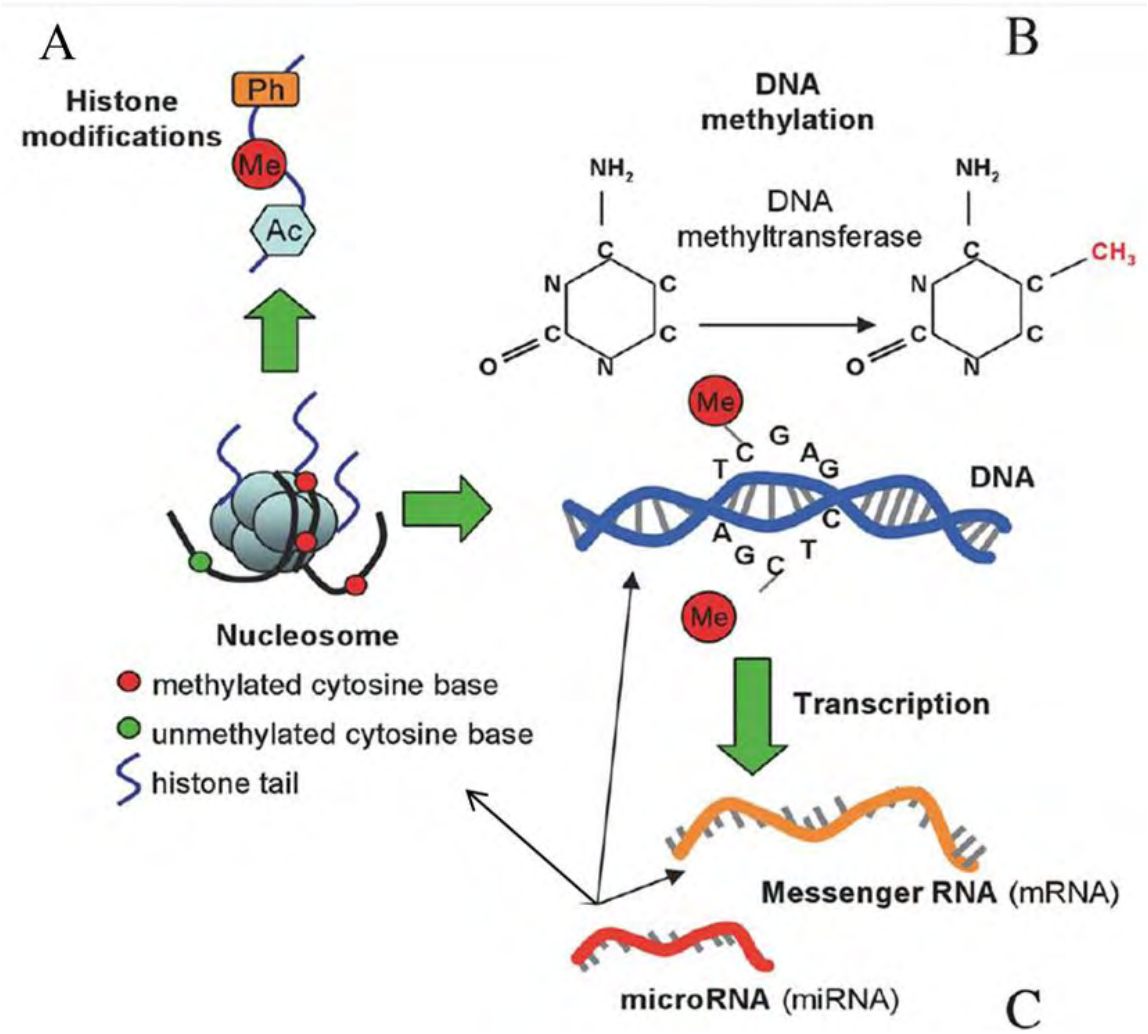



**Figure C3-12.** Types of epigenetic modifications. (A) Histones can undergo phosphorylation (Ph), methylation (Me), and acetylation (Ac), among other chemical modifications. These modifications are involved in chromatin remodeling and transcriptional regulation. (B) DNA molecules are methylated by the addition of a methyl group to carbon position 5 on cytosine bases, a reaction catalyzed by DNA methyltransferase enzymes, which maintains repressed gene activity. (C) Noncoding RNA (ncRNA) regulate these processes. (Adapted from Gomez-diaz et al. 2012).

or without PopP2 and modification of the expression level of associated genes will also validate our data.

- Susceptible plants expressing the RRS1-S: Dam fusions have also been generated. A comparative analysis in plantlets expressing or not Pop P2 will indicate if specificity of response is putatively driven at least in part at a transcriptomic level by RRS1.

Our future prospects aim:

(i) At the identification of RRS1-R and RRS1-S specific DNA binding sites in adults plants: on a global genome scale, under different environmental conditions, in plants resistant, or not, to pathogenic bacteria.

(ii) At the understanding of transcriptional dynamics associated to RRS1 proteins by high throughput RNA sequencing. These approaches will be developed in root-specific tissues where RRS1 genes have been shown to be mainly expressed. Indeed due to the soil born nature of R. solanacearum and to its mode of invasion, it is highly probable that important components of the interaction are present at the root level. Interestingly, RRS1-R and RRS1- S genes are mainly expressed in roots, more precisely in pericycle cells and in some endodermal cells located at the protoxyleme poles, known to be, from cytological observations, the preferential access points of bacteria to xylem vessels. Focusing especially at target loci in these cells should give a pertinent and sensitive picture of RRS1 binding to DNA in the context of immunity against *Ralstonia solanacearum*. Our objective is to use plants expressing RRS1-R or RRS1-S fused to Dam to develop the INTACT system (Deal e Henikoff, 2010; Deal e Henikoff, 2011). This latter strategy will enable us to purify, using the strong biotin-streptavidin interaction property, labeled nuclei from specific tissues, through transgenic expression of a nuclear targeting fusion protein (NTF) in the root pericycle and in the endoderm. The NTF protein is a chimeric protein, composed of a domain necessary for association with the nuclear envelope, GFP for visualization and a biotin ligase recognition peptide. It will be expressed under the control of the RRS1 promoter which is active in these tissues. In planta biotinylation of nuclei will be achieved by the constitutive expression of a Biotin ligase (BirA). In order to determine the optimal conditions (in particular the sampling times) to perform these experiments, the binding of

RRS1-R to some of its identified loci targets will be analyzed during the interaction with R. solanacearum by looking at methylation status of these loci during a time course experiment. In parallel, transcript accumulation corresponding to the studied genes will be quantified by qRT-PCR experiments.

Although many studies have been devoted to transcriptional changes in response to plant pathogens, a tissue-specific link between chromatin occupancy and transcription would provide unprecedented insight into the dynamics of transcription regulation in response to biotic stress. After all, by using similar approaches it will be possible to perform comparative analyses of target genes more generally under any biotic or abiotic stress.

# Chapter IV

## *Hrp* mutant bacteria as biocontrol agents

## 4.1 Introduction

Bacterial wilt, caused by the *R. solanacearum* species complex, inflicts severe economic losses in many crops worldwide. Because of its aggressiveness, large host range, broad geographical distribution and long persistence in soil and water environment, *R. solanacearum* ranks among the most devastating pathogens in *solanaceous* crops. (Elphinstone, 2005; Genin e Denny, 2012). Host resistance to this pathogen remains the most effective control strategy against this disease. However resistance genitors released to date are not stable over regions, due to the huge phenotypic and genomic plasticity of the pathogen, its great variability across sub-regions and to the significant genotype x environment interactions in the resistance expression. Therefore, alternative control measures to these bacteria, such as biological control which consists in applying living organisms called biocontrol agents, have been investigated with an increased interest. More especially biocontrol experiments with Hrp- mutants of *Ralstonia solanacearum* that are still able to colonize plants and to multiply to some extent without causing disease have been undertaken (Trigalet e Demery, 1986). The authors showed that a preinoculation with hrp mutants of *R. solanacearum* lead to high rate of protection against a subsequent inoculation with a virulent strain on tomato (Trigalet e Trigaletdemery, 1990). These results motivated further research on the mechanisms involved in protection. This system was therefore adapted from tomato to *A. thaliana* to facilitate the study of the mechanisms underlying protection (Feng *et al.*, 2012). These results motivated a writing of a review focusing on on induced resistance obtained by plant inoculation with bacteria mutated in *hrp* genes (Hrp mutants).

# *Hrp* mutant bacteria as biocontrol agents: towards a sustainable approach in the fight against plant pathogenic bacteria

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**Keywords:** *hrp* mutant, phytopathogen, biological control, ABA, basal resistance, bacteria.

**Abbreviations and acronyms used throughout the text:**

ABA**,** Abscisic acid

*A. thaliana, Arabidopsis thaliana*

BCA, Biological Control Agent

*E. amylovora, Erwinia amylovora*

*Hrp,* Hypersensitive Response and Pathogenicity

*P. syringae, Pseudomonas syringae*

PAMP, Pathogen-Associated Molecular Pattern

PTI, Pamp Trigerred Immunity

*Ralstonia solanacearum, R. solanacearum*

*T3SS,* Type Three Secretion System

*X. campestris, Xanthomonas campestris*

**Abstract.**

Sustainable agriculture necessitates development of environmentally safe methods to protect plants against pathogens. Among these methods, application of biocontrol agents has been efficiently used to minimize disease development. Here we review current understanding of mechanisms involved in biocontrol of the main Gram- phytopathogenic bacteria-induced diseases by plant inoculation with strains mutated in *hrp* (*hypersensitive response and pathogenicity*) genes. These mutants are able to penetrate plant tissues and to stimulate basal resistance of plants. Novel protection mechanisms involving the phytohormone abscisic acid appear to play key roles in the biocontrol of wilt disease induced by *Ralstonia solanacearum* in *Arabidopsis thaliana*. Fully understanding these mechanisms and extending the studies to other pathosystems are still required to evaluate their importance in disease protection.

## Introduction.

Diseases have a major impact on plant yield, quality and safety. Disease control constitutes therefore a major challenge for agriculture. One option for controlling plant disease consists in developing synthetic chemicals respecting public health and environment. Alternatively, using living organisms called biocontrol agents (BCA) constitutes a way to biologically control pests or pathogens and is a potentially important component of sustainable agriculture.

Prior exposure to eliciting organisms renders frequently plants more tolerant to subsequent infection. Non-pathogenic Rhizobacteria termed Plant-Growth-Promoting Rhizobacteria (PGPR) induce the well documented induced systemic response (ISR) (Lugtenberg e Kamilova, 2009). Systemic acquired resistance (SAR) is another well-known form of resistance induced *via* local inoculation of a pathogen and provides long-term resistance to subsequent attack (Durrant e Dong, 2004).

In contrast, resistance induced by plant inoculation with bacteria mutated in *hrp* genes (for *h*ypersensitive *r*esponse and *p*athogenicity), namely *hrp* mutants, remains poorly documented.

To successfully infect a plant, bacterial pathogens have to counteract plant defense mechanisms and redirect host metabolism for nutrition and growth. Type III Secretion System (T3SS) is a major determinant of pathogenicity of many Gram-negative bacteria. It allows delivery within plant cells, of a battery of proteins so-called type III effector proteins known to collectively suppress plant defence and to favour bacterial multiplication and nutrition (Hueck, 1998; Galan e Collmer, 1999). *Hrp* genes, required to set up a functional T3SS, are necessary for disease development in susceptible plants and elicitation of the hypersensitive response in resistant plants. They are highly conserved across the main Gram-negative phytopathogenic lineages and exhibit extensive homologies with their animal counterparts, thus establishing a link between plant and animal pathology (Lindgren, Peet e Panopoulos, 1986). These genes have been grouped in three classes. The first class includes genes highly conserved among diverse animal and plant pathogenic bacteria and are named *hrc* (*hrp*-conserved). The second class contains transcriptional regulators of

T3SS regulon genes whereas the third one includes structural proteins and some secreted proteins like chaperone or other post-transcriptional regulatory proteins. *Hrp* genes clustered in pathogenicity islands have been subjected to intensive mutagenesis leading, in most of the cases, to loss of pathogenicity (Tampakaki *et al.*, 2010).

The great majority of studies on *hrp* mutants aimed at a better understanding of the role of *hrp* genes. This review will focus on the plant responses to *hrp* mutants in order to gain some insights on their protective effect against virulent bacteria.

*Hrp* mutants were indeed used to reduce or completely abolish disease symptoms caused by virulent bacteria in several pathosystems involving the main Gram-negative phytopathogenic bacteria (*Pseudomonas syringae, Ralstonia solanacearum, Erwinia amylovora, Xanthomonas campestris species)*. Natural occurrence of *hrp* mutants in the environment was recently demonstrated, making them potential interesting BCA (Demba Diallo *et al.*, 2012).

In this review, we first describe how *hrp* mutants colonize plants and induce host responses, focusing on the bacterial species mentioned above. The molecular mechanisms underlying biocontrol exerted by the *R. solanacearum hrp* mutants in the model plant *A. thaliana* are then presented in more details.

**Plant/*Hrp*- mutant bacteria interactions.**

**Infectiveness and invasiveness of *hrp* mutants:**

*Hrp* mutants are prototrophic and generally not impaired in their ability to grow in culture (Lindgren, Peet e Panopoulos, 1986). Most of them are able to colonize and invade, to some extent, plant tissues (Lindgren, 1997). *hrp* mutants are generally able to enter into the apoplastic compartment, and to invade plant tissues although their multiplication in a susceptible host is affected.

A well-documented example concerns the vascular pathogen *R. solanacearum,* the causative agent of bacterial wilt disease, that infects plants through root tips and lateral root cracks and reaches xylem vessels leading to their spread throughout the host (Yabuuchi *et*

*al.*, 1995). Most *R. solanacearum* mutants altered in different *hrp* genes could be detected, after tomato root inoculation, within similar tissues than wild type strains, i.e root tips, lateral root emergence sites and root xylem vessels. However, they propagated only in the lower part of the stem and did not reach the fruits (Frey *et al.*, 1994). Bacteria numeration in root collar and stem, as well as microscopic observations, showed that some of the *hrp* mutants were significantly impaired in their ability to multiply and colonize tomato plants (Vasse *et al.*, 2000). On petunia, *R. solanacearum hrp* mutants failed to induce the formation of root lateral structures that constitute efficient colonization sites allowing extensive bacterial multiplication (Zolobowska e Gijsegem, 2006).

*Hrp* mutants from other Gram-negative phytopathogenic bacteria that are able to invade their hosts have been also described. In the case of *E. amylovora*, the agent of fire blight, bacteria penetrate the plant apoplast primarily *via* natural openings in flowers or through wounds on young aerial vegetative parts (Billing *et al.*, 1983). *E. amylovora hrp* mutants could be detected in xylem vessels but formation of lysigenous cavities (structures appearing in the later stages of infection and filled with bacteria) were never observed (Faize *et al.*, 2006). *P. syringae* bacteria, that elicit leaf spots and other foliar necroses in host plants, enter *via* stomata or wounding sites. Then bacterial colonization becomes systemic *via* the host vascular system (Hirano e Upper, 1990). In *A. thaliana* leaves, efficient multiplication of *P. syringae hrp* mutants was impaired in comparison to wild type strain multiplication (Hauck, Thilmony e He, 2003). In cantaloupe, *P. syringae hrp* mutants inoculated in seedlings, were detected in plant tissues but population stabilized around the initial size after inoculation (Demba Diallo *et al.*, 2012). *X. campestris* virulent bacteria, infect plants through hydathodes at the leaf margins or through stomata and colonize the vascular system (Hayward, 1993), causing tissue necrosis and severe leaf wilting symptoms (Williams, 1980; Onsando, 1992). Similarly, *X. campestris hrp* mutants failed to grow to the extent of wild type in plant tissues as attested by population counts or microscopy observations (Bonas *et al.*, 1991; Brown *et al.*, 1998).

**Plant responses to *hrp* mutants:**

Although *hrp* mutants do not trigger any disease or HR symptoms, inoculated plants often

display important developmental, molecular and biochemical alterations, thereby suggesting the elaboration of plant defense responses.

Following pathogen attack, the first line of active plant defense, called basal defense or PTI (Pathogen-associated molecular patterns -PAMP- triggered immunity), involves plant pathogen recognition receptors, the pattern-recognition receptors (PRRs) that recognize PAMPs. This perception triggers many signalling events through cGMP, mitogen-activated protein kinases (MAPKs), $Ca^{2+}$ and $H^{+}$ influxes, early accumulation of reactive oxygen species, cell-wall thickening leading in some cases to papillae formation, and altered expression of many genes (Zipfel e Robatzek, 2010). Proteins involved in primary metabolism, redox modulation, molecular chaperoning and cytoskeleton rearrangement are some of the key components of the PTI (Zimaro *et al.*, 2011). In addition, PAMPs modify mitochondrial and chloroplast proteome and reconfigure proteins into membrane rafts enabling efficient host signal transduction and downstream responses after the initial recognition (Jones *et al.*, 2004; Jones *et al.*, 2006).

Cellular, molecular and metabolic changes observed upon inoculation by *hrp* mutant strains, clearly indicate that basal defense mechanisms are generally highly induced.

In different host plants, localized strengthening of cell walls due to the accumulation of hydroxyproline-rich glycoproteins, phenolics and callose is often detected in cells adjacent to the inoculation sites of *X. campestris* and *P. syringae hrp* mutants (Brown *et al.*, 1998; Hauck, Thilmony e He, 2003). In Lettuce, in response to *P. syringae hrp* mutants, cell wall alterations were associated with $H_2O_2$ accumulation and increases in peroxydase activity, which probably strengthens plant cell wall structures (Bestwick, Brown e Mansfield, 1998). In *A. thaliana* tissues responding to *P. syringae hrp* mutant, a rapid flux of indole carboxylic acid compounds to the cell wall correlates with a limitation of bacterial multiplication (Forcat *et al.*, 2010). In response to inoculation by *R. solanacearum hrp* mutants, vascular coating, a non-specific plant defense reaction, was observed on tomato roots (Vasse *et al.*, 2000).

Changes in chloroplastic and mitochondrial leaf nuclear proteomes were also described in *A. thaliana* after *P. syringae hrp* mutant inoculation, which reveals a regulation of primary

metabolism through redox-mediated signaling components and the existence of a rapid communication system between organelles (Jones *et al.*, 2006).

Plant gene expression was monitored following *hrp* mutant inoculation in several pathosystems. Pioneering work by Jakobek and Lindgren identified defense-associated transcripts, such as phenylalanine-ammonia-lyase (PAL), chalcone synthase, chalcone isomerase and phytoalexins, accumulating in bean following challenge by a *hrp* mutant of *P. syringae* (Jakobek e Lindgren, 1993). More recently, several studies established that inoculation with *hrp* mutants leads to an extensive reprogramming of gene expression, a requirement for elaboration of immune responses during plant–pathogen interactions (Kazan *et al.*, 2001; Truman, De Zabala e Grant, 2006; Feng *et al.*, 2012). In the study of Truman et al, a set of genes induced by *hrp* mutants whose expression is also modulated in response to many PAMPs and to virulent *P. syringae* strains, was proposed to represent the primary host response to bacterial infections(Truman, De Zabala e Grant, 2006). Transcriptional reprogramming was also investigated in *A. thaliana* following root inoculation with a *R. solanacearum hrp* mutant strain. Despite the absence of apparent symptoms, in response to *hrp* mutants, many plant genes were regulated in a similar way than after inoculation of a susceptible plant with a *R. solanacearum* virulent strain (Hu *et al.*, 2008; Feng *et al.*, 2012). 27% of the up-regulated genes are related to abscisic acid (ABA) biosynthesis and signalling according to Li et al (Li *et al.*, 2006). Additionally, several *A. thaliana* mutants altered in the biosynthesis (*aba1-6*) or signalling (*abi1-1, abi2-1*) associated to this hormone exhibit an altered response to *R. solanacearum* (Hernandez-Blanco *et al.*, 2007). Interestingly, among these ABA-related genes, several genes are also responsive to *P. syringae hrp* mutants in the early stages of infection (Kazan *et al.*, 2001), suggesting that ABA signaling is also associated in plant response to *P. syringae hrp* mutants (our unpublished observations). It is noteworthy that according to genetic approaches, the limited multiplication of *P. syringae hrp* mutants monitored in *A. thaliana* leaves was not related to SA- or ethylene-mediated mechanisms (Hauck, Thilmony e He, 2003). Actually, the effect of ABA in this process remains to be evaluated. The importance of ABA in plant responses to *hrp* mutant is also strengthened by the fact that it does positively regulate callose deposition, a plant basal defense response-related which is stimulated following *hrp* mutant inoculation (Trigalet e Trigaletdemery, 1990; Wilson *et*

*al.*, 2002).

Altogether, these data support well the enhancement of plant basal defenses in response to *hrp* mutant inoculation. Molecular mechanisms underlying this response remain to be fully elucidated and one can question their importance in protecting plants against virulent bacteria. Actually, *hrp* mutants have been successfully used in bioprotection experiments. For instance, when *X. campestris pv. vesiculata hrp* mutants were inoculated on tomato leaves prior to inoculation with wild type virulent strains, disease severity was reduced, both under controlled and field conditions (Moss *et al.*, 2007). *Hrp* mutants of *P. syringae* pv. *tomato* strain DC3000 were also able to provide significant reductions in bacterial speck severity on tomato caused by a subsequent inoculation with wild type bacteria, under greenhouse conditions (Wilson *et al.*, 2002). *E. amylovora hrp* mutants were effective in controlling fire blight disease when inoculated on apple seedlings or apple flowers (Faize *et al.*, 2006). *Hrp* mutants of *R. solanacearum* were able to protect susceptible tomatoes from virulent strains under growth chamber conditions or green-house conditions (Trigalet e Trigaletdemery, 1990; Frey *et al.*, 1994; Etchebar *et al.*, 1998). Molecular mechanisms occurring after inoculation of protected plants with virulent *R. solanacearum* bacteria have been investigated in *A. thaliana* (Feng *et al.*, 2012). The following chapter will focus on biocontrol resulting from *R. solanacearum hrp* mutant inoculation which it is to date the best documented interaction.

**Plant protection against *R. solanacearum* triggered by *hrp* mutants.**

Wilt disease caused by the soil-borne bacteria *R. solanacearum* is of substantial economic importance due to its broad host range, aggressiveness and long persistence in soils. Means to control this disease are limited. Thus, alternative ways to control disease such as biological control have been investigated with an increasing interest. In this context, mutant strains able to colonize tomato plants without causing disease symptoms have been tested for their protective effect (Trigalet e Demery, 1986). The authors showed that root pre-inoculation with a *hrp* mutant leads to high protection rate against a subsequent inoculation with virulent strains (Trigalet e Trigaletdemery, 1990). Furthermore, this strategy provided a durable protection by persisting several months within the plant without affecting fruit

number and weight (Frey *et al.*, 1994). Protection was also achieved in the model plant *A. thaliana* using a similar approach (Feng *et al.*, 2012). *A. thaliana* plants were inoculated with a *hrpB* regulatory mutant and simultaneously or subsequently challenged with the wild type virulent *R. solanacearum* strain. HrpB regulatory activity is well characterized and its contribution to *R. solanacearum* virulence resides essentially in the control of T3SS function (Genin e Denny, 2012). Simultaneous root inoculation by both the wild type and *hrp* mutant strains did not induce protection, although the mutant strain was favored by a high mutant to wild type strain inoculum ratio. These results suggested that protection may not be caused by a spatial competition between the two strains as previously proposed (Etchebar *et al.*, 1998). Indeed, when both *hrp* and virulent *R. solanacearum* strains were co-inoculated in tomato, they colonized separate xylem vessels (Etchebar *et al.*, 1998). (Similar observations had been made in apple seedlings inoculated simultaneously with a *hrp* mutant and a wild type strain of *E. amylovora* (Faize *et al.*, 2006). On the other hand, a subsequent inoculation with the virulent strain allowed a high protection rate associated with a decrease in the multiplication of the virulent strain. The delay required between *hrp* mutant and wild type strain inoculations suggested that some plant signalling pathways had to be established before inoculation of virulent bacteria. Heat-killed *hrp* mutant bacteria were also able to induce resistance but to a lower extent than live ones, which suggested that an active metabolism for both partners was required for full protection. Genetic analyses established that, despite the fact that this mode of protection by root inoculation resembles ISR, neither jasmonic acid, nor ethylene participated in the establishment of this resistance which rather relies on ABA signaling (Feng *et al.*, 2012). As previously mentioned, *hrp* mutant inoculation in *A. thaliana* led to extensive genome re-programming (Feng *et al.*, 2012). Subsequent inoculation of protected plants with the virulent strain indeed reversed the expression of 70% of the genes whose expression was altered by the *hrp* mutant pre-inoculation. This reprogramming affected many ABA-related genes, associated with disease development. Thus, upon inoculation of protected plants by a virulent *R. solanacearum*, the pattern of modulation of gene expression is opposite to the pattern of expression observed after infection of unprotected plants. Regulation of disease-associated genes in *hrp* mutant protected plants may have generated a hostile environment for the invading pathogen and a priming of resistance through stimulation of yet unknown

pathways by *hrp* mutants cannot be excluded.

## Opening questions:

Mechanisms underlying the biological control using *hrp* strains remain poorly understood. By using *R. solanacearum*, a soil borne vascular pathogen, it was shown that the molecular basis for *hrp*-induced protection differs from the well-studied mechanisms underlying SAR and ISR and has yet to be fully explored.

The prominent role of ABA in this process requires additional studies. This phytohormone has emerged as a crucial actor in plant stress monitoring (Kim, 2012). A model has been proposed involving ABA as a multifaceted actor, depending on the phase of the infection and the nature of a given microorganism (Ton, Jurriaan, Flors, Victor e Mauch-Mani, Brigitte, 2009). Its intricated role in the plant response to pathogens, driving increased resistance or increased susceptibility depending on the case, is documented in a recent publication (Denance *et al.*, 2013). Typically, it is plausible that this phytohormone whose role in water stress responses is well known, plays an important function in plants exposed to water deprivation due to the vessel obstruction following *R. solanacearum* invasion and facing simultaneously abiotic and biotic stresses. In this context, it should be of interest to test if ABA signalling is more generally associated to vascular pathogens. A specific role for ABA in the plant response to soil borne pathogens such as *R. solanacearum* can be also questioned. ABA mutants impaired in biosynthesis or signalling in the model plant *A. thaliana*, could help to address these points.

Several studies illustrate indeed the role of ABA in response to various root-applied stresses. Its synthesis, and transport through xylem vessels up to the aerial parts of the plant, is induced by several abiotic stresses applied on roots (*e.g.* salt stress, ammonium nutrition, phosphate and potassium deficiencies) (Jiang e Hartung, 2008). Soil attackers also influence ABA signalling in plants. For instance, ABA acts as an important signal to prime above ground defenses during below ground aggressions by herbivorous (Erb *et al.*, 2009). Soil application of the chemical B-aminobutyric acid (BABA) induced resistance through ABA-dependent signalling (Ton e Mauch-Mani, 2004; Van Der Ent *et al.*, 2009). It is noteworthy that plants treated with *R. solanacearum hrp* mutant exhibit an increased

resistance to *P. syringae,* a foliar pathogen whose entry through stomata is prevented by ABA-mediated basal defences (Cao, Yoshioka e Desveaux, 2011; Feng *et al.*, 2012). This observation suggests that, following *R. solanacearum hrp* mutant inoculation, a signal migrates from roots to leaves leading to protection against *P. syringae*.

Another interesting point concerns the possible inheritance of the protective effect. Priming against environmental challenges may be inherited in the progeny of the primed plants (Slaughter *et al.*, 2012). Epigenetic components acting on gene expression regulation and more largely on chromatin structure and organization contribute to plant stress responses (Gutzat e Scheid, 2012). ABA signalling pathways appears to be connected to chromatin remodelling complexes (Saez *et al.*, 2008). It might therefore be interesting to check whether *hrp*-induced protection is inherited in the progeny of protected plants.

Despite an obvious lack of knowledge on the molecular mechanisms supporting the ABA-dependent biocontrol observed with *hrp* mutant bacteria, this strategy of natural vaccination of plants that requires further investigations from scientists working in this field, could provide a sustainable approach in the battle against plant pathogens.

# Chapter V
# Conclusion and perspective

# Conclusion and perspectives

Bacterial wilt, caused by the soil-borne bacterium *R. solanacearum*, inflicts severe economic losses in many crops worldwide, especially in *Solanaceaous* plants (Potato, Tomato, Eggplant...). Several strategies, including treatment with biocontrol agents, were developed with some success to control the disease mostly on tomato plants. For example, treatment of tomato roots with the non-pathogenic *Pythium oligandrum* (PO) (Butt e Copping, 2000; Brozova, 2002), induced resistance to the bacteria (Hase *et al.*, 2006; Hase *et al.*, 2008). This increased resistance was accompanied with the systemic activation of the JA- and ET-signaling pathways as demonstrated by analysis of global gene expression of PO-treated tomato roots (Takahashi *et al.*, 2006). Some beneficial microorganisms also possess the ability to control bacterial wilt in tomato. The colonization of *Pseudomonas fluorescence* FPT9601-T5 indeed suppressed the appearance of bacterial wilt symptoms (Aino *et al.*, 1997). As reviewed in the last chapter of this work, biocontrol through inoculation of *hrp*- bacteria was also successfully used to reduce wilt disease caused by virulent strains on tomato and on Arabidopsis. These promising approaches lead generally only to a partial reduction of the disease and the success depends in a large part of the environmental conditions, and in particular humidity and temperature that impact on the outcome of the interaction (Hayward, 1993). Host resistance remains so far the most effective strategy to control this disease. However, in many cases, the considerable variation among pathogen strains that progressively adapts to host plants breaks down the resistance. Further studies aimed at a better understanding of the still largely unknown molecular mechanisms underlying bacterial control are required.

Several years ago, a resistance gene with an atypical modular TIR-NBS-LRR-WRKY structure, *RRS1-R*, was cloned from Arabidopsis resistant plants. This receptor allows the establishment of effector-triggered immunity (PopP2) and fully protects plants against wilt disease (Deslandes *et al.*, 2002). More recently, it was shown that *RRS1 and RPS4*, two TIR-NB-LRR genes, are both required for disease resistance against multiple pathogen isolates including *R. solanacearum* (Gassmann, Hinsch e Staskawicz, 1999; Deslandes *et al.*, 2002; Narusaka *et al.*, 2009). The unique structure of the RRS1-R resistance protein

provides a shortcut in signaling from perception of bacterial effector to plant cell responses and constitutes an ideal tool to study a major component of stress responses**:** gene reprogramming. The advent of genomics and transcriptomics provided a comprehensive description of the magnitude of the transcriptional reprogramming that occurs in cells responding to perceived effectors (Tao *et al.*, 2003; Caldo, Nettleton e Wise, 2004; Adams-Phillips *et al.*, 2008; Moscou *et al.*, 2011). Nevertheless, the molecular mechanisms that cause and underlie this reprogramming remain obscure.

A Dam ID approach has been initiated which will allow a sensitive view of chromatin occupancy by RRS1-R in resistant Nd-1 plants. The production of susceptible Col-0 plants expressing RRS1-S fused to DAM was also initiated, which will render possible a comparative analysis of the RRS1-R and RRS1-S direct targets.

These tools open the opportunity to identify targets of various DNA-binding proteins under different environmental conditions. Indeed, fingerprints of DNA binding sites can be mapped by simple molecular approaches. Thus, the effect of biotic and abiotic stresses on the dynamics of target occupancy by the RRS1 proteins can be easily addressed. According to our study, RRS1 gene expression is probably dependent upon environmental conditions and therefore, the binding sites occupancy by the RRS1 proteins may be affected by changes in the environment. A tissue-specific identification of the RRS1 binding sites can also be considered. It should be worth focusing on root cells expressing RRS1 genes since *R. solanacearum* is a root pathogen. In parallel, the impact of RRS1 binding on gene transcription could be monitored by performing transcriptomic analyses on the same tissues.

Last but not least, numerous studies during the last few years have revealed that WRKY transcription factors physically interact with a wide range of proteins involved in signalling, transcription, and chromatin remodelling (Chi *et al.*, 2013) .Interestingly, interactions between WRKY have been reported and functions of the corresponding complexes vary, depending upon the WRKY partners. The importance of such associations is illustrated by the crucial role among AtWRKY18, AtWRKY40 and AtWRKY60 proteins in the ABA signal transduction (Xu *et al.*, 2006; Chang *et al.*, 2009; Liu *et al.*, 2012). Remarkably, following PopP2 induction, transcription of many WRKY genes (21 genes) is affected (unpublished results from the group). It will be also quite interesting to scan FARM

sequences, obtained from the whole genome analysis, in order to identify regulatory *cis*-elements associated to W boxes, which may indicate preferential associations with other transcription factors in the formation of DNA-binding regulatory complexes under our experimental conditions.

Altogether, these studies will probably reveal new partners and mechanisms involved in transcriptional dynamics sustaining plant immunity.

In conclusion, the deciphering of plant cellular processes leading to resistance or susceptibility to the bacterium *R.solanacearum* through the identification of genes whose regulation is dependent upon RRS1-R and RRS1-S via their WRKY domain, could be a crucial step to elaborate novel strategies to control bacterial wilt. Manipulation of these genes may lead to increased resistance to *R.solanacearum* but also to *Pseudomonas syringae* and *Colletotrichum higginsianum* since RRS1 and its partner resistance gene RPS4 are able to confer resistance also to these pathogens. In addition, this could provide new data on the molecular mechanisms occurring at the root level during plant pathogen interactions, an area of research still poorly documented.

# Chapter VI
# Material and Methods

# 6.1 Materials

## 6.1.1 Bacteria

*E. coli* strains: DH5α ($F^-$ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^-$ $m_K^+$), λ–) competent cells were used for standard cloning. DB3.1 ( F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20($r_B^-$, $m_B^-$) ara14 galK2 lacY1 proA2 rpsL20($Sm^r$) xyl5 Δleu mtl1) competent cells were used for cloning Gateway Donor and Destination vectors (Invitrogen)

Rosetta™ 2(DE3) ($F^-$ ompT $hsdS_B$($R_B^-$ $m_B^-$) gal dcm λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysSRARE ($Cam^R$) ) competent cells were used for protein expression.

*Agrobacterium tumefaciens*: GV3101 (Rifampycin resistance) or GV3103 (streptomycin/spectinomycin resistance)/ pMP90 (Gentamycin resistance) strains were used for stable transformation of *A. thaliana* and transient expression in *N. benthamiana*.

## 6.1.2 Bacterial pathogen

*Ralstonia solanacearum* Strain *GMI1000* (Deslandes *et al.*, 1998) or derivative mutant were used for root inoculation.

## 6.1.3 Plant Material

All plants used in this study originate from *A. thaliana* Col-0, or Nd-1 accessions. Col 0/iPop P2 line and Nd-1/iPop P2 line, correspond respectively to Col-0 and Nd-1 plants containing an estradiol inducible gene (pLexA*:PopP2::GUS*). CH1-2 is a transgenic line, containing RPS4 and *RRS1*-R in a Col-0 genetic background, resistant to *R.solanacearum*.

## 6.1.4 Oligonucleotides

Primers used in the present study were synthesized by SIGMA (Table 1).

## 6.1.5 Plasmids

**Table C6-1.** Primers used in this thesis

| **Primers** | **Primer sequence 5' → 3'** |
|---|---|
| AttB1-WRKY Forward | GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGGTTTCGATATATGTTATATC |
| AttB2- RRS1-R* | GGGGACCACTTTGTACAAGAAAGCTGGGTCAAAAGTAAAAATTATAATCATCGAA |
| AttB2- RRS1-S* | GGGGACCACTTTGTACAAGAAAGCTGGGTCAGCAGATGGAGGAGGAAGT |
| AdRt | CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGA |
| AdRb | TCCTCGGCCG |
| AdPCR primer | GGTCGCGGCCGAGGATC |
| Dam05 | AAACAAATTGCGCCGAGGTTT |
| Dam06 | CCGAACGGCACGTTAAACTCA |
| EF-1$\alpha$ F | CTGGAGGTTTTGAGGCTGGTAT |
| EF-1$\alpha$ R | CCAAGGGTGAAAGCAAGAAGA |
| AdPCR primer | GGTCGCGGCCGAGGATC |
| AttB1-PBS1-like | GGGGACAAGTTTGTACAAAAAAGCAGGCTTATTATCTCCCAAGTCAACCCACA |
| AttB2-PBS1-like | GGGGACCACTTTGTACAAGAAAGCTGGGTCAAGGACACGAGAACTGAGACA |
| At5g45260-250R | GGGGACCACTTTGTACAAGAAAGCTGGGTCCCACGAATTCCTCATCCTTTTC |
| At5g45260-3000F | GGGGACAAGTTTGTACAAAAAAGCAGGCTTATCAGTTCTTGAAGAGCTTTCAG |
| AT5G45260-R-F-250 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTATGGAAATAGACGATGTCTCCA |
| AT5G45260-F-250 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTATGGAAATAGATGATGTCTCCAT |
| AT1G73680 For qPCR | AAAAATCAATTCAGCCAAAA |
| AT1G73680 Rev qPCR | GGTGAATTTCGGTTATAATA |
| AT2G26980 For qPCR | GAATCGATGGTCACATTG |
| AT2G26980 Rev qPCR | TTATGCAACTTCCTTCCAT |
| AT2G40460 For qPCR | TAAAGGAATTTCACATAACT |
| AT2G40460 Rev qPCR | CAATGCCTAGAAGAGAGTA |
| AT2G44290 For qPCR | TAAGTGTTTGGCTAGTTAA |
| AT2G44290 Rev qPCR | CTCATGTTTTGTAGTATAGTAT |
| AT3G55450 For qPCR | CCCATCCCATGATAC |
| AT3G55450 Rev qPCR | AAGTAGGTAAGAAAAATAA |
| AG5G01810 For qPCR | GCTAATTTAATTTGGTAACC |
| AG5G01810 Rev qPCR | CACACAAGAATCATCAATC |
| ATCG00690 For qPCR | GGTGCGGCCGCAGGA |
| ATCG00690 Rev qPCR | GGTTTTCCTGTACGTTCA |
| AT1G06130 For qPCR | AATGGATGATGATAACTAG |
| AT1G06130 Rev qPCR | TATATACAAGTTATAGAG |
| AT2G38310 For qPCR | TCTCGTCCTAGATATACCT |
| AT2G38310 Rev qPCR | CTCGTATATCAACACTAGA |
| AT3G03470 For qPCR | AAGCTATTGTTTATTGTTTATA |

| | |
|---|---|
| AT3G03470 Rev qPCR | AATATTTTCACCAACTAAAA |
| AT3G03830 For qPCR | CCCTGTCATAGTCTGTC |
| AT3G03830 Rev qPCR | TTGGAAGTTTCTGTGGTC |
| AT5G66310 For qPCR | TTAGTAGCACATCACAATC |
| AT5G66310 Rev qPCR | AAGTAATGTTAGTTGGTAG |
| AT5G47640 For qPCR | TTGACCGAAGGGTCGT |
| AT5G47640 Rev qPCR | AAACACGGAGAAGATA |
| AT5G67390 For qPCR | ACTTGGACTCTTCTCA |
| AT5G67390 Rev qPCR | CTAATGGAGAGTACAAG |
| AT2G36630 For qPCR | CCTTGTAAGTATATGTCAA |
| AT2G36630 Rev qPCR | TTTGTGTGGTGGGGC |

Plasmids used in this thesis are shown in Table 2.

### 6.1.6 Buffer and solution

Buffers and solutions that were used in this thesis are shown in Table 3.

### 6.1.7 Media and Antibiotics

Sterile media (prepared in deionized water) were used for the growth of bacteria and for *in vitro* culture of *A. thaliana* as follows.

#### 6.1.7.1 Media for *E. coli* culture and transformation

Luria-Bertani (LB) broth (5g yeast extract, 10g trypton, 10g N, pH=7.5 per liter) or agar plates were used for culture and SOC medium (5g yeast extract, 20g trypton, 20 mM glucose, 0.5g NaCl, 2.5 mM CaCl2, pH=7.5) for transformation.

#### 6.1.7.2 Media for Agrobacterium tumefaciens culture and transformation

YEB broth liquid medium **(**10g yeast extract, 10g peptone, 5g NaCl per liter)or LB Agar plates (5.0 g tryptone**,** 2.5 g yeast extract, 5.0 g NaCl, 7.5 g agar per liter).

#### 6.1.7.3 Media for Arabidopsis thaliana culture

MS medium (4.7g MS salt supplemented with vitamins, 5g glucose, 15 g agar, pH 5.7~5.8).

#### 6.1.7.4 Antibiotics

Carbenicillin: 50mg/mL in Ethanol
Chloramphenicol: 30 mg/mL in Ethanol
Gentamycin: 30mg/mL in DMF
Kanamycin: 50 mg/mL in H2O
Stock solution were stored at –20°C

## 6.2 Methods

### 6.2.1 Plant growth conditions

**Table C6-2.** Plasmids used in this thesis

| Plasmids (vector-insert) | Recipient bacterium |
|---|---|
| *pDON$^{TM}$207 (Invitrogen) Gent$^{R}$* | DH5α |
| *pAM-PAT 35S- GW* | DB3.1 |
| *pKGWFS7* | DB3.1 |
| *pBin -GW LR* | DB3.1 |
| *pAM-PAT- GW* | DB3.1 |
| *pGEX-GW* | DB3.1 |
| *pAM-PAT 35S - RRS1-R$_{cDNA}$ flag* | GV3003 |
| *pAM-PAT 35S -RRS1-S$_{cDNA}$ flag* | GV3003 |
| *pAM-PAT 35S -Pop P2 3xHA* | GV3003 |
| *pER8- Pop P2 3xHA* | GV3003 |
| *pAM-PAT -PBL1prom :GUS* | GV3003 |
| *pBIN-250RR1-Rprom RRS1-R$_{geno}$ 3xHA Dam-$_{RRSI-R}$Terminator* | GV3001 |
| *pBIN-250RRS1-Rprom-$_{RRSI-R}$Terminator* | GV3001 |
| *pBIN-250RR1-Sprom RRS1-S$_{geno}$ 3xHA Dam-$_{RRSI-R}$Terminator* | GV3001 |
| *pBIN-250RRS1-Sprom -$_{RRSI-R}$Terminator* | GV3001 |
| *pBIN-$_{RPS4}$Terminator-RPS4-RRS1-R$_{geno}$ 3xha Dam-$_{RRSI-R}$Terminator* | GV3001 |
| *pKGWFS7-p250R* | GV3001 |
| *pKGWFS7-p3000R* | GV3001 |
| *pKGWFS7-p250S* | GV3001 |
| *pKGWFS7-p3000S* | GV3001 |
| *pGEX-WRKY-R-his* | Rosetta-DE3 |
| *pGEX-WRKY-S-his* | Rosetta-DE3 |
| *pGEX-WRKY-R::Dam-his* | Rosetta-DE3 |
| *pGEX-WRKY-S::Dam-his* | Rosetta-DE3 |

Seeds were sterilized for *in vitro* growth of *Arabidopsis thaliana*. The appropriate amount of seeds was placed in a 1.5 ml microcentrifuge tube. 1ml JAVA (chlorine bleach) solution with 0.1% Tween-20 was added. Seeds were vortexed for 5 seconds. After 10 minutes, seeds were centrifuged for a few seconds and the solution discarded. Seeds were rinsed 5 times by water and sowed on MS-medium containing plates or selective MS-medium plates. Sterilized seeds were stored in the dark for 2 days at 4℃ then incubated in a growth chamber under the following conditions: temperature 20°C, 16h light (250 µE/m2s). Plantlets were transferred to Jiffy pots (Jiffy France, Lyon France) after 7 days in the growth chamber. The plants were then grown for 3 weeks under the following conditions: 22°C, 10h light (250 µE/m2s). Plants were transferred to 9 cm square pots in the green house for transformation and seed production.

### 6.2.2 *A. thaliana* floral dip stable transformation

The protocol used for *Agrobacterium*-mediated stable transformation of *A. thaliana* is based on the floral dip method. Before transformation, *Agrobacterium* strains from -80 ℃ glycerol stock were streaked onto a fresh selective YEB medium plate and incubated at 28 ℃ for 2 days. In the morning, a freshly grown *Agrobacterium* colony was collected and dissolved in 10 ml YEB liquid medium and incubated at 28 ℃ for several hours. The pre-culture was used to inoculate a 1L flask containing 250ml of YEB medium. The culture was incubated at 28 ℃ overnight. When the $OD_{600}$ was between 1 and 2, the culture was transferred into a centrifuge bottle, and cells were pelleted by centrifuging at 8000rpm for 10min at room temperature. During the centrifugation, a sucrose solution (50g/L) was prepared using sterile water. Pelleted cells were resuspended with the 5% sucrose solution in order to have a final culture with $OD_{600}$=1. 100 µl/L of Silwett L77 was added and the resulting solution mixed gently. Approximately 5 *A. thaliana* plants were used for transformation. The first inflorescence shoots were cut to induce the growth of additional inflorescences. Plants were used for transformation when a maximum number of young flower heads were present. Plants to be transformed were dipped in the bacterial suspension for 10 seconds with gentle agitation. After drying, the plants were covered with a plastic film to maintain high humidity and put them away from direct light for 24 hrs. The next day, the plastic films were slashed and the plants were put away from direct light for 24hrs.

Afterwards, plastic films were removed and pots transferred to greenhouse until the appearance of seeds.

### 6.2.3 Transient expression in N. benthamiana

*Agrobacterium* strains containing the constructs of interest were cultured overnight at 28 ℃ in 10 ml of selective liquid YEB media. The culture was spun down and the bacterial pellet was resuspended in the Agromix infiltration buffer (10 mM MES; 10 mM $MgCl_2$/KOH pH 5.6; 150 μM Acetosyringone) to an $OD_{600}$ of 0.5. The *Agrobacterium* bacterial solution was incubated at room temperature at least 2 hrs. Young *N. benthamiana* leaves were hand-infiltrated with a needle-less 1 ml syringe on the underside of the leaf. Infiltrated leaves were used for protein extraction and western blots 48 hrs after infiltration. Genomic DNA was extracted by CTAB 48 hrs after infiltration for digestion.

### 6.2.4 RNA extraction

NucleoSpin RNA II Kit (MACHEREY-NAGEL, Germany) extracted the plant RNA according to the recommended protocol. Sample was grinded (up to 100 mg of tissue or plantlet) in liquid nitrogen. RNA was eluted in 35 μL RNase-free water and centrifuge at 11,000 g for 1min. An additional DNA Digestion was performed with 2 μL DNase I (Ambion, TURBO) at 37°C for 1 hour. After DNase inactivation RNA was transferred to a new tube (AXYgen RNase free tube).

### 6.2.5 RNA reverse transcription into cDNA

Fist-Strand cDNA synthesis was performed using SuperScript II RT (Invitrogen, USA) according to the following protocol.

-Pre-mix 1 μg total RNA into RNase free $H_2O$ up to final volume 10 μL.

-Add 5.5 μL following pre-mix components for each sample: 1 μL Oligo $(dT)_{17}$, 1 μg/μL; 0.25 μL RNase inhibitor; RNase free $H_2O$ 4.75 μL.

-Heat total RNA to 65°C for 5min in PCR machine and quick chill on ice.

- Add 9.5 μL following pre-mix components for each sample: 5 μL 5X First-Stand Buffer; 2.5 μL 0.1 M DTT; 1.5 μL dNTP mix; 0.5 μL SuperScript II RT.

- Incubate at 42°C for 1 hour.
- Inactivate the reaction by heating at 70°C for 15 min.
-Quick chill on ice for 5min and keep in -20°C.

### 6.2.6 Extraction of plant genomic DNA

Plant tissues were ground in 1.5 or 2 ml microcentrifuge tube. Then, 400 µl of 2 X CTAB DNA extraction buffer (100 mM Tris Ph 8.0; 1.4 M NaCl; 20 mM EDTA; 2% CTAB; β-mercaptoethanol) were added and the solution vortexed for 5 to 10 seconds. The solution was incubated for 30 min at 65 ℃. One volume of chloroform/isoamyl alcohol (24: 1) was added, and the solution vortexed 5 to 10 seconds. The solution was centrifuged at 13,000 rpm for 10 min and a volume of 300 µl from the supernatant was transferred into a clean tube and. 0,8 volume of cold isopropanol was added. The solution was centrifuged at 13,000 rpm for 10 min and the pellet was washed twice with 70% ethanol. The dried pellet was resuspended in 50 µl water containing RNase. The DNA was stored at -20 ℃ and used for PCR amplification. For each PCR reaction, 1µl DNA solution was used.

### 6.2.7 Plasmid DNA isolation from bacteria

Standard alkaline cell lysis minipreps of plasmid DNA were performed using the Wizard® Plus Minipreps DNA Purification System (Promega) according to the manufacturer's instructions.

### 6.2.8 Protein extraction for SDS-PAGE and western blotting

Total protein extraction of leaf: Four leaf discs (7mm) or 20 seven-day old plantlets were ground in liquid nitrogen. The ground tissue was resuspended in 100µl of Laemmly extraction buffer 2X (0.125 M Tris-HCl, 4% SDS, 20% Glycerol, 0.02% Bromophenol blue, 0.2 M DTT pH 7.5) and heated at 95 ℃ for 3 minutes. The solution was then centrifuged at 13000rpm for 1min. Samples were stored at -20 ℃ or directly loaded onto 10% SDS-PAGE gels

Total protein extraction of *E.coli*: Proteins were expressed in *E. coli*, bacteria were centrifuged and the pellet was suspended in 100µl of Laemmly extraction buffer 2X. The protocol is the same as above.

### 6.2.9 Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Prepare polyacrylamide gel according to standard protocol. Use 1X Laemmly running buffer to fresh well and load 20 µL samples. Run at 80V through the stacking gel then run at 100V until the dye migrates about two-thirds of the way down the gel.

### 6.2.10 Immunoblot analysis (Western blotting)

Proteins are separated by SDS-PAGE. Then, the proteins are transferred to Nitrocellulose Protran Membrane at 100V for 1hrs in 1X Transfer buffer. After transfer the membrane is stained with Ponceau. The blot membrane is incubated with a 5% generic protein (milk protein) in 1X TBS buffer at least 1hr before incubation incubated in primary antibody solution (dilution in 1X TBS-T buffer) at room temperature 2~3 hrs or overnight at 4°C. The membrane is washed 3 times in 1X TBS-T buffer for 5~10min/each time. And then, the membrane is incubated in secondary antibody solution (dilution in 1X TBS-T buffer) at room temperature 1~2 hrs. The membrane is washed 3 times in 1X TBS-T buffer. Proteins are detected by HRP Western Blot Detection System.

### 6.2.11 GATEWAY Cloning Technology

**BP Recombination Reaction**

-1 µL of pDON 207 (50 ng/µL, AttP1-GWY-AttP2)
-X µL of PCR product (Gel purified, AttB1-pcr product-AttB2)
-1 µL of BP clonaseII
-Add $H_2O$ to final volume 5 µL.

-Incubate at 25°C for 1-2 hours. Add 4 µL of $H_2O$ and 1 µL of proteinase K. Incubate at 37°C for 10 min. Dialyseon 0.05 µm membrane filters (MILLIPORE) for 40 min.

-Use 1 μL to electroporate 50 μL DH5α. Cells are resuspend with 950 mL SOC media. Incubate with shaking (~150 rpm) at 37°C for 1.5 hours. Plate on gentamycin (15 μg/ml) LB plates.

**LP Recombination Reaction**

-1 μL of pENTR (50 ng/μL, AttL1-GWY-AttL2)
-1 μL of pDEST vector (50 ng/μL, AttR1-gene-AttR2)
-1 μL of LP clonaseII
-Add $H_2O$ to final volume 5 μL.

-Incubate at 25°C for 1-2 hours. Add 4 μL of $H_2O$ and 1 μL of proteinase K. Incubate at 37°C for 10 min. Dialyse on 0.05 μm membrane filters (MILLIPORE) for 40 min.

-Use 1 μL to electroporate 50 μL DH5α. Cells are resuspend in950 mL SOC media. Incubate with shaking (~150 rpm) at 37°C for 1.5 hours. Plate on appropriate selective LB plates. Streak the colonies on a fresh selective plate and Genta (15 μg/ml) plate in parallel.

### 6.2.12 Histochemical staining for GUS Activity detection

- *A.thaliana* plantlets (leaves of N.*benthamiana* or other tissue) were placed in a small Petri plate, a multiwell plate or a microfuge tube containing GUS substrate solution (X-Gluc stock were diluted 50X with GUS staining buffer).
-Infiltrate tissue 3 times for 1 minute, each time "gently" releasing the vacuum.
-Incubate at 37°C for 4-5 hours, blue color will appear; (Staining can last overnight if necessary).
-After we mounted in water for microscopic obsevations.

### 6.2.13 Electrophoretic mobility shift assays

#### 6.2.13.1 Construction of the plasmids for WRKY and WRKY::Dam expression in *E.coli*

Primers AttB1-WRKY Forward and AttB2- RRS1-R(or)S* (*mean without stop codon) were used to amplify the cDNA fragments corresponding to the WRKY domain of RRS1-

R(or)S. Each AttB1-WRKY-AttB2 fragment was used to construct expression vectors pGEX-GST::WRKY-R/S::6xHis and pGEX-GST::WRKY-R/S::Dam::6xHis. Both of these vectors were transfered into *Rosetta*™ 2 (DE3) *E.coli* Competent Cells for protein expression.

### 6.2.13.2 Expression of WRKY and WRKY::Dam

-Growth 1 colony into 5 ml LB containing Carbenicillin 50 μg/mL and Chloramphenicol 30 μg/mL. Incubate at 37°C with shaking (~180 rpm) overnight.
-Add 2.5 mL overnight liquid culture into 50 mL (3 bottles) fresh LB containing Carb 50 μg/mL and Cam 30 μg/mL. Grow with sharking at 37°C for 3 hrs (OD=0.6)
-Use 10 μM IPTG to induce the protein at 16°C for 20~24 hrs
-Harvest E.*coli* cells by centrifugation at 4,000 rpm for 10 min at room temperature. Cells were directly used to extract proteins or kept at -20°C.

### 6.2.13.3 Protein extraction and purification for Gel shift assay

- *E.coli* cells were resuspended with 4.5 mL 1X PBS buffer containing 1mM PMSF.
-Cells were disrupted by French pressure cell press (FRENCH® Press, Thermo). -The crude extract was centrifuged at 100,000rpm for 30 minutes.
-The supernatant was transferred to a new microcentrifuge tube and purified by Glutathione Sepharose™ 4B (GE Healthcare, Sweden) as follow:
-Add 400ul 50% GSH resin into the supernatant on wheel (20 rpm) at 4°C for 4h30.
-Add 1mL 1X PBS buffer containing 1mM PMSF into column to wash the resin. Repeat this step 4 times.
-Add 40 uL of 40 mM reduced glutathione elution buffer (Reduced glutathione dissolved in 50 mM Tris-HCl, pH=8.0). Incubate at RT for 10 min. Centrifuge at 500 × g for 5 minute.
- Check protein concentration using Nano-Drop. (The concentration of GST-tagged proteins can be estimated by measuring the absorbance at 280 nm. The GST-tag can be approximated using the conversion; A280 ~ 1 corresponds to ~ 0.5 mg/ml.)
-Add glycerol to adjust the concentration to 10% in elution buffer. (Protein is more stable in glycerol solution. 1X binding buffer also has 10% glycerol). -The protein could be used for Gel shift assay and stored at -80°C. About 1 μg protein was used for EMSA.

## 6.2.14 Electrophoretic Mobility Shift Assay (EMSA)

### 6.2.14.1 Prepare and Pre-Run Gel:

Prepare a 6.5% native polyacrylamide gel in 0.5X TBE

| | |
|---|---|
| 10X TBE | 600 µL |
| 24% Acryl/bis (39/1) | 3.3 mL |
| 60% glycerol | 500 µL |
| 20% APS | 36 µL |
| TEMED | 12 µL |
| $H_2O$ | 7.6 mL |

### 6.2.14.2 Pre-Run the gel

Fill an electrophoresis unit with 0.5X TBE running buffer. Flush wells and pre-run the gel for 60 minutes at 120 V. This pre-run step removes ammonium persulfate from the gel.

### 6.2.14.3 Prepare and Perform Binding Reactions

Prepare binding reactions as following order while gel is pre-running.

Set up 15µL binding reactions in 0.5mL microcentrifuge tubes:

| | |
|---|---|
| $H_2O$ | 5 µL |
| 5X Binding buffer | 3 µL |
| 1 µg/ µL Poly dIdC | 1 µL |
| Unlabeled W box | 20pmol (100X) 50pmol (250X) |
| Mutant W box | 20pmol (100X) 50pmol (250X) |
| Protein Extract | 1~5 µL > 1ug |
| Biotin Lab eled W box | 200fmol |

Incubate samples for 20 minutes at room temperature.

### 6.2.14.4 Gel electrophoresis

-Switch off current to the electrophoresis gel, and change new cold 0.5X TBE running buffer.

-Very carefully load the samples directly (i.e., no dye added) onto the bottom of the wells of native polyacrylamide gel. Add a small volume (e.g., 5 µL) of DNA-loading buffer containing bromophenol blue to one lane that does not contain a reaction, as a marker to follow migration in the gel.
-Run electrophoresis at 200V for 10minutes in cold room (4°C), then change to 160 V until the bromophenol blue dye has migrated about 2/3 to 3/4 down the length of the gel.

#### 6.2.14.5 Transfer of Binding Reactions to Nylon Membrane

-Soak nylon membrane in 0.5X TBE for at least 10 minutes.
-Sandwich the gel and nylon membrane in a clean electrophoretic transfer unit according the manufacturer's instructions.
-Fill the transfer unit with cool 0.5X TBE and transfer at 100 V for 30 minutes.
-When the transfer is complete, place the membrane with the bromophenol blue side up on a dry paper towel. (There should be no dye remaining in the gel.) Allow buffer on the membrane surface to absorb into the membrane. This will only take a minute. Do not let the membrane dry.

#### 6.2.14.6 Cross-link Transferred DNA to Membrane

Cross-link at 120 mJ/cm$^2$ for 45-60 second exposure using the auto cross-link function of a commercial UV-light cross-linker instrument. After the membrane is cross-linked, directly detect biotin-labeled DNA by Chemiluminescence.

#### 6.2.14.7 Detect Biotin-labeled DNA by Chemiluminescence (LightShift Chemiluminescent *EMSA* Kit, Pierce)

-Gently warm the Blocking Buffer and the 4X Washing Buffer to 37-50°C in a water bath until all particulate is dissolved.
-To block the membrane and 15 mL of Blocking buffer and incubate for 15minutes with gentle shaking.
-Prepare conjugate/blocking buffer solution by adding 14.2 µL Stabilized Streptavidin-Horseradish Peroxidase Conjugate to 5mL Blocking Buffer. Decant blocking buffer from

the membrane and replace it with the conjugate/blocking solution. Incubate membrane for 15 minutes with gentle shaking.

-Prepare 1X wash solution by adding 40 mL of 4X Wash Buffer to 120 mL ultrapure water. Transfer membrane to a new container and rinse it briefly with 20 mL of 1X wash solution. Wash membrane four times for 5 minutes each in 20 mL of 1X wash solution with gentle shaking.

-Transfer membrane to a new container and add 30 mL of Substrate Equilibration Buffer. Incubate membrane for 5 minutes with gentle shaking.

-Prepare Substrate Working Solution by adding 1 mL Luminol/Enhancer Solution to 1 mL Stable Peroxide Solution.

-Remove membrane from the Substrate Equilibration Buffer, carefully blotting an edge of the membrane on a paper towel to remove excess buffer. Place membrane in a clean container or onto a clean sheet of plastic wrap placed on a flat surface.

-Pour the Substrate Working Solution onto the membrane so that it completely covers the surface. Alternatively, the membrane may be placed DNA side down onto a puddle of the Working Solution. Incubate membrane in the substrate solution for 5 minutes without shaking.

-Remove membrane from the Working Solution and blot an edge of the membrane on a paper towel for 2-5 seconds to remove excess buffer. Do not allow the membrane to become dry.

-Wrap the moist membrane in plastic wrap, avoiding bubbles and wrinkles. Expose membrane to an appropriately equipped CCD camera for 2-5 minutes.

## 6.2.15 DNA adenine methyltransferase identification (DamID)

### 6.2.15.1 Plasmid Constructions

-Dam sequence was amplified from E.coli DNA; a fragment containing a triple HA-DAM coding sequence fused to the RRS1-R terminator was PCR amplified. A gateway cassette was inserted in front of the construct to produce a gateway destination vectors. This plasmid was double digested with Pme I and EcoR I I (NEB, USA). The fragment (2723bp) of 3Ha-Dam-Rterm was recovered by gel purification system.

-The pBINGW-LR vector was ordered from the Arabidopsis Biological Resource Center (ABRC) and double digested with Xmn I and EcoR I (NEB, USA). The fragment (11887bp) was recovered by gel purification system.
-These two fragments were ligated at room temperature for 3h by T4 ligase (Promege, USA). The ligation product was transfer into DB3.1 competent cell.
-The plasmid of pBINGW-LR-3Ha-Dam-Rterm vector was extracted from liquid culture of DB3.1 and kept in -20°C for Gateway LR reaction.
-The promoter (-250bp) fromRRS1-R or RRS1-S and promRRS1-R: RRS1-R genomic sequence or promRRS1-S: RRS1-S genomic sequences were amplified with AtttB1/AttB2 primer pairs.
-These fragments were cloned into pDON207 vector by Gateway BP reaction, and then were used to make the LR reaction with pBINGW-LR-3Ha-Dam-Rterminator vector.
-The pBIN:35S::RRS1-R::Dam:35Sterm and pBIN:35S::RRS1- S::Dam:35Sterm were also constructed by making an LR reaction with a pBIN:35S-GW-3Ha-Dam-Rterminator vector (Figure C6-1).

### 6.2.15.2 Selection of transgenic plant and checking Dam and EF-1α expression level

- pBIN-promRR1-R: RRS1-R::3Ha::Dam-RRSI-RTerminator and pBIN-promRR1-R: 3H::Dam-RRSI-RTerminator constructs were introduced into Nd1 line and Nd-1/i Pop P2 line (Figure C6-2).
-T0 generation seeds were selected on Kanamycin resistance MS plate.
- Plant material was harvested from T1 or T2 generation plantlets grown on plate 10 days, tissues were grinded in Liquid nitrogen and material was shared to prepare DNA and RNA from the same samples. RNA was used for cDNA preparation and Dam fusion and elongation factor 1-alpha (*EF-1α*, *At*5g60390) expression level were checked by qRT-PCR with primer: Dam05/Dam06; EF-1α F/EF-1α R.

### 6.2.15.3 Control of PopP2 expression in plants

-The plants Nd-1 and Nd-1/iPop P2 transgenic line were grown on MS plate with 10 μM estradiol.

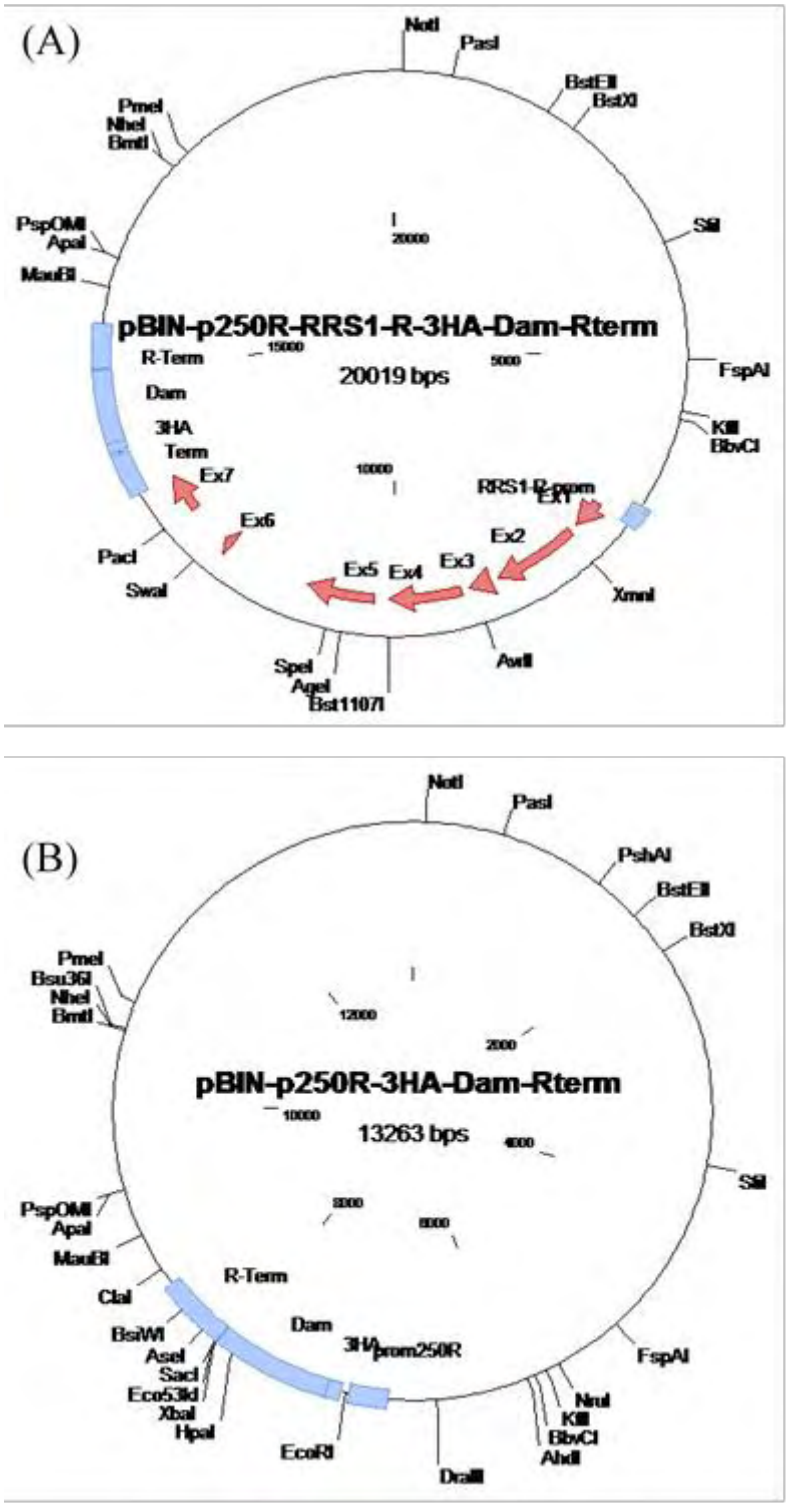


**Figure C6-1.** The map of constructions for DamID. (A): *pBIN::RRS1-Rprom:RRS1-R::3HA:Dam:Rterm*. (B): *pBIN::RRS1-Rprom::3HA:Dam:Rterm*.

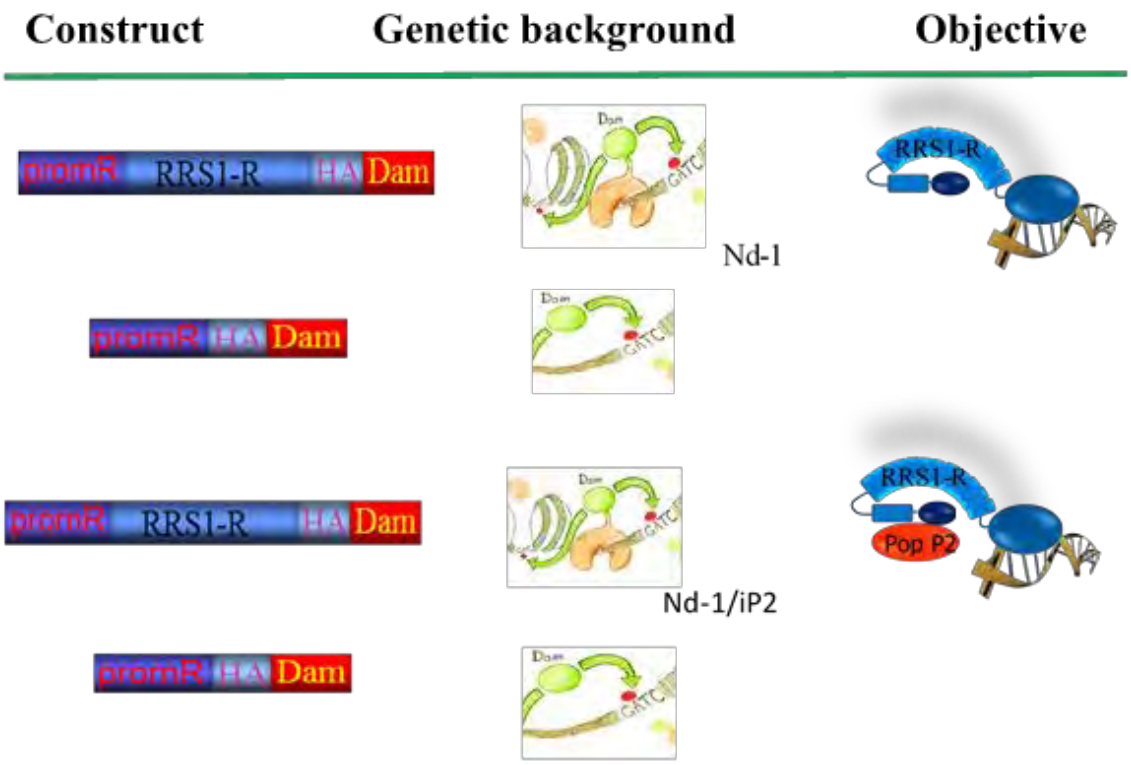


**Figure C6-2.** Construct for stable expression of *RRS1-R::Dam* in *A. thaliana*. The *pBIN::promR:RRS1-R::HA :Dam* and *pBIN::promR::HA :Dam* were constructed, the promoter RRS1-R was used to drive the gene expression. Both construct were transferred into Nd-1 line and Nd-1/ Estradiol inducible Pop P2 (Nd-1/iP2) line. *pBIN::promR:RRS1-R::HA:Dam* were used for target identification, and *pBIN::promR::HA:Dam* as nonspecific methylation background reference. Some experiments were performed with *RRS1-S*.

From a single plate, some plantlets were grinded and material was used to control by western blot the Pop P2 expression, others were recovered for GUS staining or for DNA (FARMs identification) and RNA extraction (Dam expression level control).

**DNA adenine methyltransferase identification (DamID)**

The Dam ID was performed according to protocols from two papers (Vogel, Peric-Hupkes e Van Steensel, 2007; Germann e Gaudin, 2011).

### 6.2.15.4 Preparation of the AdR double stranded adaptors (50 µM)

50 µL of AdRt (100 µM) and 50 µL primer (100 µM) were mixed in a 1.5 mL Eppendorf tube. The tube was incubated in boiling water for 1min. Water was cooled down slowly to room temperature in order to anneal the two primers into the AdR double-stranded adaptor (Germann e Gaudin, 2011).

### 6.2.15.5 Amplification of FARMs

Genomic DNA was extracted using the CTAB method and 2.5 µg of DNA was digested with DpnI (NEB) at 37°C overnight. DpnI was then Heat-inactivated for 20 min at 80°C.
-Digestion products were purified as PCR products by Wizard® SV Gel and PCR Clean-Up System (Promega).
-The AdR double stranded adaptor was ligated to DpnI digested sites by T4 ligase (Promega) at 15°C for overnight.
-Ligation products were purified as PCR products by Wizard® SV Gel and PCR Clean-Up System (Promega).
-DpnII digestion was conducted for 4h or overnight at 37°C.
-Digestion products were purified as PCR products by Wizard® SV Gel and PCR Clean-Up System (Promega). DNA concentration was checked on nanodrop.
-100ng DNA was used for amplification by Go Taq (Promega) with AdRb oligonucleotide under the following conditions in 80 µl.

| | |
|---|---|
| 72 °C for 10 min, fill gap of adaptor | 1cycle |
| 95 °C for 2 min | 1cycle |
| 94 °C for 1 min, 65 °C for 5 min, 72 °C for 2 min | 3cycle |

94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min 30cycle
72 °C for 5 min
18 °C for o/n

7 µl were used for FARM cloning in pGEMT. More than 1 µg DNA (dosage by PicoGreen) were used for whole genome analysis by high-throughput sequencing.

## 6.2.16 Microplate fluorometric GUS assay

### 6.2.16.1 Protein extraction for fluorometric GUS assay

8 fresh leaf discs (7mm) were ground by the TissueLyser II (QIAGEN, Germany): (30 seconds/frequency 30) in 2ml microcentrifuge tubes while kept in frozen tubes. The powder was directly added to 100µl of GUS buffer 1X and vortexed until complete dissolution. The samples were then centrifuged at 13,000 rpm for 15 minutes at 4 ℃. The supernatant were transferred to a new microcentrifuge tubes. The sample could be used for fluorometric GUS assays or stored at -80 ℃.

### 6.2.16.2 Fluorometric GUS assay: BSA standard curve preparation

-First, use BSA (stock 10 µg/µL) to prepare BSA standards:

| **Final Qty µg/10µL** | **0** | **0.5** | **1** | **2** | **4** | **6** | **8** | **10** |
|---|---|---|---|---|---|---|---|---|
| BSA (1 µg/µL) | 0 | 5 | 10 | 20 | 40 | 60 | 80 | 100 |
| Water | 100 | 95 | 90 | 80 | 60 | 40 | 20 | 0 |

-Prepare spectrophotometer cuve with appropriate designation of each standard.

| | |
|---|---|
| Standard | 10 |
| GUS Buffer 1X | 10 |
| Biorad 1X reagent | 480 |

-Mix gently by hand after addition of Biorad 1X reagent.

-OD were measured at 595nm by spectrophotometer. (Reading must be done no later than 5 min after the last sample is reacted with Biorad 1X reagent)
-Establish the BSA coefficient standards curve

#### 6.2.16.3 Total sample protein preparation

-Total sample proteins were diluted 10X (5 µL total protein extraction into 45 µL GUS buffer 1X). Add the followings to the cuve in given order.

| | |
|---|---|
| Total sample protein | 10 |
| Biorad 1X reagent | 490 |

- OD were measured at 595nm, total protein were quantified according to BSA standards curve

#### 6.2.16.4 Plate design

Design the plate to contain the followings (200 µL final volume).
-Blank (3 measures): 100 µL GUS Buffer 1X, and 100 µL 2 mM 4-MUG.
-Negative (3 measures): 200 µL GUS Buffer 1X alone.
-Positive (2 measures): 100 µL GUS Buffer 1X, 100 µL 2 mM 4-MUG, and GUS enzyme.
-Standard (2 measures for each standard): 200 µL different 4-MU concentration (100 µM, 50 µM, 25 µM, 10 µM, 5 µM, 1 µM in GUS Buffer 1X).
-Samples: 100 µL GUS Buffer 1X containing 1 µg of total protein and 100 µL 2 mM 4-MUG in GUS Buffer 1X.
Substrate must be added just before loading the plate into the fluorimeter (LUMIstar OPTIMA Upgradeable Microplate Luminometer (BMG LABTECH, Germany). Fluorescence was measured at 37°C.

### 6.2.17 *R. solanacearum* inoculation

Plant root inoculations and bacterial internal growth curves were performed as previously described (Deslandes et al,. 1998). Plant phenotypic responses were scored daily, using a

disease-index scale ranging from 0 to 4, according to the percentage of wilted leaves (0 = no wilt, 1 =1 to 25%, 2= 26 to 50%, 3 = 51 to 75%, 4 =.75%).

### 6.2.18 Micoscopic analyses

For transversal sections, hypocotyls or roots were embedded in Technovit 7100 resin (Hareus Kulzer) before making 10-μm-thick sections with a Reichert-Jung microtome. Observations were made with a bright-field Axiophot microscope. Digital images were taken with a Leica camera and software.

**Table C6-3.** Buffers and solutions used in this thesis

**For transient expression in N.*benthamiana***

Agromix infiltration buffer:

| Agromix infiltration buffer: | |
|---|---|
| MES | 10 mM |
| $MgCl_2$/KOH pH=5.6 | 10 mM |
| Acetosyringone | 150 µM |

CTAB DNA extraction buffer 2 X

| | |
|---|---|
| Tris-HCl | pH=8.0 |
| NaCl | 1.4 M |
| EDTA | 20 mM |
| CTAB | 2% |
| β-mercaptoethanol | 0.2% |

**For SDS-PAGE**

4 pieces Mix gels:

| | |
|---|---|
| Mix gel 10% | 20 mL |
| 30% Acrylamide/Bis-Arylamide | 10mL |
| 20% APS | 300 µL |
| TEMED | 10 µL |

4 pieces Gels Stacking:

| | |
|---|---|
| Mix Stacking | 10 mL |
| 30% AA/ABA | 2 mL |
| 20% APS | 120 µL |
| TEMED | 15 µL |

Stock Solution Mix Gel 10%:

| | |
|---|---|
| H20 | 80 mL |
| Tris HCl 1.5 M pH=8.8 | 50 mL |
| SDS 20% | 1 mL |

Stock stacking:

| | |
|---|---|
| H2O | 63 mL |
| Tris HCl 1M pH=6.8 | 11.4 mL |
| 20% SDS | 450 µL |

Laemmly running buffer 1X:

| | |
|---|---|
| Tris | 2.9g |
| SDS | 1g |
| Glycin | 14.4g |

Laemmly extraction buffer 2X:

| | |
|---|---|
| Tris-HCl pH=7.5 | 0.125 M |
| SDS | 4% |
| Glycerol | 20% |
| DTT | 0.2 M |
| Bromophenol blue | 0.02% |

Transfer buffer 10X:

| | |
|---|---|
| Tris | 58.2g |
| Glycine | 29.3g |
| SDS | 3.75g |
| Adjust volume to 800 mL H2O | Don't adjust pH |

Transfer buffer 1X:

| | |
|---|---|
| Transfer buffer 10X | 100 mL |
| Transfer buffer 10X | 100 mL |
| 96.6% Ethanol | 200 mL |
| H2O | 700 mL |

TBS-T buffer 1X (1 L):

| | |
|---|---|
| Tris-HCl pH=7.5 | 10 mL |
| NaCl | 8.7g |
| 20% Tween | 1 mL |

**For GUS staining of plant:**

GUS staining buffer:

| | |
|---|---|
| NaPi* pH=7 | 50mM |
| EDTA | 5mM |
| Ferrocyanide | 0.5mM |
| Ferricyanide | 0.5mM |

(*) NaPi* pH=7 solution: Mix 42.3 mL 0.5M $NaH_2PO_4$ and 57.7 mL 0.5M $Na_2HPO_4$. Add $H_2O$ to final volume 1L.

X-gluc stock: The X-Gluc (bromochloroindoyl-b-glucuronide) is dissolved in dimethyl formamide (Final concentration 25mg/mL) and stored in the dark at -20 ℃.

**For Electrophoretic Mobility Shift Assay (EMSA)**

5X EMSA binding buffer:

| | |
|---|---|
| Hepes KOH PH=7.8 | 100mM |
| Glycerol | 50% |
| DTT | 5mM |
| EDTA | 0.5mM |
| KCl | 250mM |

6.5% native polyacrylamide Gel in 0.5X TBE:

| | |
|---|---|
| 10X TBE | 600 µL |
| 24% Acryl/bis (39/1) | 3.3 mL |
| 60% glycerol | 500 µL |
| 20% APS | 36 µL |
| TEMED | 12 µL |
| H2O | 7.6 mL |

**For Microplate fluorometric gus assay**

GUS protein exaction buffer 1X:

| | |
|---|---|
| NaPi* pH=7.5 | 50mM |
| 2-mercoapto | 10mM |
| NaEDTA. pH=8.0 | 10mM |
| Triton** | 0.10% |
| Na-L.Sarco. | 0.10% |

(*) NaPi* pH=7.5 solution: to be prepared from 16% 0.2M $NaH_2PO_4$ and 84% 0.2M $Na_2HPO_4$.
(**) Triton stock solution should be prepared from 100% commercial solution.

Substrate: 4-MUG (2 mM):

| MW=388 | ForX final volume of 2 mM 4-MUG, use (mg) | | | |
|---|---|---|---|---|
| | X=1 mL | X=5 mL | X=10 mL | X=50 mL |
| 4-MUG (mg) | 0.776 | 3.88 | 7.76 | 38.8 |

Standard solution 4-MU (1 mM). Always use fresh solution:

| MW=198 | For X final volume of 1 mM 4-MU, use (mg) | | |
|---|---|---|---|
| 4-MU (mg) | X=10 mL | X=20 mL | X=50 mL |
| | 0.776 | 3.88 | 7.76 |

# Chapter VII
# Reference

# Reference

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# ABSTRACT

In nature, plants are constantly exposed to microbial pathogens and have evolved an effective and dynamic immune system in order to survive. *R. solanacearum*, the causing agent of wilt disease, is a soil-borne bacteria pathogenic on more than 200 plant species. Bacteria enter roots, invade xylem vessels and then spread rapidly to aerial parts of the plant through the vasculature. In *A. thaliana* Nd-1 plants, *RRS1-R*, with its partner *RPS4* allows resistance to strains of *R. solanacearum* that deliver PopP2, a type III effector, into the plant cells. Previous studies showed that RRS1 and RPS4 are two NBS-LRR receptor proteins involved in the perception of the effector. Interestingly, RRS1 also harbors a WRKY transcription factor domain in its C-terminal end. In a susceptible *Arabidopsis* ecotype Col 0, *RRS1-S* is an allelic gene of *RRS1-R*, which encodes a similar structure. The recognition of bacterial and plant proteins leads to RRS1 protein accumulation in the nucleus, triggering possibly transcriptional gene regulation. Important genomic reprogramming of the infected plant cells has indeed been shown.

My work shows that the *RRS1-S* and *RRS1-R* genes are expressed mainly in mature roots and basal hypocotyls, in pericycle cells and passage cells from the endoderm. These cells correspond to entry sites of the invading *R. solanacearum* bacteria within the vascular tissues. We also demonstrated the binding of WRKY domain of RRS1-R and RRS1-S, *in vitro*, to W boxes which are *cis*-regulatory elements recognized by WRKY transcription factors.

In order to identify the *in vivo* target sequences of RRS1-R and RRS1-S, a DamID (DNA adenine methyltransferase IDentification) approach, detecting transitory DNA-protein associations was developed. DamID is based on the fusion of a protein of interest to a DNA Adenine Methyl-transferase from *E. coli,* which will methylate DNA in the vicinity of the binding sites of this protein. The fingerprints can be further mapped by DNA restriction with methylation sensitive enzymes, and cloned or directly sequenced. Analysis was focused on RRS1-R, by cloning FARMs (Fragment Associated to RRS1 driven Methylation) from Nd-1 plants expressing or not an inducible *PopP2* gene. This allowed the identification of several putative targets of RRS1-R and led us to propose a model for its function as a transcription factor. High throughput sequencing was then initiated at a whole genome scale analysis. The function and transcriptional regulation of a putative RRS1 target gene was evaluated.

Taken together, the results of this study illustrate the important role of RRS1-R in the regulation of the plant response to *R. solanacearum*.

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**TITRE : Identification et caractérisation des gènes cibles de *RRS1-R*, une protéine conférant la résistance d'*Arabidopsis thaliana* à la bactérie pathogène *Ralstonia solanacearum***

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**LIEU ET DATE DE SOUTENANCE : INRA, Castanet-Tolosan, Le 14 Février 2014**

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**RESUME :**

*Ralstonia solanacearum*, agent du flétrissement bactérien, affecte près de 200 espèces végétales. Les gènes RRS1-R confèrent à l'écotype d'A. thaliana Nd-1 une résistance à différentes souches de *R. solanacearum*. RRS1-R code une protéine de structure modulaire associant les domaines typiques de nombreuses protéines de résistance TIR-NBS-LRR et un domaine signature de facteurs de transcription WRKY. Dans l'écotype sensible Col-0, le gène RRS1-S code pour une protéine qui présente une structure très semblable. Au cours de ce travail, nous avons montré que les gènes RRS1-R et RRS1-S s'expriment essentiellement dans les cellules du péricycle et les cellules de passage de l'endoderme des racines matures et de la base de l'hypocotyle, cellules qui correspondent aux sites de pénétration des bactéries dans le système vasculaire où elles se multiplient. Nous avons montré que les deux domaines WRKY des protéines codées par ces gènes se fixent spécifiquement aux boites W, reconnues par les facteurs de transcription de la famille WRKY. Nous avons par la suite développé une approche DamID (DNA adenine methyltransferase IDentification) visant à identifier les gènes cibles des protéines RRS1-R et RRS1-S in vivo. L'analyse a été focalisée sur l'identification des gènes cibles de RRS1-R, dans le fond génétique résistant Nd-1 exprimant, ou pas, la protéine d'avirulence PopP2 sous contrôle d'un promoteur inductible. Dans chacun des cas le séquençage d'une centaine de FARMs (Fragments Associated to RRS1-driven Methylation) a permis de proposer des cibles potentielles et un modèle de fonctionnement de RRS1-R comme régulateur transcriptionel. Ce travail se poursuit par une analyse globale au niveau du génome, grâce au séquençage haut débit des FARMS et par l'étude de la fonction dans la réponse de la plante et de la régulation transcriptionelle de quelques cibles d'intérêt. Les résultats de ce travail illustrent dans leur ensemble l'importance de RRS1-R pour réguler la réponse des plantes à R.solanacearum.

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**MOTS-CLES : *Arabidopsis thaliana*, *Ralstonia solanacearum*, TIR-NBS-LRR, RRS1, DNA adenine methyltransferase Identification.**

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