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MONTSERRAT SOLÉ CASTELLVÍ

Facultat de Biologia

Departament de Genètica

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Memòria presentada per Montserrat Solé Castellví per tal d'optar al títol de Doctora expedit per la Universitat de Barcelona. Tesi doctoral realitzada sota la direcció de Marc Valls i Matheu al Departament de Genètica de la Facultat de Biologia (UB).

El director, L'autora,

Marc Valls i Matheu Montserrat Solé Castellví

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"Sail away from the safe harbor. Catch the trade winds in your sails.

Explore. Dream. Discover."

Mark Twain

ABBREVIATIONS

3-OH-PAME	3-Hydroxypalmitic acid methyl ester
ABA	Abscisic acid
AD	GAL4 activating domain
AIP	2-Aminoindan-2-phosphonic acid
AtMC	*Arabidopsis thaliana* metacaspase
ATP	Adenosine triphosphate
avr	Avirulence
AWR	Alanine-tryptophan-arginine motif
BD	GAL4 binding domain
bp	Base pair
C-	Central
C-/C(t)	Carboxyl (terminal)
CC	Coiled-coil
cDNA	complementary DNA (from mRNA template)
CDPK	Calcium-dependent protein kinase
CEL	Conserved effector locus
CFU	Colony-forming unit
CHS	Chalcone synthase
Col-0	*Arabidopsis thaliana* columbia ecotype
cv.	Cultivar
CyaA	Adenilate cyclase
DAB	3,3'-Diaminobenzidine
DAMP	Damage-associated molecular pattern
DMR6	Downy mildew resistane 6 protein
DNA	Deoxy-ribo-nucleic acid
EF-Tu	Elongation factor Tu
EFR	EF-Tu receptor
EPS	Exopolysaccharide
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
EV	Empty vector
Flg22	Flagellin 22-amino acid sequence
FLS2	Flagellin-sensitive-2 (receptor)
GC %	Guanine-cytosine content (percentage)
GST	Glutathion S-transferase
Gus	Beta-glucuronidase gene
GWY	Gateway cloning system
H_2O_2	Hydrogen peroxide
HA	Human influenza hemagglutinin
His	Histidine
HR	Hypersensitive response

Hrc	Hypersensitive response conserved
hrp	Hypersensitive response and pathogenicity
ISR	Induced systemic resistance
JA	Jasmonic acid
Kb	Kilobase
Kda	Kilodalton
KIN10	Snf1-related kinase
LPS	Lipopolisaccharide
LRR	Leucine-rich repeats
LSU	Low sulphur protein
MAPK/MPK	Mitogen-activated protein kinase
Mb	Megabase
MBP	Maltose-binding protein
N-/N(t)	Amino (terminal)
NB	Nucleotide binding domain
NO	Nitric oxid
O.D.	Optical density (Absorvance)
ORF	Open reading frame
PAL	Phenylalanine ammonia lyase
PAMP/MAMP	Pathogen/microbe-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PR	Pathogenesis-related
PRR	Pattern-recognition receptor
PTI	PAMP-triggered immunity
pv.	Pathovar
R	Resistance (gene or protein)
RLK	Receptor-like kinases
RNA	Ribo-nucleic-acid
ROS	Reactive oxygen species
RRS1	Resistant to *Ralstonia solanacearum* 1
SA	Salycilic acid
SAR	Systemic acquired resistance
SD	Shine-Dalgarno sequence
SN	Supernatant
sp.	Species (undetermined)
spp.	Species (several)
SUMO	Small ubiquitin-related modifier
T1SS	Type I Secretion System
T2SS	Type II Secretion System
T3	Type III
T3E	Type III-effector proteins
T3S	Type III Secretion
T3SS	Type III Secretion System

T4SS	Type IV Secretion System
T5SS	Type V Secretion System
T6SS	Type VI Secretion System
TAL	Transcription activator like
TB	Trypan blue dye
TIR	Toll-interleukin 1 receptor domain
TLR	Toll-like receptor
WB	Western-blot
WNK8	Serine-threonin-protein kinase
WT	Wilt-type
X-α-GAL	5-bromo-4-chloro-3-indolyl alpha-D-galactopyranoside
Y2H	Yeast-two-hybrid
YFP	Yellow fluorescent protein

CORE GLOSSARY

Avirulence: Inability of a pathogen to cause a compatible reaction on a host cutivar with genetic resistance.

Biotroph: pathogen that require living host tissue to complete their life cycle.

Callose: a plant cell-wall polysaccharide produced in response to wounding or infection by pathogens.

Chlorosis: Abnormal yellowing of the normal green color of leaves due to loss of chlorophyll.

Compatible interaction: takes place when pathogen infects a susceptible host and disease develops.

Cultivar (cultivated variety): plant or group of plants selected for desirable characteristics that can be maintained by propagation.

Effector protein: bacterial protein translocated to host cell by the Type III Secretion System in order to alter host cell structure and function.

Effector-triggered immunity: strong plant defences triggered by R-proteins upon effector recognition or its activity that often lead to a hypersensitive response.

Exopolisaccharide: are high-molecular-weight polymers that are composed of sugar residues and are secreted by microorganisms into surrounding environment.

Hemibiotroph: biotroph pathogen that induce substantial host cell death at the end of the infection cycle.

Homologues: the term designs genes or proteins that share an arbitrary threshold level of similarity and with a common origin.

Host range: the variety of plant genera and species that a pathogen can infect.

Host resistance: takes place when a host plant for a particular pathogen encodes one or few proteins capable of avoiding compatible plant-pathogen interaction.

Hypersensitive response: active defense mechanism that limits the progression of the infection by the rapid death of few host cells (programmed cell death), mainly due to pathogen avirulence gene recognition.

Incompatible interaction: takes place when a pathogen cannot infect a non-host plant or a resistant cultivar and thus disease cannot develop.

Indentity: quantitative term that defines the degree of identical sequence (same aminoacid) match between two aligned sequences.

Infection: invasion by and multiplication of pathogenic microorganisms in a tissue/organism that lead to a disease process.

Koch's postulates: set up criteria by Robert Koch to determine if a given bacteria is the cause of a given disease.

Lateral/horizontal gene transfer: any process in which an organism incorporates genetic material from another organism without being the offspring of that organism.

Lignin: complex organic material derived from phenylpropane that imparts rigidity and strength to woody tissues.

Necrosis: death of plant tissue in a localized area resulting in a black or brown lesion followed by a tissue collapse.

Necrotroph: pathogen that can live on dead tissues.

Non-host resistance: refers to the phenomenon that most plant species are typically resistant to the pathogens of other plant species. It is the most durable and broad-spectrum type of plant resistance.

Ortholog: homologous sequences that were separated by a speciation event.

Oxidative burst: a rapid and transient production of huge amounts of reactive oxygen species.

PAMP-triggered immunity: plant immune responses that are elicited upon detection of conserved molecules from pathogens.

Paralogue: homologous genes that result from divergent evolution from a common ancestral gene produced by gene duplication.

Parcial resistance: pathogen reproduction or symptom production occurs but to a less extend than in susceptible plants.

Passive defence: mechanisms that are already present before pathogen recognition.

Pathogen: organism that incites or causes a disease process with a host.

Pathogen-associated molecular pattern: molecules associated with groups of pathogens that are recognized by cells of the innate immune system.

Pathogenesis-related proteins: synthesis and accumulation of defense-related proteins following pathogen infection of plants.

Pathogenicity: the ability of a pathogen to interfere with one or more functions within a plant.

Pathology: science branch that studies causes and effect of diseases.

Pathovar: bacterial strain or set of strains with the same or similar characteristics but with different pathogenicity to one or more plant hosts.

Phylogeny: the sequence of events involved in the evolution of a species, genus, etc.

Programmed cell death: active defense mechanism where the attacked cell and several plants cells around it die in response to signals preventing pathogen spreading.

Race: population of pathogens isolates that have the same virulence.

Reactive-oxygen species: chemically-reactive molecules containing oxygen.

Resistance: ability of the host to reduce pathogen growth, reproduction, and/or disease-producing activities.

R-protein: plant resistance protein that directly or indirectly recognise an effector bacterial protein.

Similarity: quantitative term that defines the degree of similar sequence match between two aligned sequences. Aminoacids are similar when they share similar chemical properties (i.e. hydrophobicity, charge etc.).

Soilborne pathogens: can survive in soil for an extended period of time in absence of host plants.

Susceptibility: inability of the host to reduce pathogen growth, reproduction, and/or disease-producing activities.

Symptom: a phenomenon that arises from and accompanies a particular disease or disorder and serves as an indication of it.

Type III Secretion System: needle-like structure found in several pathogens that serves for secretion of effector proteins into the host cell.

Virulence: the rate or how well a pathogen is able to manipulate host cell functions.

Xylem: a tissue in vascular plants that carries water and dissolved minerals from the roots and provides support for softer tissues.

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CHAPTER 1
HOW PATHOGENS ATTACK

Bacteria appear to be the most abounding life organisms on earth. Although most species are not harmful (and many are even beneficial), some can produce diseases that affect not only the human population but also our sustaining food. In this chapter, some of the requirements for a bacterium to become a successful pathogen will be discussed.

1.1. Plant pathology in context

Infections caused by fungi are the most common plant disease and have had the greatest impact on population. For instance, in the 1840 decade around 1 million people died in Ireland and another million migrated to USA because of the famine, as a consequence of an outbreak of potato blight. Some years later, in 1861, DeBary demonstrated that *Phytophtora infestans*, was the causal agent for the potato blight disease (Agrios, 2004), a scenario that promoted the study of plant pathology.

In 1878, following the discovery of bacteria as the disease-causative agent for anthrax by Pasteur and Koch (1876), Burrill describes for the first time a bacterial disease of plants: the fireblight of apple and pear trees. Soon after, establishment of agar culture media and Koch's postulates represented a step forward in the pathology field since permitted the identification of a causal agent for a given disease (Agrios, 2004). From these novel discoveries and until the late 1980s, much effort was devoted to study the physiology of disease by determination of virulence factors from biologically active cell-free-extracts. However, these studies did not provide much information about biotrophic pathogens (Keen, 2000; Schneider and Collmer, 2010).

A new era based on single-gene molecular genetics was born after the gene-for-gene hypothesis postulated by Flor (Flor, 1955). Plants and pathogens were studied in an integrative view and it was found that both resistance and infection ability are genetically controlled. Pathogens that produce an avirulence gene (avr) cannot infect plants that express a resistance (*R*) gene, which display resistance towards the pathogen. The discoveries of (1) the double helix structure of DNA by Watson and Crick, (2) *Agrobacterium tumefaciens* Ti plasmid by Schilperoort and Schell and (3) polymerase chain reaction (PCR) by Mullis and collaborators, facilitated the switch from physiological studies to molecular genetics in order to genetically

characterize the virulence factors involved in disease establishment, mainly by gain/loss-of-function methods (Keen, 2000; Schneider and Collmer, 2010). Martin and collaborators managed to introduce for the first time in 1993 the tomato *R* gene *Pto* that confers resistance to the pathogen *P.syringae* pv. *tomato* carrying the *avrPto* avirulence gene (Martin et al., 1993). The genomic era began when the genome sequence of the first phytopathogen, *Xylella fastidiosa* (causative of citrus variegated chlorosis) was released in 2000 (Simpson et al., 2000). A constant improvement and cost reduction of genome sequencing techniques has favoured the sequencing of a vast number of plant pathogens in the last years. Currently, more than 2000 bacterial pathogens genomes have been released. An exhaustive bioinformatical sequence analysis enabled the successful determination of new pathogenesis determinants and also the prediction of their function by conserved motif search or by comparison to previously described virulence factors from other sequenced pathogens (Vinatzer and Yan, 2008; Schneider and Collmer, 2010). In 2000, the *Arabidopsis thaliana* genome sequence became available (AGI, 2000) and consolidated it as a strong model to further dissect plant-pathogen interplay mechanisms.

1.2. Bacterial phytopathogens

Plants are constantly exposed to pathogens and infection is a rare event, therefore, pathogens are more an exception than a rule. In addition, disease is dependent on three components that confer the disease triangle: the host (growth stage, general heath and susceptibility), the pathogen (presence or absence, pathogenicity and fitness) and the environment (temperature, soil properties, humidity). Plant disease is caused by organisms of all kingdoms including Animalia, Stramenophila, Fungi, Prokaryotae, Plantae and Protozoa (Trigiano et al., 2008) as shown in Figure 1. Most plant pathogens are grouped in the gram-negative bacteria, being proteobacteria its major subgroup. This represents the largest and most phenotipically heterogenic bacterial lineage and is divided into four different classes according to rRNA sequences: alpha, beta, gamma and epsilon. While plant pathogens from the genera *Ralstonia*, *Burkholderia* and *Acidovorax* belong to β-proteobacteria, *Xanthomonas* and *Pseudomonas* belong to γ-proteobacteria (Ecker et al., 2005; Falkow et al., 2006).

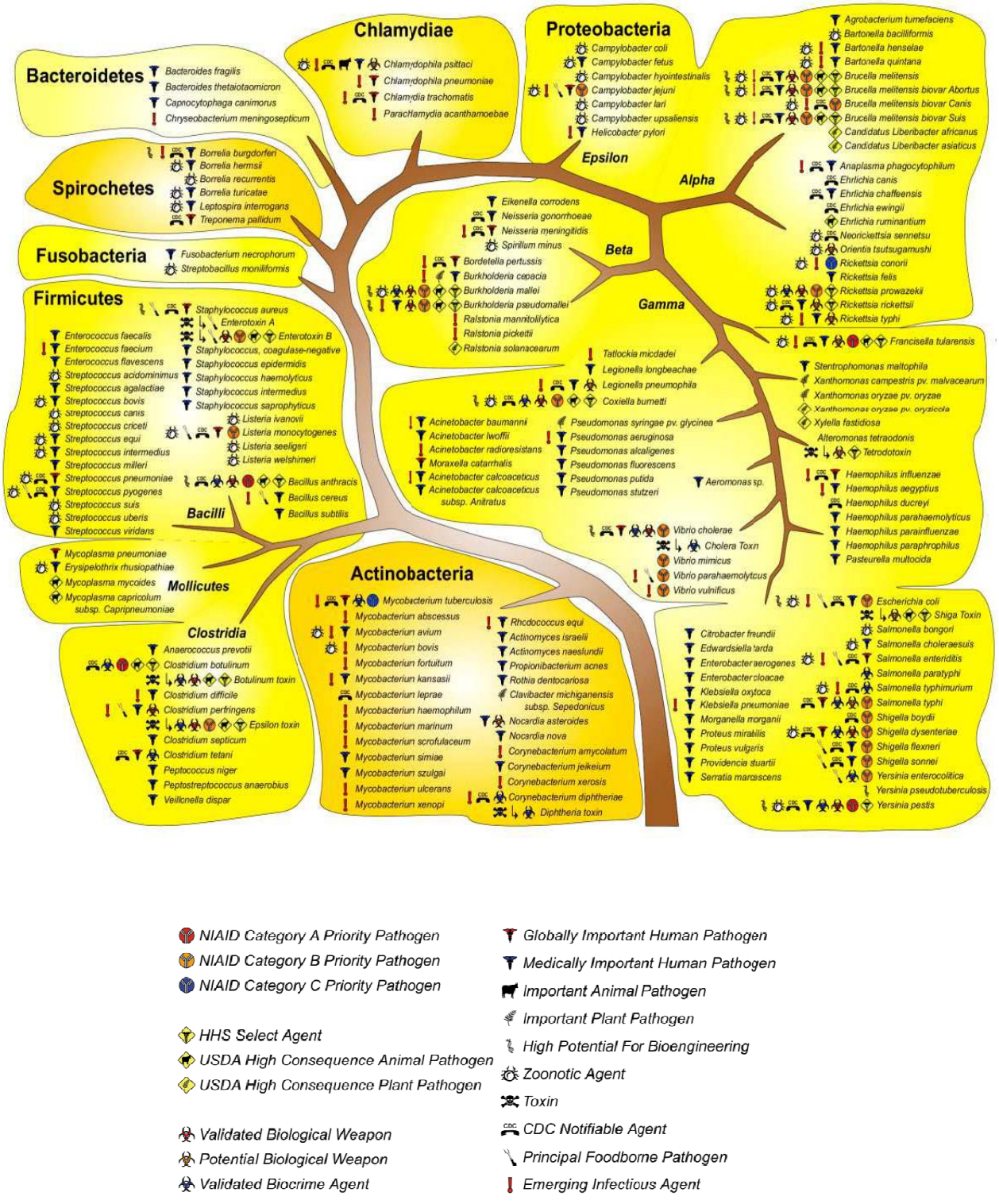


Figure 1. Distribution of bacterial pathogens in the different kingdoms.

Image obtained from (Ecker et al., 2005).

Bacteria cause a severe economical impact on agriculture by producing diseases ranging from spots, mosaic patters or chlorosis on leaves or fruits, tuber rots or even the plant death (Trigiano et al., 2008). Major diseases caused by pathogens that are relevant for crop production are listed in Figure 2.

Plant disease	Crop affected	Causal agent
Bacterial blight (A)	Cassava	*Xanthomonas axonopodis* pv. *manihotis*
Stewart's wilt	Maize	*Erwinia stewartii*
Corn stunt disease	Maize	*Spiroplasma kunkelii*
Halo blight (B)	Oats	*Pseudomonas syringae* pv. *coronafaciens*
Bacterial wilt (C)	Potato	*Ralstonia solanacearum*
Bacterial soft rot (D)	Potato	*Erwinia carotovora*
Common scab	Potato	*Streptomyces scabies*
Bacterial ring rot	Potato	*Clavibacter michiganensis* subsp. *sepedonicus*
Bacterial leaf blight	Rice	*Xanthomonas oryzae* pv. *Oryzae*
Bacterial pustule	Soybean	*Xanthomonas axonopodis* pv. *phaseoli*
Soil rot (E)	Sweet potato	*Streptomyces ipomoea*
Bacterial leaf streak	Wheat	*Xanthomonas translucens* pv. *undulosa*

Figure 2. Description of bacterial diseases that produce a major impact in crops.

Some of the causal agents of important crops such as potato, oats or maize and the consequences of infection as exemplified in the images. Adapted from (Strange and Scott, 2005).

1.3. What makes a pathogen a pathogen: virulence determinants

Some pathogens can survive in different environmental conditions, as benign epiphytes or soil saprophytes until they accidentally infect a host plant. For successful pathogen survival, expression of virulence factors requires extreme coordination as it is important for energy saving, proper disease development, evasion or even pathogen dispersion (Mole et al., 2007; Wu et al., 2008). Only success on coordination of all these factors will facilitate disease establishment through a victorious infection and progression. Discovery of pathogenesis determinants, and their function, is crucial for understanding the interaction between bacterial pathogen and host and thus be able to combat bacterial disease in a not too far future. Although many virulence determinants are shared in both plant and animal pathogens (Wu et al., 2008), only those from phytopathogens will be discussed here. Bacterial “weapons” include exopolysaccharides, secretion systems, hormones and toxins, adhesion molecules, and

signalling components (Boucher et al., 2001; Mole et al., 2007; Wu et al., 2008), some examples of which will be discussed below.

A first kind of pathogenicity determinants are proteins related to cell membrane, which are important for contact with host cells as they might be needed for host perception, modulation of cell adhesion or successful colonization. For example, PrhA membrane receptor in *R.* solanacearum perceives an undefined plant substance and activates the infection machinery (Aldon et al., 2000). Adhesin-like proteins present in pathogens such as *Xanthomonas* or *Xylella* for adhesion in xylem vessels play also a pivotal role in virulence (Agrios, 2004). With a different functionality, the outer-membrane proteins *TolC* from *Erwinia amylovora* mediate resistance towards plant phytoalexins (Al-Karablieh et al., 2009).

Hormonal signalling is vital for proper plant welfare and some bacteria aim to destabilize it. Striking examples are production of the phytohormone auxin by *Pseudomonas syringae* pv *savastanoi* (Smidt and Kosuge, 1978) and of cytokinins by *A. tumefaciens* (Akiyoshi et al., 1984). In addition, several pathovars of *P. syringae* produce coronatine, an analogue of jasmonic acid that also interferes with plant hormone balance (Glazebrook, 2005). Apart from hormones, phytotoxins are harmful products for plants and can be freely diffused to the plant cell tissues as for the case of phaseolotoxin produced by *P. syringae* pv. *phaseolicola* that produces a systemic chorosis in beans (Bender, 1999).

Production of huge amounts of exopolysaccharides is also a key factor for general virulence. These substances protect the bacterium by keeping moisture or directly obstructing the xylem as it has been described for *R. solanacearum* and *X. campestris* (Büttner and Bonas, 2009). Regarding *Ralstonia,* its exopolysaccharide (EPS) is composed in equimolar ratios of *N*-acetylgalactosamine plus two amino sugars 2-*N*-acetyl-2-deoxy-galacturonic acid and 2-*N*-acetyl-4-N-(3-hydroxybutanoyl)-2,4,6-tri-deoxy-D-glucose (Orgambide et al., 1991). Strains lacking EPS are seriously impared in pathogenicity (Denny and Baek, 1991). The more complex polysaccharide xanthan gum is present in *X. campestris* and it is also important for pathogenesis (Katzen et al., 1998). It has been also pointed out that exopolysaccharide production could be important for biofilm formation in *Xillela fastidios*a (Roper et al., 2007).

Another important virulence signalling in bacteria is the quorum sensing to control cell-density. This perception is often coupled with regulation of the infection process and further detection to verify cell density population, which will switch a change in protein expression needs. An example is the secretion of *N*-acylated homoserine lactones (Kumari et al., 2006). An

alternative quorum sensing system is controlled in *R. solanacearum* by 3-hydroxypalmitic acid methyl ester (3-OH-PAME) (Flavier et al., 1997).

Secretion systems are also very important for bacterial fitness and proper host defense subversion depends on them. In gram-negative bacteria, at least six different systems of secretion have been described so far. A general description on each type of secretion and molecules secreted through is presented in Table 1. A remarkable feature is that up to three existing systems, the Type III secretion system (T3SS), the Type IV (T4SS) and the Type VI (T6SS), translocate DNA and/or proteins into eukaryotic cells (Büttner and Bonas, 2009).

System	Description	Transport across inner membrane	Secreted proteins
T1SS	ABC* transporter in IM, periplasmic membrane fusion protein, OM channel	Sec-independent	Toxins, degradative enzymes
T2SS	At least 11 components in IM, periplasm and OM; predicted periplasmic pseudopilus	Mediated by Sec or TAT system	Toxins, degradative enzymes
T3SS	Evolutionary related to bacterial flagellum; at least 20 components in IM, periplasm and OM; extracellular pilus (plant pathogens) or needle (animal pathogens)	Sec-independent	Extracellular components of T3SS, effector proteins
T4SS	Evolutionary related to bacterial conjugation system; spans both bacterial membranes; extracellular pilus	Sec-independent	Extracellular components of T4SS; DNA and/or proteins
T5SS	Protein channel in OM; autotransporters and two-partner secretion systems	Sec-dependent	e.g. adhesins
T6SS	Multicomponent secretion machinery, evolutionary related to phage tail-associated protein complexes	Presumably Sec-independent	Hcp and VgrG, which contains C-terminal actin crosslinking domain

*ABC: -binding cassette; IM: inner membrane; OM: outer membrane

Table 1. Protein secretion systems from Gram-negative bacteria.

In bacteria, there are up to six different ways of secreting proteins and their role in attachment and pathogenesis has been extensively contrasted. Adapted from (Büttner and Bonas, 2009).

Type I and Type II secretion system (T1SS and T2SS) are involved in secretion of extracellular enzymes, proteases, lipases and toxins (Omori and Idei, 2003; Agrios, 2004). For example, secretion of some plant cell-wall-degrading enzymes will help to nourish the bacteria in the hostile environment and hence favour pathogen progression. Pectinases and cellulases have been described in most bacterial pathogens and shown to assist pathogenicity, but it is in the rot-producing bacteria, like *Erwinia carotovora,* that these enzymes are most important as virulence determinants (Bell et al., 2004).

On the other hand, the Type IV secretion system (T4SS) consists in a pilus-like structure and, apart from the secretion of a wide range of products, it is known to play a role in conjugation and attachment. For instance, *A. tumefaciens* possesses a type IV transporter (also known as VirB complex) to transfer the T-DNA segment of the Ti plasmid and virulence proteins into host cells (Shirasu and Kado, 1993). T4SS is also required for *R. solanacearum* natural transformation (Kang, 2002). In *X. campestris* pv. *hyacinthi it* was shown that pilus was implicated in the bacterial attachment to hyacinth leaves (Ojanen-Reuhs et al., 1997) whereas in *X. campestris* pv *vesicatoria* is important for bacterial aggregation that contributes to UV protection (Van et al., 1994). In *Azoarcus*, this secretion system seems to be crucial for colonization of grass roots (Dörr et al., 1998).

Type VI secretion system (T6SS) has been recently discovered in animal pathogens (Mougous et al., 2006; Pukatzki et al., 2006) and since then it has been found in many pathogenic bacteria, including those infecting plants. The role of T6SS in pathogenesis has become increasingly clearer although this secretion might be also important for other functions apart from those related with pathogenesis (reviewed in Jani and Cotter, 2010).

Most pathogenic gram-negative bacteria, which include pathogens such as *Ralstonia, Pseudomonas* or *Xanthomonas*, use the Type III secretion system (T3SS) that mediates injection of bacterial proteins directly to the host cytoplasm. Traditionally, these translocated proteins have been called effectors as they produce an effect on the host. Generally, the main objective of effector proteins is to manipulate host defences to favour bacterial infection. In most cases, the T3SS is the major virulence determinant, as bacteria devoid of a functional T3SS are no longer able to produce disease on the plant or animal host (Alfano and Collmer, 2004). The T3SS machinery and its associated effectors will be discussed in more detail below.

1.4. The Type III Secretion System

The term T3SS was first used by Salmon and collaborators to describe a syringe-like membrane appendix, and has since become one of the major topics in pathogenicity (Salmond and Reeves, 1993). It is a highly conserved secretion system and plays a central role in pathogens, being necessary for both virulence and host-specificity (McCann and Guttman, 2008). Moreover, the T3SS is not only present in animal or plant pathogens but also in symbiotic bacteria where, as found in *Rhizobium,* it is important for plant nodulation (Viprey et al., 1998). Hence, the T3SS is a valuable macromolecular tool controlling different types of bacterial-host communication.

Despite the existence of many secretion complexes in bacteria, the T3SS is the most sophisticated, as at least 25 different proteins are required for its assembly and probably also the most fast-evolving one due to its constant interaction with host organisms (Cornelis, 2006; McCann and Guttman, 2008).

T3SS families

Phylogenetic studies of several conserved proteins required to build the T3SS injectisome indicate that there are 7 different families of T3SS: that of the Chlamydiales, Hrp1 (e.g. *E. amylovora* or *P. syrinage*), Hrp2 (e.g. *R. solanacearum*, *B. pseudomallei* or *X. campestris*), SPI-1 (e.g. *B. pseudomallei, S. flexneri* or *S. enterica*), SPI-2 (e.g. *E. Coli* EPEC, *Y. pestis* or *S. enterica*), the rhizobium T3SS and Ysc type (e.g. *Y. pestis* or *P. aeruginosa*). In general, type Hrp1 and Hrp2 are the most prevalent in plant pathogens whereas Ysc, SPI-1 and SPI-2 occur in free-living animal pathogens (Troisfontaines and Cornelis, 2005; Cornelis, 2006). Genes encoding the T3S apparatus in plants are often clustered together and are named *hrp* (hypersensitive response and pathogenicity) since they are important for both disease and resistance in plants as it will be described in the Chapter 3.

Structure of T3SS

The T3S apparatus is composed of two main parts: the basal body that spans through the inner and outer bacterial membrane with ring-like anchoring structures, and the external appendage that will serve as a bridge between bacteria and host for correct protein translocation (Figure 3A) (Galan and Wolf-Watz, 2006). The basal body is highly conserved among pathogens whereas the appendage varies depending on the type of pathogen: animal pathogens use a needle-like structure for secretion while plant pathogens employ a larger appendix that resembles a pilus (Figure 3A). This is not surprising as plant pathogens require a longer structure to be able to traverse the plant cell wall, which is not present in animal cells (Galan and Collmer, 1999; Galan and Wolf-Watz, 2006; Buttner and He, 2009). Although most of the advances in characterization of the T3S injectisome have been made in the animal pathogens *Salmonella* or *Shigella*, the T3S-associated filamentous structure was first discovered in the phytopathogen *P. syringae* (Roine et al., 1997).

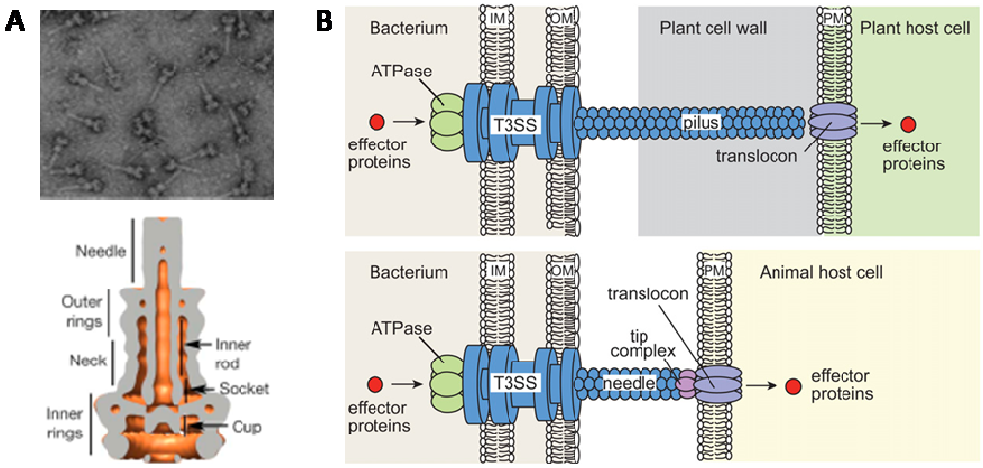


Figure 3. T3SS in animal and plant pathogens.

Although basal structure of T3SS is shared between plant and animal pathogens, external appendages differ. A) Electron micrographs of purified needle complexes from *Salmonella typhimurium* and basic structure of T3SS apparatus (Galan and Wolf-Watz, 2006). B) Schematic representation of the main differences in T3SS apparatus between animal and plant pathogens (Buttner and He, 2009).

The **basal structure** is composed of some inner rings and outer rings, joined by a rod, that anchor the injectisome to the bacterial membrane and it is thought to be the first element to be assembled. Among all proteins involved in injectisome formation, nine that belong to this basal body are extremely conserved in all pathogens and they are hence named *Hrc* (hypersensitive response conserved) and include an ATPase (HrcN), a secretin (HrcC), rings (HrcJ and HrcQ) or other structural inner-membrane proteins (HrcR, HrcS, HrcT, HrcV and HrcU (Cornelis, 2006). In addition, most of them are similar to those described for the flagellum basal body and they might be assembled and export in an analogous way. A deep debate rose to determine whether the T3SS derived from bacterial flagellum or if they share a common ancestor. While it is easy to think that the bacteria needed first a mobility system rather than a well-structured infection mechanism for eukaryotic cells (bacteria evolved millions of years before the origin of eukaryotes), implying that T3SSs evolved recently from flagella, the phylogenetic analysis does not point towards this direction. Today, the most plausible scenario is that the T3SS and the flagellar system diverged as a result of gene duplication from a common ancestor (Gophna et al., 2003; He et al., 2004; Cornelis, 2006; McCann and Guttman, 2008). However, contrary to the flagellar system, the T3SS are often encoded in extra-chromosomal loci or placed in pathogenicity islands (characterized by a differential GC content or presence of transposable elements), The fact that these regions are absent in non-pathogenic related species corroborates that they might be mobilized and transferred horizontally between bacteria sharing same habitat (Galan and Collmer, 1999, Cornelis, 2006 #78). This idea is reinforced by the fact that some bacteria possess more than one secretion

system, as is the case for some *Yersinia* or *Burkholderia* species and the fact that the T3SS phylogenetic tree does not match the species divergence tree (Cornelis, 2006; Lipscomb and Schell, 2011).

As mentioned before, the T3SS basal structure is coupled to an **external appendix** that will connect bacteria with the host cell (allowing protein passage from the bacterial cytosol to the eukaryotic cytosol) and its length and shape depends on the host cell type. In animal pathogens, the extracellular structure is called needle (around 80 nm long and 7-8 nm width, 2 nm inner diameter, in *S. enterica*), it shares similarities with the flagellar hook and has been far more studied than the supramolecular structure from plant pathogens (He et al., 2004; Cornelis, 2006). The final length of the needle matters to ensure a correct injection and several hypotheses have been proposed for its regulation. An example is the ruler model in *Y. enterocolitica* in which the protein YscP modulates needle subunits export and terminates it by its own secretion (Journet et al., 2003). In plant pathogens and symbionts, a pilus-like structure is coupled to the basal body. This appendage is much longer but with similar diameter than that of animal pathogens and it is encoded by *hrp* genes. HrpA in *P. syringae* or *E. Amylovora*, HrpE in *X. campestris* and HrpY in *R. solanacearum* have been described as component proteins of the Hrp pili (Roine et al., 1997; Van Gijsegem et al., 2000; Jin et al., 2001; Weber et al., 2005). It has also been proven that the pili are required for correct protein delivery to host cells and disease establishment. Appendages have been also postulated to work as sensors of cell contact since the T3SS apparatuses are scattered on the entire bacterial surface (Galan and Wolf-Watz, 2006).

Accessory proteins for the T3SS

The first proteins to be secreted through the basal body are the outer proteins that act as subunits to form the pilus or needle structure. Afterwards, for correct injection through the eukaryotic membrane, some accessory proteins called **translocators** seem to be needed too (e.g. PopF1 and PopF2 from *R. solanacearum* described in Meyer et al., 2006). Apparently, the function of the translocators is to create a channel, in the host cell membrane, continuous to that of the appendage and thus directly connect the host and pathogen cytosols. In some bacteria, it appears that a tip complex mediate the connexion between appendage and translocon (Figure 3B, (Buttner and He, 2009)).

Another group of important T3SS accessory proteins are the **chaperones**, which are small, acidic and dimeric proteins that specifically bind to the type III effectors (T3Es) and might regulate their secretion. Several functions have been attributed to them, including effector

unfolding, stabilization or even controlling the sequence of effectome secretion (He et al., 2004; Galan and Wolf-Watz, 2006; Buttner and He, 2009). In general, chaperones are specific to a certain protein and its genes are often placed close to the effector in the genome, but some of them interact with several secreted proteins. As the pore in the molecular syringe is relatively small, it is thought that chaperones could carry effectors to the **ATPase subunit** placed at the bottom of the basal body. ATPase-mediated effector unfolding could take place and energy release helps somehow the secretion process (Akeda and Galan, 2005; Lorenz and Buttner, 2009). Back in the eukaryotic cell, effectors may be folded again. It has been also speculated that chaperones could also prioritize effector secretion.

T3-secretion pathway is a sequential process involving first basal body assembly, followed by the appendage formation, secretion of accessory proteins such as translocators and it culminates with effector secretion. This implies a constant substrate switch that could be mediated by other accessory proteins called **T3S substrate specificity switch proteins**. These proteins might interact with C-terminal region of proteins in the bacterial inner membrane to modulate substrate specificity (He et al., 2004). Some proteins named **harpins** might be also secreted through T3SS although it appears that, contrary to effectors, they do not need to be injected into the host cell to exert their function. However, their role in pathogenesis or in secretion-assistance remains still unclear (Buttner and He, 2009).

1.5. Type III- effector proteins

Assembly of the T3SS apparatus and secretion of proteins through it requires a very tight regulation since it is a highly energy-consuming process. As commented before, genes coding for a functional injectisome are grouped together in gene clusters to coordinate its transcriptional regulation (Tang et al., 2006). In addition, effector secretion and translocation is only triggered when a host cell is perceived to avoid unnecessary energy expenses. In addition, while the T3S apparatus is well conserved among pathogens, secreted proteins are very heterogeneous (McCann and Guttman, 2008). Genes encoding Type III-secreted effectors (T3Es) display some common characteristics to T3SS-encoding genes: they are located in regions with a biased GC content, alternative usage of codons or close to transposable elements. This scenario has prompted to assume many events of horizontal gene transfer between bacteria to acquire novel effectors (Hueck, 1998; Petnicki-Ocwieja et al., 2002; Genin and Boucher, 2004; Schulze-Lefert and Panstruga, 2010).

Contrary to the Sec-dependent secretion pathways, T3 effector proteins do not bear any cleavable signal peptide. This fact has triggered an agitated debate on whether the secretion signal is present in the protein itself or at mRNA level. The most accepted view is that the N-terminal amphipathic nature and amino acid composition are crucial for secretion signalling (He et al., 2004). For instance, in *P. syringae* pv. *tomato* several features of effector proteins were identified in their first 50 amino acids: (1) solvent-exposed amino acids in the first five positions, (2) absence of Asp or Glu residues in the first 12 and (3) amphipathicity of these first 50 aminoacids. Genome analysis following these criteria lead to the identification of 38 putative effectors in this bacterium as shown in Petnicki-Ocwieja et al., 2002, which were experimentally confirmed. Furthermore, this criterion has been extremely helpful to predict the number of putative effectors in a pathogen, from a genome sequence analysis, as the first step towards effector discovery and its function characterization afterwards.

The size of the effector repertoire depends on each bacterium and even each strain. For instance, in *X. campestris* pv. *vesicatoria* around 20 effector proteins have been confirmed and in *P. syringae* pv. *tomato* DC3000 the number is estimated around 30. In *R. solanacearum* GMI1000 some 30 genes have been proven to encode effector proteins translocated through the T3SS, although around 40 more have also been predicted (Cunnac et al., 2009; Kay and Bonas, 2009; Mukaihara and Tamura, 2009; Poueymiro and Genin, 2009; Mukaihara et al., 2010; Remenant et al., 2010) (Table 2)

Bacterial species	**Repertoires of effectors proven to be secreted/injected**
P. syringae	HopK1, HopY1, HopAS1, HopU1, HopF2, HopC1, HopAT1, HopG1, HopD1, HopQ1, HopR1, HopAM1, HopN1, HopM1, AvrE, AvrB3, HopB1, HopX1, HopZ3, HopAB2, AvrPto, HopE1, HopV1, HopAQ1, HopG1, HopI1, HopX1, HopO1, HopT1, AvrRpt2, AvrA, HopW1, HopD1, HopQ1, AvrD1, AvrB2, HopAR1
Xanthomonas spp.	AvrBs1, AvrBs2, AvrBs3, AvrRxo1, AvrRxv, AvrXccC, AvrXv3, Ecf, HpaA, XopJ, XopX, XopB, XopC, XopD, XopE, XopF, XopN, XopO, XopP, XopQ
R. solanacearum	GALA1-7, SKWP1-6, HLK1-3, RipB, PopW, PopP, PopC, RipT, AvrA, PopB, RipA (many others predicted)
E. amylovora	DspE, HrpN, HrpW, HopPtoC, AvrRpt2, EopB
Rhizobium spp.	NopI, NopP, NopJ, NopM, NopT, NopB, NopN
Pantotea spp.	WtsE, PthG, HsvG, HsvB

Table 2. Effectors known to be transclocated in plant pathogens.

Many effectors have been described for several plant pathogens, being *P. syringae* the pathogen with the best characterised effectome. Adapted from (Dean, 2011).

Recently, a tremendous effort has been made to increase the knowledge of effector dynamics and its particular implications in disease. High redundancies in the effector repertoire have been described in several pathogens in the sense that several effector deletions are required

for causing a detrimental effect on bacterial virulence (Cunnac et al., 2004; Kvitko et al., 2009; Lindeberg et al., 2009). As depicted in many papers, it seems that effectors present in a particular bacterium can be grouped into 2 types: those highly conserved among pathovars in the same species that evolve slowly, and those that are far less conserved and are thought to evolve considerably faster. While the first group is referred to as the core effectome and might be important for virulence independently to the host, the second group could be implicated in host specificities (Guidot et al., 2007; Hajri et al., 2009; Schulze-Lefert and Panstruga, 2010). Lately, discovering the minimal effector repertoire to cause disease has become an interesting field of study and great advances have been achieved in the *P. syringae* and *R. solanacearum* pathogens (Guidot et al., 2007; Cunnac et al., 2010). Constant selection forces against or in favour of T3 effectors are applied as a result of plant-pathogen interactions and effectors can be considered as a modular system. While a particular subset of effectors (or module) might be useful for a particular host-pathogen context, other subsets could be key for interaction in another context. This fact could explain the high number of T3Es present in pathogenic bacteria and their apparent redundancy.

Although a vast amount of translocated proteins have been described so far, most of their targets or biochemical functions remain still elusive and knowledge is restricted mainly to the pathogens *P. syringae and Xanthomonas* species (Büttner and Bonas, 2009; Bogdanove et al., 2010; Block and Alfano, 2011). According to the biochemical functions already solved, effectors tend to mimic host genes as a strategy to manipulate cellular functions by interfering with host cell processes. T3 effectors have been proven to function as transcription factors, SUMO proteases, cysteine proteases, ubiquitin ligases, kinases, phosphatases and acetyltransferases, as reviewed elsewhere (da Cunha et al., 2007; Speth et al., 2007; Block et al., 2008; Lewis et al., 2009). Focusing on plant processes, effectors can modulate protein turnover and host gene transcription, cellular trafficking, defense-related pathways including MAPK and hormone signalling as is represented graphically in Figure 4.

Recent research has led to the notion that the main role of most bacterial effectors is to touch down plant defences to facilitate pathogen invasion. Precise interplay between T3Es and their targets in the plant will be covered in Chapter 3. On the other hand, plants present a wide arsenal of defence mechanisms against bacteria to avoid succumbing to manipulation by the pathogen and to disease establishment. All well characterized lines of defence will be deeply discussed in the next chapter.

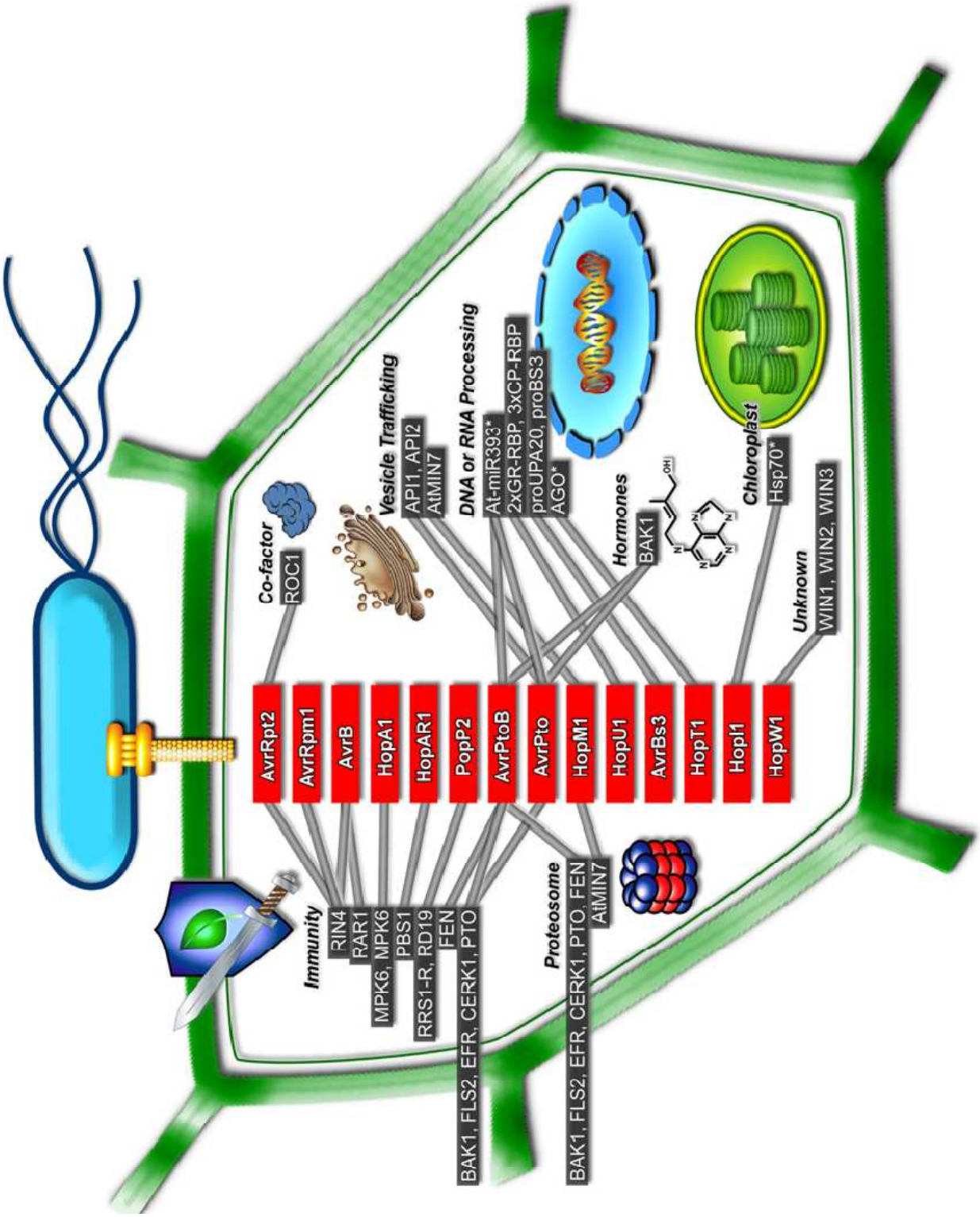


Figure 4. Schematic representation of the main processes targeted by T3E in plant cells.

Bacterial effector proteins interfere with several plant processes: plant immunity, protein-degradation, trafficking or hormone signalling. Image obtained from (Lewis et al., 2009).

CHAPTER 2: How plants defend

CHAPTER 2
HOW PLANTS DEFEND

Plants are sessile organisms that are incessantly in contact with a huge array of pathogens. However, and contrary to what had been thought for long, plants possess a notably extended weaponry to fight against harmful bacteria. This chapter will focus on the displayed plant basal defences and immunity used to successfully withstand and fight back pathogen incursions.

2.1. Overview

Plant defence strategies can be grouped into passive or active as it is represented in Figure 5 (Trigiano et al., 2008; J.A.Gatehouse, 1999; Robinson, 1999; Maarten J.Chrispeels, 2003).

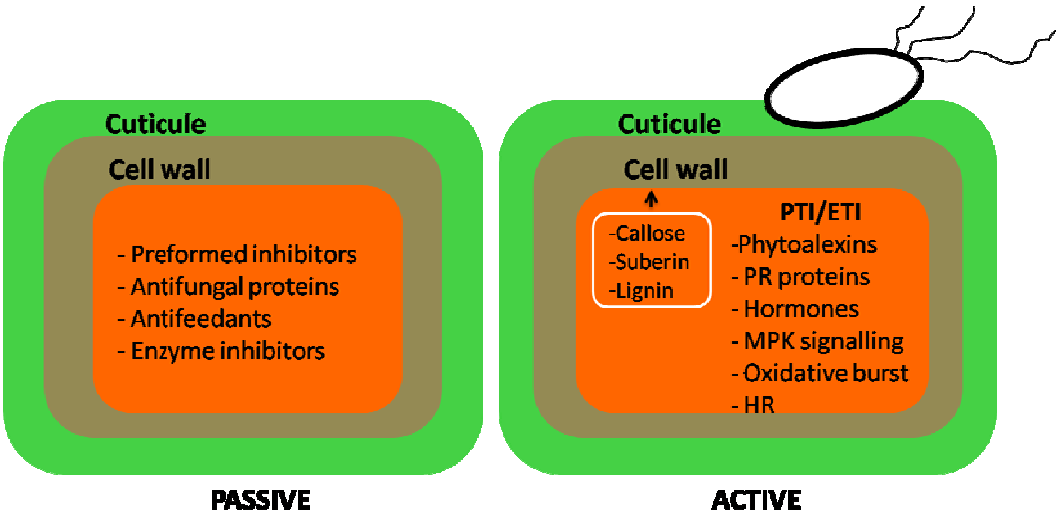


Figure 5. Schematic view of the defence systems in plants.

Plant defences can be classified into passive defences that are pre-existing to the pathogen incursion and active defences that are induced upon pathogen detection.

Passive defences

Passive defences include all those mechanisms inherent to the plant, prior to pathogen perception. Normally, this type of defence is important in the pre-penetration or penetration stage of the pathogen but it loses its importance when the disease cycle is more advanced (Gohre and Robatzek, 2008; Trigiano et al., 2008). For example, the physical structure of plant surfaces, previous colonization of non-pathogenic bacteria (involving nutrient-competition) or host antimicrobial substances released will be crucial for pathogen penetration. Normally, physical barriers such as the cuticle of leaves or external thickenings (e.g. suberization) will

prevent bacterial entrance forcing the pathogen to rely on stomata opening or wound tissues to penetrate (Gohre and Robatzek, 2008). Moreover, if the pathogen manages to reach the apoplast, another structure prevents further spread: the plant cell wall. Only those pathogens adapted to gain access to the plant cytoplasm to nourish, block plant defences and proliferate will successfully persist in the battle (Gohre and Robatzek, 2008; Trigiano et al., 2008).

Active defences

On the other hand, an array of defences is also activated upon contact with the pathogen (Gohre and Robatzek, 2008; Trigiano et al., 2008). In general, plant metabolism is altered in order to cope with bacterial intrusions. Major changes in metabolism are a decrease in photosynthesis and an increase of sink metabolism and sugar accumulation (Berger et al., 2007).

Pathogen/microbe-associated molecular patterns (PAMP/MAMP), such as the flagellum, are perceived by **pattern-recognition receptors (PRRs)** present in the host membrane and a set of specific responses are triggered (Jones and Takemoto, 2004; Gohre and Robatzek, 2008). Pathogen-derived signalling might include ion fluxes, oxidative burst, protein phosphorylation and synthesis of defence-related hormones such as ethylene, jasmonic acid and salicylic acid. In response to these signals, a set of genes will be transcribed including PR (pathogenesis-related) defense genes and genes that aim at strengthening structural barriers such as callose deposition, lignification and suberization to avoid pathogen penetration or progression (Jones and Takemoto, 2004; Mysore and Ryu, 2004; Gohre and Robatzek, 2008; Trigiano et al., 2008). This process is also known as **PAMP-triggered immunity (PTI)** and it is universal and not pathogen or race specific.

Some pathogens are capable to surpass this first defence barrier, hence making colonization possible. In the case of bacterial pathogens, effector proteins injected in the plant cell through the type III secretion system (T3SS) are key to render the plant susceptible. A second defence barrier is present in the plant host to specifically detect these translocated proteins (or their effects) through resistance proteins (**R-proteins**) (Gohre and Robatzek, 2008; Boller and Felix, 2009; Wang et al., 2010). This type of defence is strain- or race-specific and it is also called **effector-triggered immunity (ETI).** Signalling followed by direct or indirect effector detection is similar to that presented in PTI but, in general, faster, stronger and more prolonged (Tao et al., 2003; Gohre and Robatzek, 2008; Boller and Felix, 2009; Wang et al., 2010; Thomma et al., 2011) as exemplified in Figure 6.

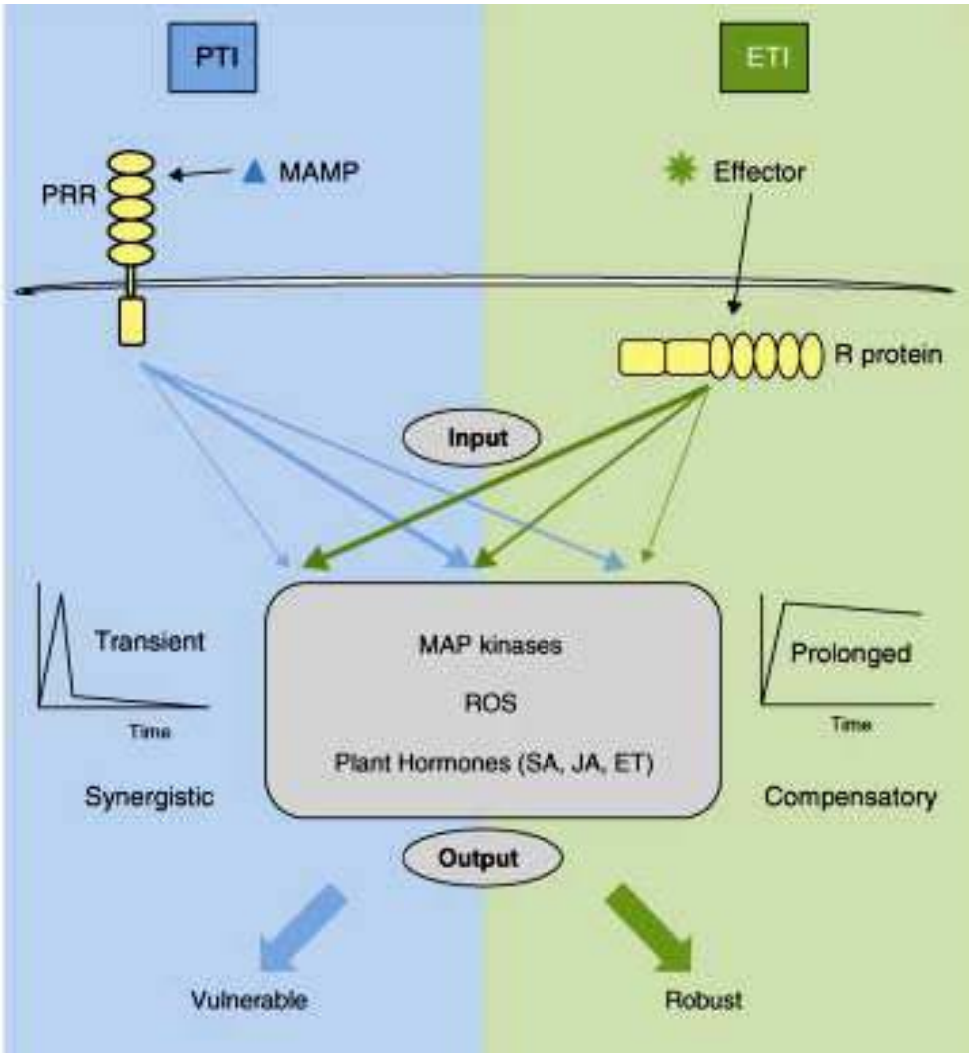


Figure 6. Signalling between PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI).

Although PTI and ETI share components of the signalling cascade, ETI induces longer and stronger responses (Tsuda and Katagiri, 2010).

ETI is more effective than PTI and in most cases ends up with a fast and confined cell death, called **hypersensitive response (HR),** surrounding the site of infection to restrict pathogen spread (Heath, 2000). These events can also activate alert mechanisms in distant parts to "immunize" the plant and thus confer **systemic resistance** (e.g. SAR/ISR) (Chisholm et al., 2006; Gohre and Robatzek, 2008; Trigiano et al., 2008).

Depending on the outcome of the mentioned defence responses and using classical definitions, interactions between plant and pathogens can result in non-host, incompatible or compatible interactions. **Non-host resistance** takes places when a virulent pathogen for a particular host is not able to infect and multiply in another type of host (Gohre and Robatzek, 2008). Normally, PTI has a relevant contribution to this type of plant resistance, but weak ETI

could also be involved. Both **incompatible** and **compatible reactions** are integrated in a host-resistance framework but whereas the first one renders the plant resistant, in the second the plant is susceptible to the pathogen infection (Tao et al., 2003). Presence of plant R-proteins that recognize bacterial effector proteins leading to a strong ETI will mediate an incompatible reaction (Gohre and Robatzek, 2008).

2.2. PAMP-triggered immunity (PTI)

Upon pathogen perception, plants exhibit a vast number of defences (independent of the type of invader) in order to avoid infection. If the bacterium is not able to beat all these active defences, PTI takes place and pathogen multiplication is avoided (Gohre and Robatzek, 2008). The term PTI emerged quite recently in substitution to the expression "basal resistance" (Chisholm et al., 2006; Jones and Dangl, 2006). PTI is considered to be the first active plant response to microbial perception and it is as important in nature as ETI (Chisholm et al., 2006; Jones and Dangl, 2006; Boller and Felix, 2009). The steps involving PTI are depicted below.

PAMPs and their detection

Nowadays, the term "elicitor" groups any molecule that promotes defence responses in plants (Hahn, 1996). These can be classified into general elicitors and race-specific elicitors. General elicitors are able to trigger resistance in both non-host and host plants (Montesano et al., 2003) and thus they are important for PTI. The other type of elicitors is strain-specific (e.g. T3 effectors or harpins) and will be discussed in the ETI section. Lately, general elicitors have also been named PAMP/MAMP which stands for Pathogen/Microbe Associated Molecular Pattern (Hann et al., 2010). PAMPs or MAMPs are molecules highly conserved in all microorganisms, not only in pathogens, that plants are able to detect and react to once they are exposed to their cell surface (Chisholm et al., 2006; Hann et al., 2010).

Most PAMPs, are part of the structures associated with the cell wall of pathogens, which has facilitated the emergence of recognition by plants. Examples comprise flagellin (protein subunit of the flagellum), lipopolysaccharide (major constituent of the outer bacterial membrane), pepdidoglycans or chitin (major constituents of the cell wall of bacteria and higher fungi respectively), among others (Table 3) (Montesano et al., 2003; Nürnberger et al., 2004; Zhou and Chai, 2008; Nicaise et al., 2009). Elongation factor Tu (EF-Tu), the most abundant bacterial intracellular protein is an exception that might act as a MAMP once released from dying bacteria (Kunze et al., 2004). While a number of PAMPs have been characterised, only few plant PRR receptors have been discovered so far (Nicaise et al., 2009).

PAMP	Pathogen(s)	Minimal structural motif required for defence activation
Lipopolysaccharide	Gram-negative bacteria	Lipid A?
Flagellin	Gram-negative bacteria	Flg 22 (N-terminal fragment)
Harpin	Gram-negative bacteria	Undefined
Cold-shock protein	Gram-negative and positive bacteria	RNP-1 motif (N-terminal fragment)
Necrosis-inducing proteins	Bacteria, fungi and oomycetes	Undefined
Transglutaminase	Oomycetes	Pep-13 motif (surface exposed epitope of the tansglutaminase)
Lipid-transfer proteins	Oomycetes	Undefined
Xylanase	Fungi	TKLGE pentapeptide (surface exposed epitope of the xylanase)
Invertase	Yeast	N-Mannosylated peptide (fragment of the invertase)
β-glucans	Fungi, oomycetes and brown algae	Tetraglucosyl glucitol-branched hepta-β-glucoside linear oligo-β-glucosides
Sulfated fucans	Brown algae	Fucan oligosaccharide
Chitin	Fungi	Chitin oligosaccharide (degree of polymerization > 3)
Ergosterol	Fungi	
Cerebrosides A, C	Fungi	Sphingoid base

Table 3. PAMPs present in several pathogens that trigger plants defences.

Plants are capable of detecting different bacterial-related molecules which among others include lipopolysaccharide, flagellin or extracel·lular enzymes and induce defences upon contact. Extracted from (Nürnberger et al., 2004).

Pattern recognition receptors (PRRs) are normally bound to the plant cellular membranes and directly detect the presence of pathogens through specific PAMP/MAMP recognition (Block et al., 2008). Interestingly, these receptors are also present in animals, where bacterial PAMP detection is carried mainly by members of the Toll-like receptor (TLR) family of PRRs that possess an intracellular Toll-interleukin 1 receptor domain (TIR) which aims to the recruiting of IRAK (harbouring non-RD domain) or RIP kinases. TLR-dependent signalling pathway activates nuclear factor-kappa B that in its turn induces a huge array of animal defence responses (Dardick and Ronald, 2006; Coll et al., 2011; Danna et al., 2011).

Plant PRRs seem to be evolutionary ancient, since they are conserved among species (Thomma et al., 2011). Several types have been described so far (Figure 7) (Segonzac and Zipfel, 2011), the most common being the receptor-like kinases (RLK), characterized by leucine-rich repeats (LRRs) and an intracellular kinase domain for signal transduction through a non-RD domain similar to animal IRAK kinases (Coll et al., 2011; Danna et al.). The most studied PRR pairs of elicitor recognition systems in plants are the FLS2 receptor for flagellin (Chinchilla et al., 2006)

and the EFR receptor for EF-Tu (elf18) (Zipfel et al., 2006). Both receptors belong to the RLK family (Nicaise et al., 2009). Despite recognizing different epitopes, flagellin detection in vertebrates by TLR5 takes place in a similar way. This reinforces the idea that PTI resembles the innate immunity in animals and probably these similarities are based on convergent evolution (Boller and Felix, 2009).

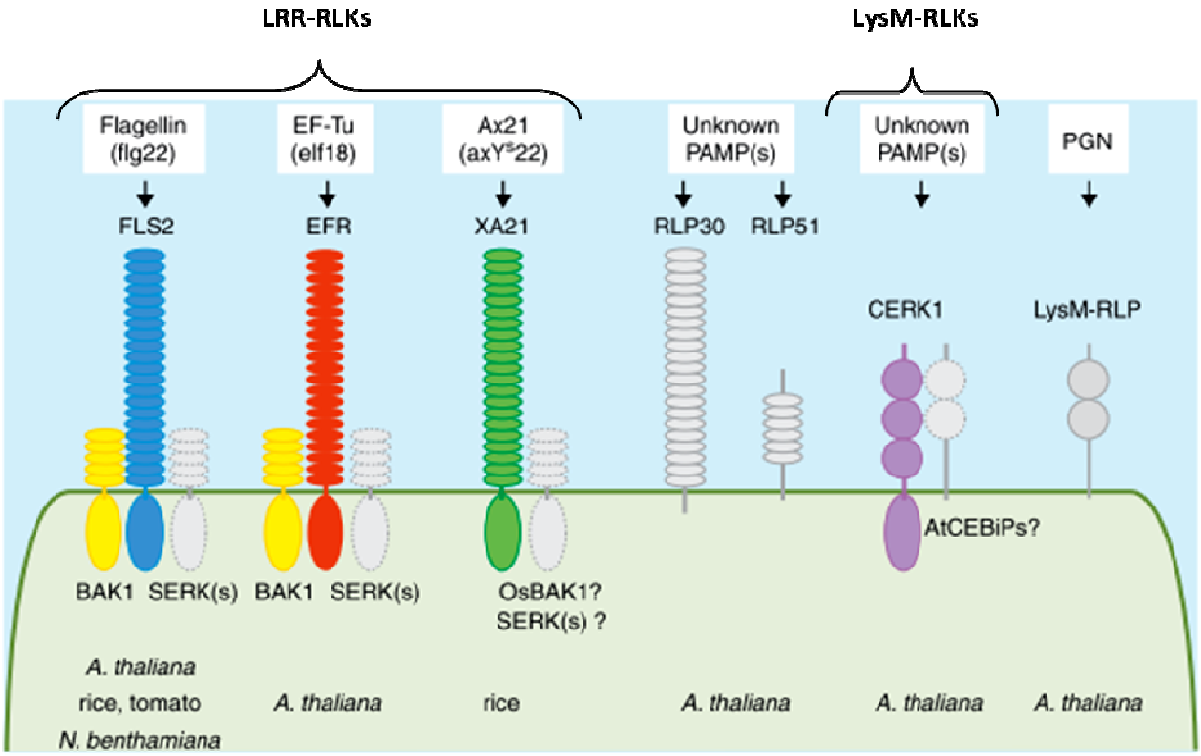


Figure 7. Different types of PRRs found in plants that respond to known or unknown bacterial PAMPs.

Most RLKs have been described in *A. thaliana* and the best characterised (FLS2, EFR and Ax21) belong to the leucine-rich group of receptors. A less characterised group is the receptors that harbour an extracellular lysine motif. Modified from (Segonzac and Zipfel, 2011).

Although FLS2 was first discovered in *Arabidopsis*, homologues have been found in several plant species. Contrary to that, EF-Tu and Ax21-dependent responses (see below) seem to be restricted to Brassicaceae and to rice, respectively. This evokes that FLS2 receptor is more anciently evolved than the others. For example, pre-treatment of *Arabidopsis* plants with flg22 restricts *P. syringae* DC3000 growth whereas FLS2 mutation renders the plants more susceptible to the infection (Zipfel et al., 2004). Regarding EFR, it was shown that *Arabidopsis* plants lacking this effector were easier to transform with *Agrobacterium* (Zipfel et al., 2006) and more susceptible to colonization of weakly virulent DC3000 strains (Nicaise et al., 2009). In addition, transgenic expression of EFR in *Solanaceae* increases resistance to a wide range of phytopathogens (Lacombe et al., 2010).

A new PAMP/RLK couple was recently described in rice (Lee et al., 2009). Ax21, a bacterial molecule thought to be related to quorum sensing, is specifically recognized by the XA21 receptor (that behaves as an R-protein). Ax21 is conserved among *Xanthomonas* species and thus considered a PAMP, although it also behaves as an avirulence gene: The presence of this receptor enables rice resistance against most *X. oryzae* pv. *oryzae* strains (Lee et al., 2009). This illustrates that PAMP/MAMP signalling may have connections and crosstalks with effector-triggered immunity. Indeed, recent experiments point out that FLS2 could also recognise Ax-21 derived peptides and trigger defence responses in a similar way as flg22 does in *Arabidopsis* plants (Danna et al.).

Another PRR receptor present in *Arabidopsis* is CERK1 which has similar intracellular domain to FLS2 but harbours LysM (lysine motif) extracellular domains instead of the typical LRRs (Segonzac and Zipfel, 2011). This receptor appears to be important for fungal chitin perception and it is suggested that it binds carbohydrate bacterial PAMPs. LysM-RLKs can also bind peptidoglycan and it has also been speculated that they could also recognise Nod factors, which are required for *Rhizobium* plant nodulation (Nicaise et al., 2009; Segonzac and Zipfel, 2011). Specific detection of other PAMPS such as RNP-1, lipopolysaccharide or transglutamases by PRRs remains still uncharacterised (Nicaise et al., 2009). In addition other potential MAMPs that have not yet been explored include the quorum sensing signals and siderophores (Boller and Felix, 2009).

Apart from the PAMPs, other damage-associated molecular patterns (DAMPs) might be also important for plant defence elicitation. All the cell wall fragments that are potentially released due to bacterial enzymatic activity belong to this group. Cutin monomers or other peptides (e.g. AtPEP1, RALF or systemin) can be liberated and act as endogenous elicitors to enhance plant defence responses (Boller and Felix, 2009).

Signalling cascade and its outcome

Upon PAMP perception through membrane receptors, a signalling cascade takes place to launch defence gene activation. Plant responses after pathogen detection can be classified in (1) early signalling or very early responses that take place after 1-5 minutes, (2) early responses that happen 5-30 minutes after perception and (3) late responses that can be experimented hours after or even days afterwards (Boller and Felix, 2009).

Among all known PTI pathways, the best characterized is the FLS2-mediated signalling cascade, summarized in Figure 8 (Segonzac and Zipfel, 2011). However, the signalling cascade appears

to be conserved for other elicitors. When a plant is challenged with PAMPs, the first event that takes place is the binding of the ligand to the receptor, which induces a change in its conformation and thus stimulates the recruitment of other membrane-associated receptors to heterodimerize (Nicaise et al., 2009; Segonzac and Zipfel, 2011). BAK1 is one such receptor, an LRR-RLK that belongs to the SERK subfamily and is required for full PAMP perception. Other SERK receptors such as the brassinosteroid receptor BRI1 play as well a major role in perception, but also BKK1 or BIR1 receptors seem to be positive regulators of PTI (Segonzac and Zipfel, 2011). Stomatal cytokinesis-defective 1 (SCD1) is another constitutive interactor of the FLS2/BAK1 complex and could modulate ROS production (Segonzac and Zipfel, 2011). After receptor recruitment and FLS2/BAK1 phosphorylation, other membrane-associated cytoplasmic kinases (BIK1 and PBS) could aggregate to the receptor complex being phosphorylated and then released to continue the cascade signalling (Segonzac and Zipfel, 2011).

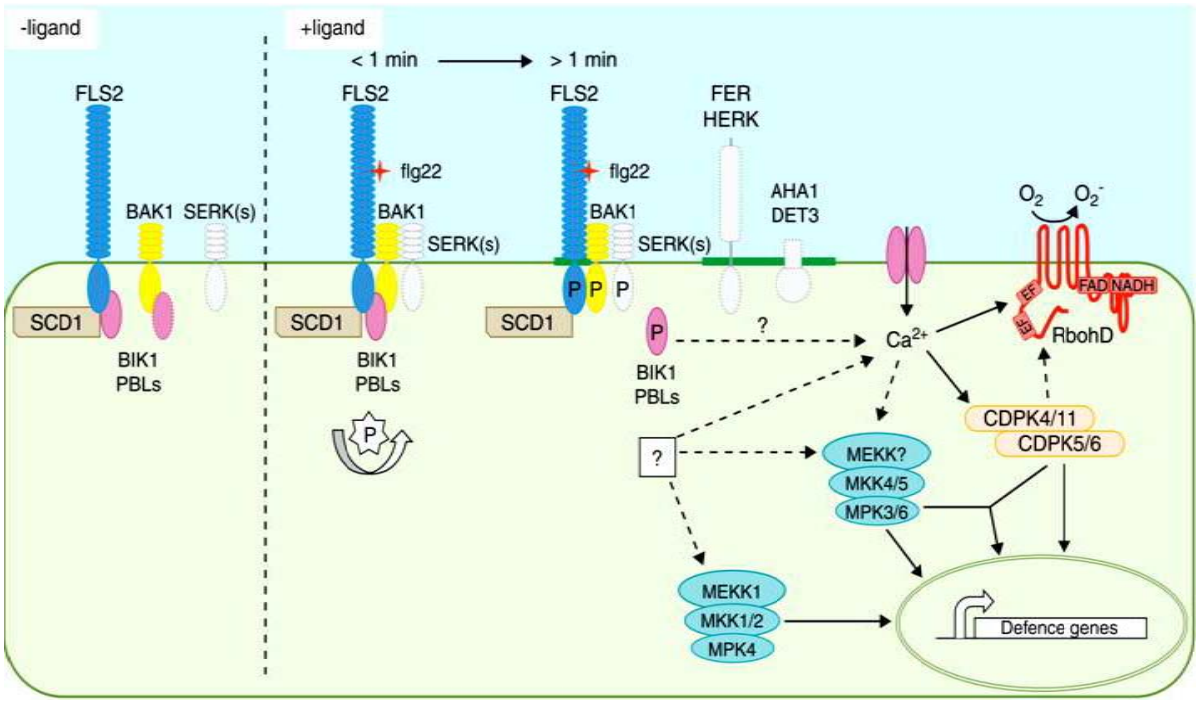


Figure 8. Scheme of events leading to PTI upon pathogen perception

Upon pathogen detection, a rapid receptor restructuration/modification takes place followed by a membrane depolarization. Signals are integrated by calcium-dependent proteins, ROS production is enhanced and MAPK will trigger defence responses. Image obtained from (Segonzac and Zipfel, 2011).

The fastest response to PAMPs seems to be a dramatic change in ion fluxes (Ca^{2+}, H^+, K^+ and Cl^-) across the plasma membrane (Nicaise et al., 2009) that leads to membrane depolarization (Boller and Felix, 2009). For example, after LPS perception, a Ca^{2+} influx takes place and increases the presence of this ion in the cytosol. This change can act as a secondary messenger

(Boller and Felix, 2009) and it is perceived by calcium-binding proteins like calmodulin and calcium-dependent protein kinases (among others). These proteins have an important role in plant defence since they control reactive oxygen species (ROS) or salicylic acid (SA) manufacture (Nicaise et al., 2009). Oxidative burst due to ROS accumulation (monitored by H_20_2 presence in the cell) is also triggered rapidly after PAMP treatment (Boller and Felix, 2009). Ca^{2+} bursts induce ROS production mainly through the membrane NADPH *AtrbohD,* produce cell wall-cross linking and act as antibiotic agents, or secondary messengers to induce a huge array of responses to cope with pathogen attack (Boller and Felix, 2009; Torres, 2010). It is not still clear whether ROS production is downstream of MAPK activation or if it is an independent pathway (Zhang et al., 2007; Asai et al., 2008). NO production might be also part of signalling elements in result of PAMP elicitation (Boller and Felix, 2009). Another early response to PAMP detection is the MAPK signalling which is excellently reviewed in elsewhere (Pitzschke and Hirt, 2006; Colcombet and Hirt, 2008; Rodriguez et al., 2010). MAP-kinase (MPK) cascades are important for numerous processes in eukaryotic cells and plant defence is amongst them (Colcombet and Hirt, 2008). Two important MPK cascades might be involved in biotic stresses: MEKK-MKK4/5-MPK3/6 could regulate positively PTI whereas MEKK1-MKK1/2-MPK4 could affect negatively PTI (Nicaise et al., 2009) as seen after flg22 treatment. In recent papers, involvement of MPK3/6 in plant defences has been described (Ren et al., 2008; Beckers et al., 2009; Bethke et al., 2009; Han et al., 2010). General activation of MPKs may produce a change in cell protein phosphorylation states (Boller and Felix, 2009) and lead to the activation of WRKY-type transcription factors that are crucial for downstream plant defence regulation (Nicaise et al., 2009).

Phytohormone production is often viewed as the next step in the PTI response cascade. Flg22 induces production of the antagonistic hormones jasmonic acid (JA) and salicylic acid (SA), but also ethylene (Nicaise et al., 2009) that are crucial for plant defences (Verhage et al., 2010). Ethylene seems to be important, directly or indirectly, for correct stomata regulation, FLS2 accumulation and ROS production during PAMP elicitation (Mersmann et al., 2010). Contrary to that, flg22 treatment induced accumulation of microRNAs (e.g. miRNA393) that negatively regulate auxin signalling, restricting pathogen growth (Navarro et al., 2006). Stomata closure is also induced through flg22 signalling cascade to avoid further pathogen penetration and it is partially dependent on abscisic acid (ABA) (Melotto et al., 2006; Melotto et al., 2008).

Up to a quarter of all *Arabidopsis* genes present differential transcript levels after pathogen challenging (Tao et al., 2003; Knepper and Day, 2010). Among all the differentially expressed genes, PR genes (pathogen-related) are overexpressed in response to pathogen infection and

encode antimicrobial compounds (e.g. PR-12 members are defensins). In general, PR genes seem to be a part of the defence signalling network that involves SA, JA and ethylene. A decrease in PR expression normally correlates to an increased susceptibility (Knepper and Day, 2010). Upregulation of specific genes, aimed at reinforcing cell walls is also triggered by PAMP perception. Two key regulatory enzymes involved in plant early responses towards pathogen attack are phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS). PAL is the first enzyme in the phenylpropanoid pathway that leads to the production of phenolic alcohols, precursors of lignin and suberin. The CHS is a branch of phenylpropanoids that leads to the production of phytoalexins which are antimicrobial flavonoids (Robinson, 1999). Several studies report the importance of lignin and callose synthesis and deposition for plant defences (Lawton et al., 1983; Bhuiyan et al., 2009; Chen and Kim, 2009; Parker et al., 2009; Huang et al., 2010; Moura et al., 2010; Naoumkina et al., 2010).

Historically, it has been stated that ETI involves the systemic-adquired resistance (SAR) and hypersensitive response (HR) whereas PTI does not. However, recent papers show that some PAMPs and harpins are capable of inducing HR and SAR in *Arabidopsis* plants and hence this event is not restricted in ETI, as reviewed in (Thomma et al., 2011).

2.3. Effector-triggered immunity (ETI)

ETI (formerly called R-gene or gene-for-gene resistance) is elicited when strain-specific effectors are translocated to the plant cytoplasm and detected by the plant. This recognition is mediated by the so-called NB-LRR proteins (Boller and Felix, 2009). Typically, ETI ends up with energetic defence reactions often leading to the hypersensitive response (HR) to restrict bacterial growth (Jones and Dangl, 2006; Boller and Felix, 2009).

Effectors and their recognition

Bacterial type-III-secreted effectors are translocated to the host cytoplasm, as described in the first chapter, in order to manipulate its defences (e.g. targeting PTI). To avoid pathogen infection, plants have developed a finely-tuned system to detect these foreign molecules and trigger an alert. Recognised effectors were formerly named avirulence proteins since a bacterium harbouring them was avirulent on a specific plant expressing a resistance protein (R-protein). This gene-for-gene model was first described by Harold Henry Flor (Flor, 1947) and it is graphically presented in Figure 9.

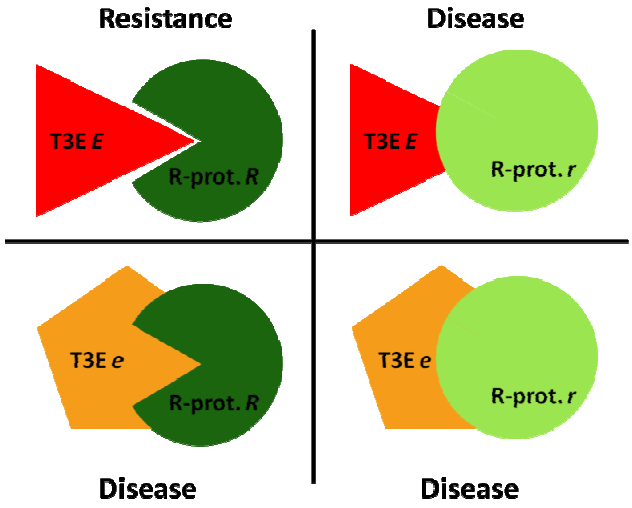


Figure 9. Gene-for-gene or Flor model

In order to overcome disease and display plant resistance, bacteria need to express an effector protein that it is specifically recognised by a plant R-protein. Otherwise, disease will establish (Flor, 1947).

The Flor model established a direct interaction between the avirulence bacterial protein and its cognate plant protein (avr/R-protein pair) strictly necessary to avoid pathogen infection. A good example that illustrates this model is the effector PopP2 present in *R. solanacearum* that targets the RRS1 R-protein from *Arabidopsis* (Deslandes et al., 2003). Nevertheless, the fact that only few cases follow this direct interaction model prompted to propose an alternative explanation: the "guard model", also compatible with Flor's observations (Van Der Biezen and Jones, 1998). According to this model, R-proteins could be guarding the effector target (guardee) and any change perceived would trigger activation of the R-protein. A paradigmatic example is the *Arabidopsis* protein RIN4, which is manipulated by three different effectors from *P. syringae* (Jones and Dangl, 2006). The guard model could explain the reduced number of putative R-proteins present in *Arabidopsis,* as different effectors might alter the same host protein/processes (Dangl and Jones, 2001; Jones and Dangl, 2006). Guardees display a function in susceptibility if the R-protein (guard) is absent, whereas they might be needed for host defence in R-protein presence. More recently, an alternative "decoy model" was also proposed based on some experimental observations. Contrary to the guard model, the decoy model is based on mimicry of the effector target to detect the pathogen. In that case, the decoy does not have any role in host resistance or in susceptibility when the R-protein is absent (van der Hoorn and Kamoun, 2008). For instance, Pto in tomato plants acts as a decoy for avrPto from *P. syringae* in the prf R-protein context (van der Hoorn and Kamoun, 2008). Both pathogen-recognition models are illustrated in Figure 10.

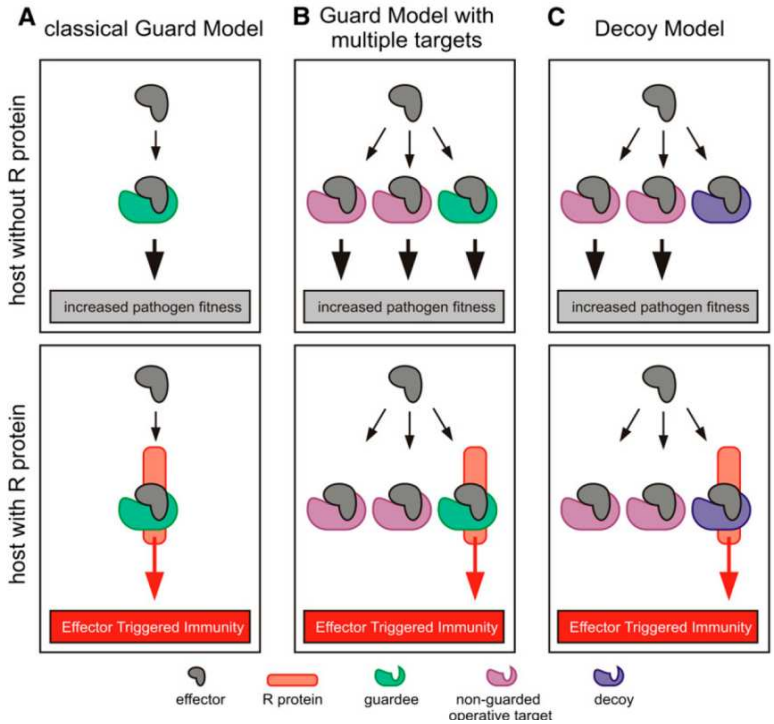


Figure 10. Comparison of the guard model and the decoy model.

The difference between the guard model and the decoy model is that, contrary to the guardee protein, the decoy protein might not have any role in resistance or susceptibility. Extracted from (van der Hoorn and Kamoun, 2008).

R-proteins, similar to PRRs, are sentinels that recognise the pathogen effectors and proceed to activate a set of responses to produce an ETI. But contrary to PRRs, R-proteins are mostly localized in the cytoplasm since most effectors are translocated there. The main features of resistance proteins are the presence of a nucleotide binding domain (NB) and leucine rich repeats (LRR) for which ther are named NB-LRR proteins (Thomma et al., 2011). These proteins are related to CATERPILLER/NOD/NLR proteins7 and STAND ATPases8 that are found in animals and their action is effective only in biotroph or hemibiotroph pathogens (Jones and Dangl, 2006). NB-LRRs present in plants can be classified in two groups depending on the structure present on N-terminal region: a Coiled-coil (CC) or a Toll and human interleukin receptor (TIR) domain (Eitas and Dangl, 2010). Best characterized R-proteins belong to the group of CC-NB-LRRs and include RPM1, Prf, Mla or RPS2. On the other hand, RPS4 and RSS1 are members of the TIR-NB-LRRs, as exemplified in Figure 11.

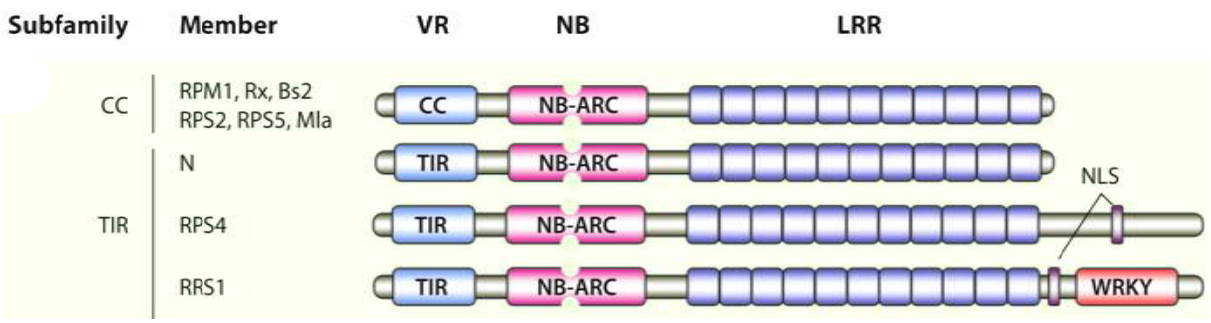


Figure 11. Types of R-proteins present in plants.

Reproduced from (Shirasu, 2009).

The LRR region appears to be involved in effector recognition (Caplan et al., 2008) but also the N-terminal domains could play an important role (Collier and Moffett, 2009). Between the N-terminal NB and the C-terminal LRR domain, there is a region called ARC that, together with the NB domain, constitutes a functional nucleotide-binding pocket (Collier and Moffett, 2009).

The ETI signalling cascade and its outcome

Contrary to PTI signalling, the responses following effector recognition by NB-LRR proteins are not well understood and we only have a blurred and scattered knowledge of the process. As stated at the beginning of the chapter, differences between PTI and ETI signalling cascade might be quantitative rather than qualitative. However, ETI is often accompanied by a hypersensitive response to restrict pathogen growth (Tao et al., 2003; Gohre and Robatzek, 2008; Boller and Felix, 2009; Tsuda and Katagiri, 2010; Wang et al., 2010; Thomma et al., 2011).

NB-LRR-recognition derives often in a programmed cell death and fine tuning of the responses is extremely important to avoid uncontrolled cell death (Coll et al., 2011). The precise way through which NB-LRR proteins are activated and thus instigate plant defences is excellently reviewed in (Caplan et al., 2008; Collier and Moffett, 2009; Lukasik and Takken, 2009; Eitas and Dangl, 2010). Basically, effectors seem to mediate the switch of NB-LRRs from an inactive state to an active conformation where NB becomes more accessible for signal transduction. It is thought that R-proteins are arrested in the plant cell and they can be activated by changes in its conformation by: (1) direct binding of the effector to the R-protein, (2) effector-mediated alteration of a host factor (or guardee) that is bound to the NB-LRR or (3) host factor binding to R-protein which is dependent on effector presence. A fourth system for pathogen detection is not related to NB-LRRs and consists of a direct transcriptional activation of an R-protein by a bacterial effector that mimics a plant transcription factor. Some NB-LRRs also possess an NLS

(nuclear localization signal) that putatively drives them to the nucleus for defence gene activation once they have been activated (e.g. RPS4, RPM1 or some MLA receptors). NB-LRR proteins require a chaperone complex composed of HSP90, SGT1 and RAR1 for its stabilization and for full plant R-resistance (Takahashi et al., 2003; Azevedo et al., 2006; Knepper and Day, 2010). According to the N-terminal domain present in the NB-LRR protein, signal transduction will follow one signalling pathway or another. Immune responses triggered by those R-proteins that harbour a CC domain will be regulated by NDR1, whereas in the TIR-NB-LRR proteins the EDS1/SAG101/PAD4 complex plays the most relevant regulation (Aarts et al., 1998). Nevertheless, this two pathways converge at some point to induce the same responses, which include changes in calcium levels, ROS production, NO signalling, MAPK cascades, SA accumulation and general transcriptional reprogramming and antimicrobial production leading to an HR and systemic acquired resistance (SAR) (Jones and Dangl, 2006). SAR confers long-lasting broad-spectrum resistance throughout the plant and it is associated with high SA levels and PR expression (especially PR-1), being NPR1 protein one of its major regulators (Vlot et al., 2008). SA production can be achieved through two different metabolic pathways, one of which initially involves the phenylalanine ammonia-lyase (PAL). HR is a programmed cell death and shares some similarities with the animal cell death process that includes cytoplasmic shrinkage, mitochondrial swelling, and chromatin condensation. At later stages, vacuolization takes place and chloroplasts are disrupted (Mur et al., 2006). Lately, a huge effort has been made to dissect all the molecular events that take place downstream of NB-LRR signalling to produce an HR. Characterization of *lsd1* mutant plants that show a runaway cell death (i.e. they are unable to arrest HR) has shed light into the process of programmed cell death (Coll et al., 2011). Recently, some *Arabidopsis* metacaspases were found to be involved in HR regulation playing an antagonistic role: AtMC1 is a positive regulator for cell death whereas AtMC2 could regulate it negatively to control HR cell confinement as shown in Figure 12. Together with AtMC2, LSD1 protein might retain AtMC1 inactive in neighbouring cells to avoid uncontrolled cell death (Coll et al., 2011).

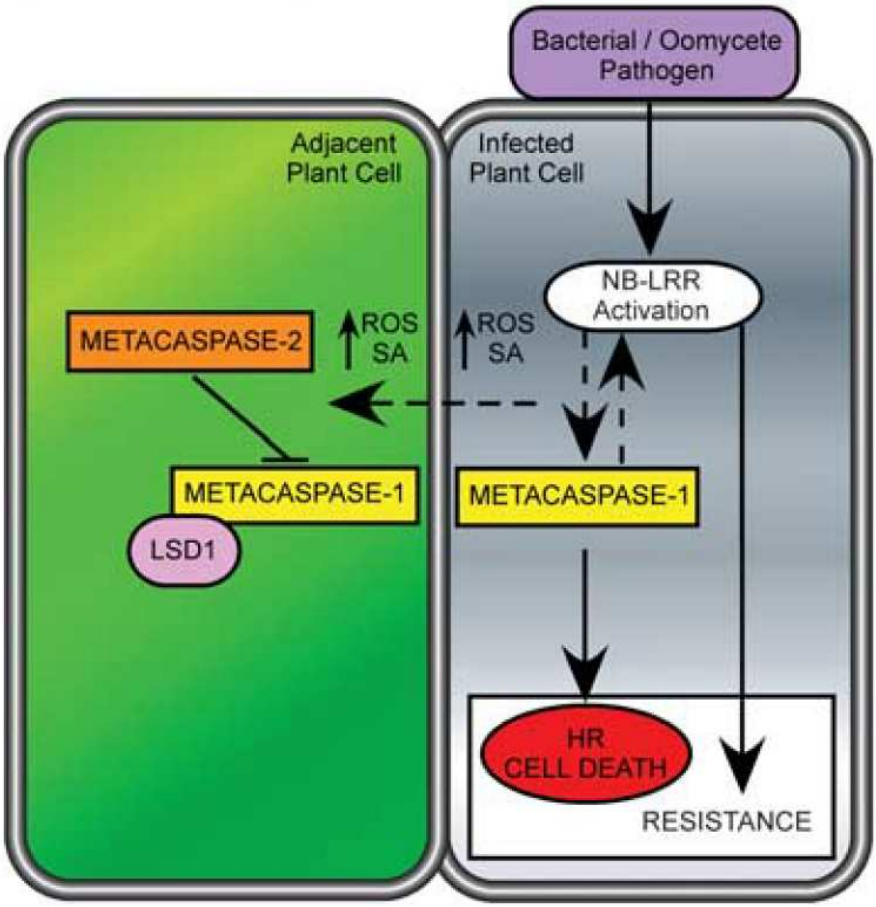


Figure 12. Metacaspase signalling and cell death in plants.

Confined cell death upon pathogen detection is an extremely regulated process where metacaspases act as positive regulators or negative regulators in adjacent cells to avoid uncontrolled cell death spread (Coll et al., 2011).

As reviewed in (Coll et al., 2011), chloroplasts are also crucial for defence responses and HR in plants since some HRs are light-dependent. Furthermore, chloroplasts can be considered as a reservoir of signalling molecules that include ROS, NO intermediates and the hormones SA and JA.

CHAPTER 3: The plant-pathogen arms race

CHAPTER 3
THE PLANT-PATHOGEN ARMS RACE

Understanding the process of plant infection requires comprehension of both plant defence and pathogen attack mechanisms. The relationship between plants and pathogens is more dynamic than it had been previously acknowledged. Disease establishment or plant resistance cannot be understood without an integrated view. Thus, in this chapter all the mechanisms of plant and bacterial pathogen interplay will be exposed.

3.1. Effectors battle against PTI, ETI and other plant defences

As commented in previous chapters, and depicted in Figure 13, bacterial effector proteins are injected to the host cytoplasm to alter its preconceived defences.

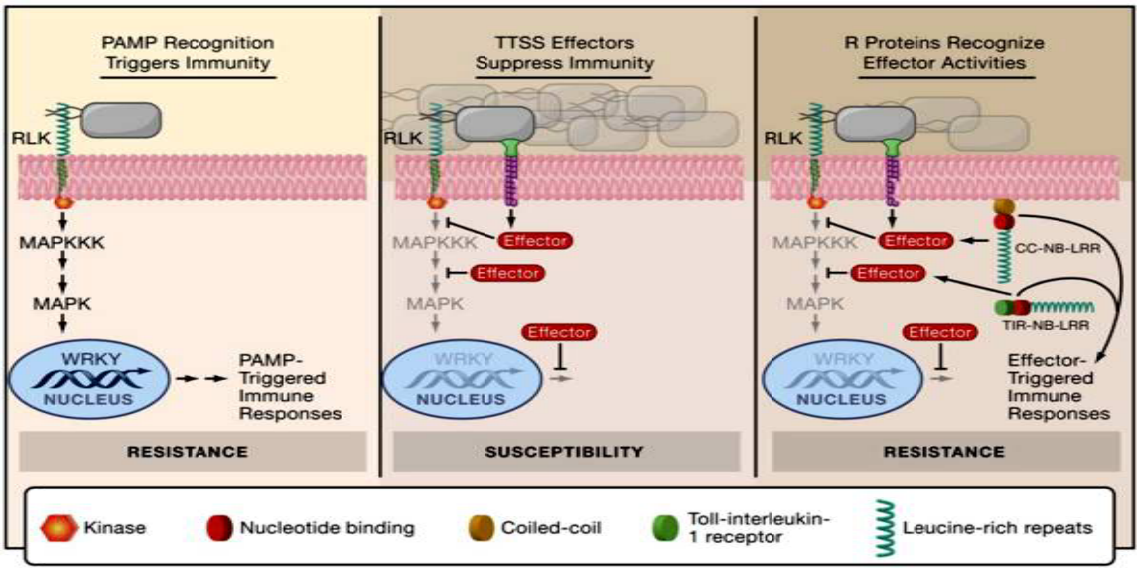


Figure 13. Molecular interplay between pathogenic bacteria and its host plant.

Depending on the resistance sources and the bacterial effectome, different scenarios can take place that will culminate with either disease establishment or plant resistance. Image obtained from (Chisholm et al., 2006).

In general, effectors tend to interfere with the PTI signalling cascade to avoid plant recognition and thus multiply and successfully infect the host. This state is also known as effector-triggered susceptibility or ETS (Chisholm et al., 2006). Alternatively, some plant proteins have acquired the capacity to specifically detect the translocated effectors or any of their operative targets, thus leading to effector triggered immunity or ETI. However, other effectors might be trained to block ETI responses in order to overcome ETI and restore the ability to multiply within the host (Jones and Dangl, 2006).

In summary, perturbation of host defences is achieved by directly targeting plant immunity (PTI and ETI) or indirectly by subversion of host ubiquitination, transcription alteration, hormone unbalancing and host protein modification, among others, as illustrated in Figure 14 (da Cunha et al., 2007; Speth et al., 2007; Lewis et al., 2009).

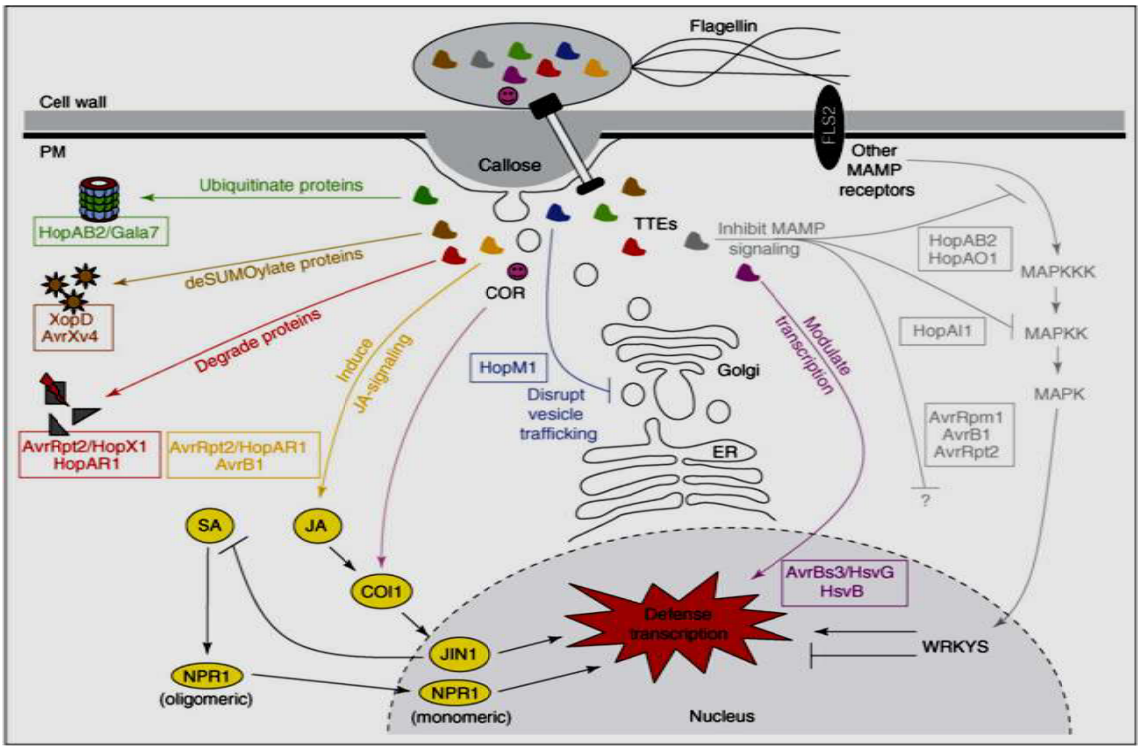


Figure 14. General host defense alteration by bacterial effectors.

Many plant pathways are altered by the T3Es to render the plant more susceptible to bacterial infection. Reproduced from (da Cunha et al., 2007).

For instance, in *P. syringae,* a large proportion of T3Es inhibit the flg22-induced PTI (Li et al., 2005). In addition, other effectors in *P.* syringae (e.g. HopU1, AvrRpm1, AvrPtoB, AvrRpt2, AvrB, HopAO1...) and *X. campestris* (e.g. XopN, XopX1...) are capable of suppressing callose deposition or defence gene expression (Cui et al., 2009). To achieve these goals, they interact directly with PAMP receptors or interfere with their signalling cascade. Alternatively, T3Es can inactivate ETI, produce hormonal alteration, alter vesicle trafficking or modulate transcriptional requirements (da Cunha et al., 2007; Block et al., 2008). Thus, functional characterization of effectors is notably unravelling the mechanisms that bacteria use to subdue host defences but is also sheding light on the plant immunity mechanisms. Some of the current knowledge on T3Es is summarised in the following Table 4 (legend p.42). *P. syringae* effectors are the most extensively characterized so far and illustrate the best models for plant-pathogen interaction.

Pathogen species	T3E	Function (Predicted or known)	Target or decoy	Host processes targeted/involved	R-protein	Refs
P. syringae	AvrB1 (AvrB)	Promotes phosphorylation	RIN4, RAR1, MPK4, HSP90	PTI, induce JA responses	RPM1, RPG1	a
	AvrPto1 (avrPto)	Kinase inhibition, PRR complex disruption	Pto, FLS2, BAK1 (?), EFR	PTI, PRR signalling, ethylene production, miRNA processing	Prf	b
	AvrRpm1	Promotes phosphorylation	RIN4	PTI	RPM1	c
	AvrRPS4	Autocleavage		PTI	RPS4, RRS1	d
	AvrRpt2	Cysteine protease	RIN4, ROC1	ETI, PTI, auxin production	RPS2	e
	HopAB2 (AvrPtoB)	Ubiquitin E3 ligase	Fen,FLS2, CERK1, EFR, BAK1, PTO	ETI, PTI	Prf	f
	HopAI1	Phosphothreonine lyase	MPK3, MPK6	PTI, MAPK signalling		g
	HopAO1 (HopPtoD2)	Protein tyrosine phosphatase	Downstream of MAPK in PTI	Suppression of cell death		h
	HopAR1 (AvrPphB)	Papain-like Cys Protease	PBS1		RPS5	i
	HopI1	J domain activity	Hsp70	SA accumulation		j
	HopM1 (HopPtoM)	Promotes degradation of host proteins (ubiquitination?)	MIN7	PTI, ETI, suppression of callose deposition		k
	HopU1 (HopPtoS2)	Mono-ADP-ribosyltransferase	GRP7 and other RNA-binding proteins	Host transcriptome alteration, PTI		l
X. campestris	AvrBsT	YopJ-like SUMO protease		PTI, ETI		m
	AvrBs2	Glycerophosphoryl diester phosphordiesterase?		Major virulence factor	Bs2	n
	AvrBs3	Transcription activator-like (TAL)	Upa20-box/Bs3	Cell size regulation	Bs3	o
	AvrBs4	Transcription activator-like (TAL)		Induces catalase crystals in peroxisomes	Bs4	p
	AvrXa27	Transcription activator-like (TAL)	Xa27		XA27	q
	AvrRxv	YopJ-like SUMO protease	ARI1 (14-3-3 protein)	Modulates defense gene expression,	Rxv	r
	AvrXv4	DeSUMOylating cysteine protease			XV4	s
	XopJ	Cysteine protease, N-myrostilation domain		Suppression of callose deposition and secretion		t
	XopD	DeSUMOylating cysteine protease, DNA-binding HLH motif	Trascription factors?	Modulates defense gene expression		u
R. solanacearum	AvrA					v
	PopP1	YopJ-like SUMO protease				w
	PopP2	YopJ-like SUMO protease	RRS1, RD19		RRS1, RPS4	x
	GALA	F-box proteins, LRR domains	SKP1-like protein	Host ubiquitin/proteasome pathway		y
E. amylovora	DspA/E		DIPM1-4 (RLKs)	PTI, suppression of callose deposition		z

←Table 4. Functions, partners and R-proteins of T3Es.

In this table, the best characterised effectors from different pathogens are described. Some of their functions include phosphorylation, ubiquitination or transcription activation of several genes in order to block plant immunity. The fact that few effectors target the same plant proteins (e.g. RIN4) explains the effector redundancy found in many pathogens. a (Mackey et al., 2002; He et al., 2004; Ong and Innes, 2006; Shang et al., 2006; Desveaux et al., 2007; Cui et al., 2010); b (Tang et al., 1996; Bogdanove and Martin, 2000; Chang et al., 2000; Hauck et al., 2003; Cohn and Martin, 2005; Lin and Martin, 2005; Anderson et al., 2006; He et al., 2006; Hann and Rathjen, 2007; Xing et al., 2007; Navarro et al., 2008; Shan et al., 2008; Xiang et al., 2008; Xiang et al., 2011); c (Ritter and Dangl, 1995; Mackey et al., 2002; Kim et al., 2005); d (Narusaka et al., 2009; Sohn et al., 2009); e (Ritter and Dangl, 1995; Chen et al., 2000; Guttman and Greenberg, 2001; Axtell et al., 2003; Axtell and Staskawicz, 2003; Mackey et al., 2003; Chen et al., 2004; Lim and Kunkel, 2004, , 2004; Coaker et al., 2005; Chisholm et al., 2005; Kim et al., 2005; Kim et al., 2005; Chen et al., 2007); f (Kim et al., 2002; Jamir et al., 2004; Abramovitch et al., 2006; Rosebrock et al., 2007; Gohre et al., 2008; Shan et al., 2008; Gimenez-Ibanez et al., 2009); g (Li et al., 2005; Zhang et al., 2007); h (Bretz et al., 2003; Espinosa et al., 2003; Underwood et al., 2007); i (Shao et al., 2002; Shao et al., 2003; Zhu et al., 2004; Ade et al., 2007); j (Jelenska et al., 2007; Jelenska et al., 2010); k (Badel et al., 2003; Badel et al., 2006; Nomura et al., 2006; Ham et al., 2007); l (Fu et al., 2007); m (Ciesiolka et al., 1999; Escolar et al., 2001; Cunnac et al., 2007; Kim et al., 2010; Szczesny et al., 2010); n (Kearney and Staskawicz, 1990; Swords et al., 1996; Gassmann et al., 2000; Wichmann and Bergelson, 2004); o (Kay et al., 2007; Romer et al., 2007); p (Schornack et al., 2004; Gürlebeck et al., 2009); q (Gu et al., 2005); r (Whalen et al., 1988; Ciesiolka et al., 1999; Bonshtien et al., 2005; Whalen et al., 2008); s (Astua-Monge et al., 2000; Roden et al., 2004); t (Thieme et al., 2007; Bartetzko et al., 2009); u (Hotson et al., 2003; Kim et al., 2008); v (Carney and Denny, 1990; Poueymiro et al., 2009); w (Lavie et al., 2002; Poueymiro et al., 2009); x (Deslandes et al., 2003; Narusaka et al., 2009); y (Angot et al., 2006); z (Boureau et al., 2006; Meng et al., 2006).

Effectors that directly target plant immunity

Some effectors target RLK complexes in membranes that recognize PAMPs to interrupt the PTI signalling cascade and avoid pathogen perception (Block and Alfano, 2011). The best characterised effectors that use this mechanism are avrPto and avrPtoB effectors from *P. syringae* DC3000, whose mechanism is exemplified in Figure 15 and explained below (Jones and Dangl, 2006).

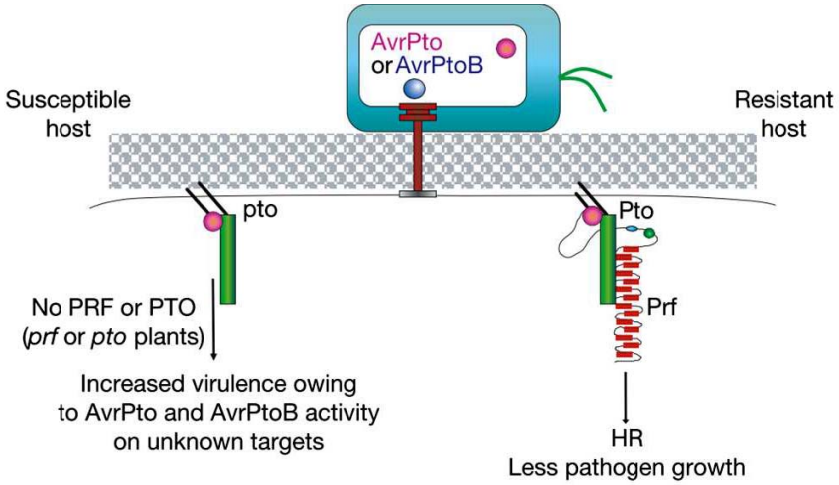


Figure 15. Partial model for AvrPto/AvrPtoB interference with the plant defences.

Obtained from (Jones and Dangl, 2006).

AvrPto, inhibits RLK kinase activity, whereas AvrPtoB ubiquitinates RLK (thanks to its C-terminal E3 ligase domain) and leads to its degradation. In both situations, the result is the uncoupling of PTI signalling. The main targets of AvrPto/AvrPtoB activity seem to be FLS2, EFR, CERK1 and BAK1 (although there is some controversy for recent experiments {Xiang, 2011 #551) (He et al., 2006; Gohre et al., 2008; Shan et al., 2008; Xiang et al., 2008; Gimenez-Ibanez et al., 2009). However, tomato plants have evolved sophisticated mechanisms to detect AvrPtoB and AvrPto and induce ETI through Prf R-protein. This ETI in tomato is mediated by the host factor PTO and/or FEN (Ronald et al., 1992; Martin et al., 1993; Salmeron et al., 1994; Kim et al., 2002; Rosebrock et al., 2007). Binding of avrPto to the PTO protein results in a conformational change that activates Prf (Xing et al., 2007). Nevertheless, avrPtoB also exhibits virulence activities: It targets the FEN kinase through its N-terminal domain and brings it to degradation thanks to its C-terminal E3 ubiquitin ligase domain to avoid ETI (Rosebrock et al., 2007). The Pto kinase in its turn, performs an extra secure level by inactivatating the avrPtoB E3 ligase so that bacteria can no longer avoid ETI (Abramovitch et al., 2006; Janjusevic et al., 2006; Rosebrock et al., 2007; Block and Alfano, 2011). This represents a paradigmatic scenario that fully illustrates the arms race between plants and pathogens.

A similar system is exploited by three other effectors: *P. syringae* AvrRpt2, AvrRpm1 and AvrB which also inhibit PTI (Mackey et al., 2002; Kim et al., 2005). In these cases, RIN4 appears to be their common host target that functions as a negative regulator downstream the PAMP-detection (see Figure 16). RIN4 is associated with the inner membrane together with the R-proteins RPS2 and RPM1 in Arabidopsis. RPM1 detects the AvrB and AvrRpm1-dependent phosphorylation of RIN4, whereas RPS2 perceives its cleavage and elimination by AvrRpt2 (Kim et al., 2005; da Cunha et al., 2007). In any case, the plant will respond with an ETI so that the pathogen is no longer able to multiply within the host. Evenmore, another effector from *P. syringae*, HopF2, recently emerged as an ETI suppressor (when induced by AvrRpt2) interfering with this same system. HopF2 is thought to bind to RIN4 preventing avrRpt2-dependent cleavage and hence avoiding Prf release (Wang et al., 2010). RIN4 has lately been considered as a decoy protein because an increase in pathogen virulence is not observed in *rin4* plants (Lewis et al., 2009).

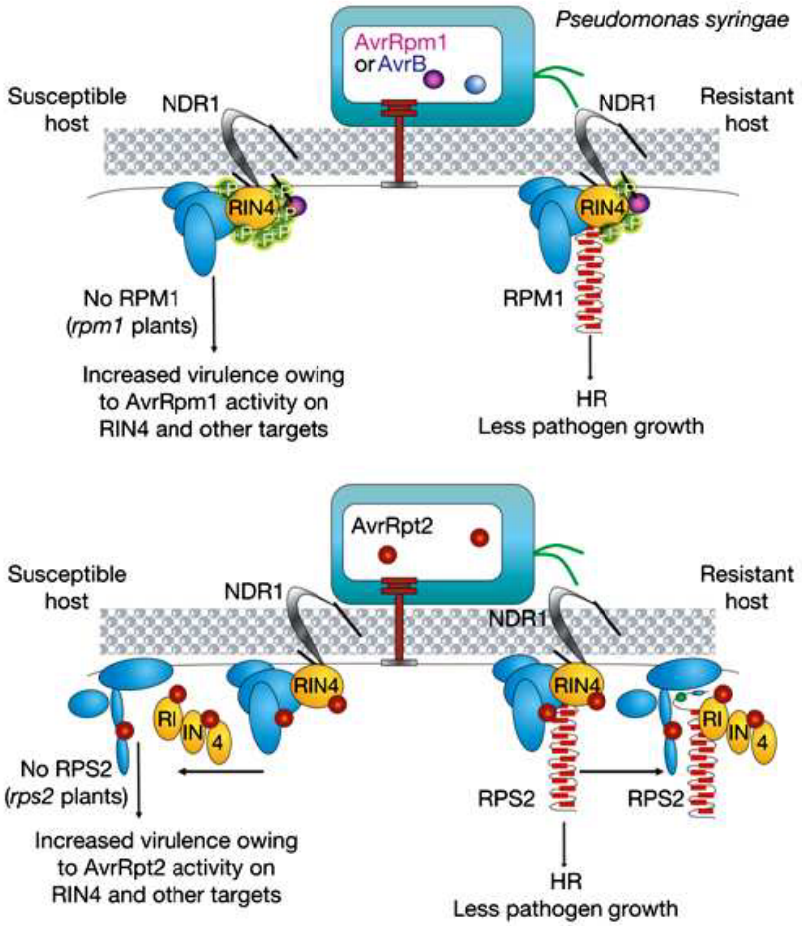


Figure 16. Partial model for RIN4 targeting by three different *P. syringae* effectors.

Obtained from (Jones and Dangl, 2006)

The virulence function of AvrB seems to be the suppression of plant immunity through RAR1 interaction which is part of the R-protein stabilization complexes (Shang et al., 2006).

Another nice example of PTI suppression has been reported for the effector HopAI1 from *P. syringae*, which displays a phosphothreonine lyase activity and irreversibly inactivates MPK3 and MPK6 involved in the downstream signalling cascade (Zhang et al., 2007).

HopAR1 (AvrPphB) from *P. syringae* also targets the ETI mechanism by cleaving the PBS1 protein kinase, which is guarded by the R-protein RPS5 (Figure 17). In resistant plants, RPS5 responds to PBS1 alteration by inactivating downstream signalling events and triggering plant defences (Shao et al., 2003).

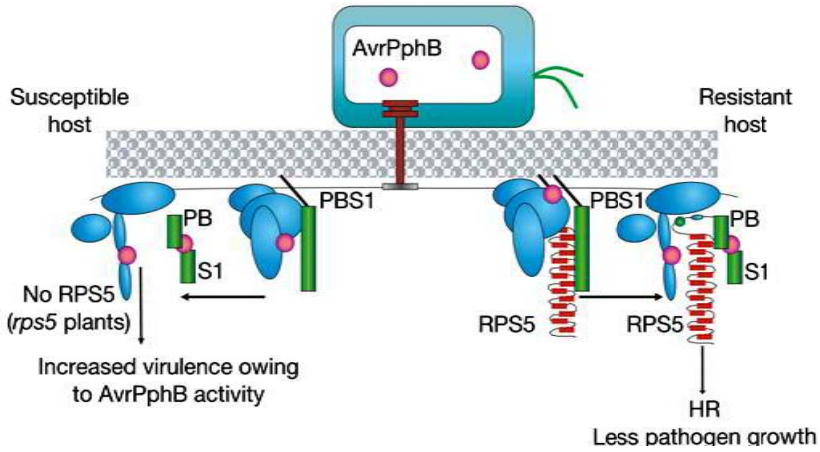


Figure 17. Model for RPS5-mediated HR through PBS1 manipulation by HopAR1 from *P. syringae.*

Reproduced from (Jones and Dangl, 2006).

In *R. solanacearum*, PopP2 is the only effector that has been described to directly bind to its cognate R-protein, in this case, RRS1. Apparently, PopP2 prevents RRS1 degradation by the proteasome pathway (Tasset et al., 2010). Upon interaction, RRS1 is directed to the nucleus where it may transcribe a set of defence genes to confer plant resistance (Deslandes et al., 1998; Deslandes et al., 2003; Bernoux et al., 2008). Autoacetylation of PopP2 and presence of its interactor protein RD19 (a cysteine protease) are both required for RRS1-mediated resistance (Bernoux et al., 2008; Tasset et al., 2010). Contrary to what has been thought until recently, more factors could be involved in this ETI, such as perception of the enzymatic activity, apart from the interaction between PopP2 and RRS1 itself.

Effectors that indirectly target plant immunity

Apart from targeting specific compounds involved in PTI or ETI, effectors can alter plant defences indirectly.

Phytohormone signalling pathways are known to be interconnected and highly regulated through positive and negative feedbacks (Figure 18) and their subtle alteration can destabilize plant defences (Pieterse et al., 2009). Some effectors as well as other virulence associated factors target hormone signalling at different levels to alter host defences and facilitate pathogen infection.

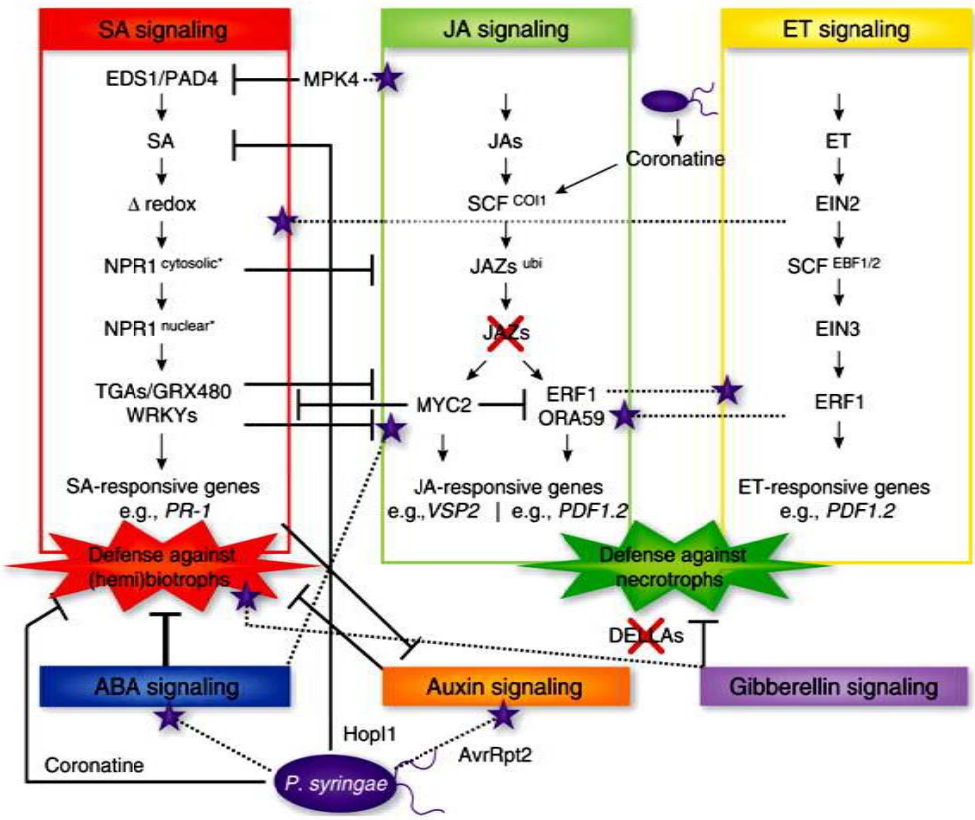


Figure 18. Phytohormone signalling networks in plants and its manipulation by bacteria.

Bacterial T3Es or toxins like coronatine aim at destabilising the plant hormonal signalling by altering the levels of hormones such as ABA, auxin, SA or JA. Positive effects are represented by stars and negative effects by a ⊥ symbol. Reproduced from (Pieterse et al., 2009).

For instance, in addition to the above-mentioned activities, AvrB can also activate jasmonate signalling in *Arabidopsis* (He et al., 2004). AvrPtoB modulates the ABA pathway and AvrRpt2 targets the auxin pathway, both to enhance host susceptibility (Truman et al., 2006; Chen et al., 2007; De Torres Zabala et al., 2009). AvrPtoB and AvrPto also affect the ethylene pathway through yet unknown mechanisms (Cohn and Martin, 2005). In the same way, HopI1 suppresses the production of SA, which is required for a successful plant resistance (Jelenska et al., 2010), and HopM1 suppreses SA-dependent responses (DebRoy et al., 2004). The phytotoxin coronatine secreted by *P. syringae*, mimics JA, suppresses SA-dependent defences and suppresses PAMP-triggered ABA signalling in stomata guard cells. This prevents stomata closure and facilitates bacterial penetration (He et al., 2004; Melotto et al., 2008; De Torres Zabala et al., 2009).

HopM1 respresents still another strategy to block plant defences. This *P. syringae* effector hijacks the proteasome, similary to what was described for AvrPtoB. The target for HopM1 is AtMIN7, an ARF GEF family protein involved in vesicle trafficking (Nomura et al., 2006). A

vesicle-trafficking impairment restricts the secretion of defence-related molecules to the plasma membrane and to the apoplast and could favour pathogen virulence (Block and Alfano, 2011).

Other effectors manipulate RNA processing, examples of which are the multifaced AvrPtoB and HopT1, both suppressors of PAMP-induced miRNAs (Navarro et al., 2006). Along the same line, HopU1 ribosylates some chloroplast RNA-binding proteins (e.g. GRP7) and this may result in virulence enhancement as a result of RNA metabolism alteration (Fu et al., 2007).

Another extremely refined system of host manipulation is targeting T3E to the plant nucleus to alter RNA transcription and achieve changes in plant metabolism or defences. This is the case of the AvrBs3 effector from *X. campestris* which harbours a nuclear-localization signal and an acidic activation domain typical of transcriptor factors (Van den Ackerveken et al., 1996; Szurek et al., 2001; Szurek et al., 2002; Schornack et al., 2006; Bogdanove et al., 2010). AvrBs3 directly binds to the UPA20 box in the plant genome and induces plant gene expression and cell expansion (Kay et al., 2007). Resistant plants contain a mimic of the UPA20 promoter, the Bs3 promoter, where AvrBs3 binds and induces the production of the Bs3 R-protein that will initiate ETI (Romer et al., 2007). This sophisticated strategy is shared by other *Xanthomonas* effectors like AvrXa27, pthXo1 etc., which all form the TAL (transcription activator like) effector family.

3.2. An eternal combat explained through evolution

Unlike animals, plants cannot move to escape pathogen attack nor display an adaptive immune system or mobile defender cells. In plants, each single cell has its own capacity to react against a pathogen and systemic responses will report the invader presence over the host to prevent penetration (Dangl and Jones, 2001; Chisholm et al., 2006). An important breakthrough in the field of plant pathology was the proposal of the zigzag model introduced by Jones and Dangl in 2006 to explain interaction between plants and pathogens in a dynamic way (Figure 19) (Jones and Dangl, 2006). The present concept of plant immunity and its components, involving PTI and ETI, was settled in their review article.

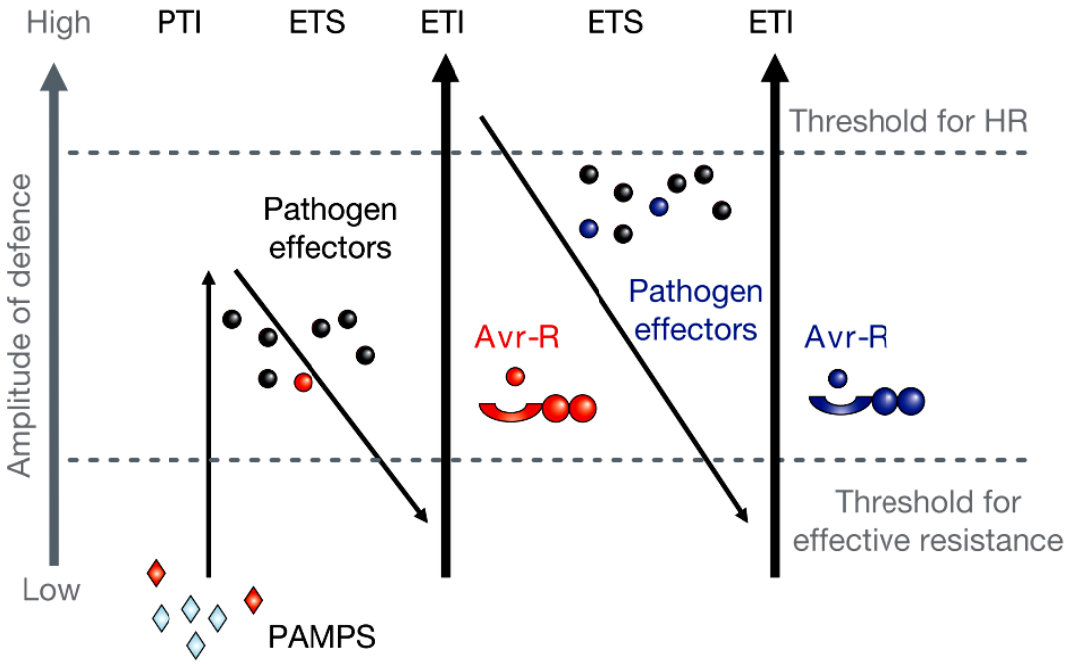


Figure 19. The zigzag model.

This model illustrates the events that may have taken place in host-pathogen interactions along time. Initially, plants learned to detect bacteria by perceiving some of their conserved molecules (PAMPs) and avoid infection by PTI. Later on, pathogens acquired effector proteins that were injected inside the plant cytoplasm aimed at blocking PTI and hence rendering the plant susceptible. Afterwards, plants developed the capacity to impede bacterial progression through specific effector recognition by R-proteins, leading to ETI. At some point in the plant-bacterial evolution, pathogen could escape recognition by modification of its effectome (Jones and Dangl, 2006).

In the zigzag model, bacterial and plant populations are considered as a whole that has been shaped during evolution. The model postulates that at an early time, plants learned to detect and respond to PAMPs through PRR-mediated PTI (Jones and Dangl, 2006). Evasion from this recognition system is very limited, as PAMPs are highly conserved among bacteria. For example, bacteria that have lost the flagellum will not be detected through FLS2 but bacterial fitness will be also diminished as bacterial mobility and attachment will be affected. In addition, some plant species will only be capable of detecting certain PAMPs, which can be different from those recognized in other species. Both PAMPs and their receptors (PRRs) will experience the forces of natural selection (Jones and Dangl, 2006). In a second phase in evolution, some bacteria managed to inject effector proteins to the host plants in order to enhance pathogen virulence by PTI inhibition, a process also known as effector-triggered susceptibility or ETS. In turn, plants developed a system to directly or indirectly sense the bacterial effectors and developed an NB-LRR-mediated effector-triggered immunity to restrict pathogen growth and avoid disease establishment. However, the effector pool in bacteria is highly dynamic so that some effectors can be lost to avoid plant recognition and new ones can

be acquired to adapt to new hosts, which in turn can be lately detected (McCann and Guttman, 2008; Schulze-Lefert and Panstruga, 2010). A diagram illustrating this selection pressure on both effectors and R-proteins is presented in Figure 20. When an effector is recognized by a plant, its frequency decreases in bacterial population because it confers a disadvantage. However, the low frequency of this effector will remarkably diminish the selective pressure in favor of the plant R gene and thus reduce its frequency (Jones and Dangl, 2006). This event leads to a resurgence of the effector due to a positive selection, since it increases bacterial fitness. The circle repeats over time and can be extrapolated to the whole effectome and pool of R-proteins. The outcome of this is the presence of a high number of effectors in pathogens that are differentially selected for upon contact with different plant hosts.

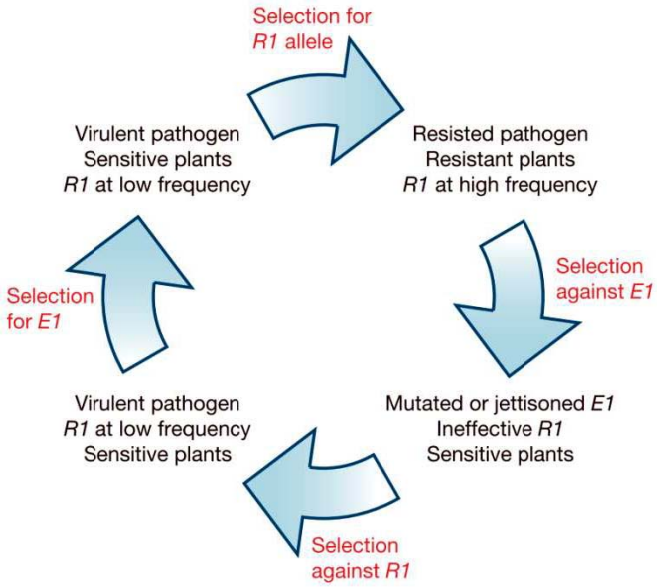


Figure 20. Diagram of selection forces displayed in nature for/against a subset of effectors (E) and R-proteins (R).

When a plant presents a resistance protein against a particular pathogen, natural selection forces it to be maintained. However, the bacterial effector protein that is detected will be negatively selected and thus its frequency in population will decrease along time, as will R-proteins also since "danger" disappeared. However, some changes in the effector could lead to a new positive selection if it confers an advantage for the bacterium. This is a cyclic scenario that perfectly illustrates the co-evolution between plants and pathogens. Diagram obtained from (Jones and Dangl, 2006).

The resulting pool of effectors present in a given bacterial strain is extremely divergent from others of the same species and probably only a subset might be needed for interaction with a particular host (Schulze-Lefert and Panstruga, 2010). On top of this, pathogens can escape the plant surveillance system by modifying its effectors. For example, AvrXa27 from *X. oryzae* (belonging to the TAL family) confers virulence on susceptible hosts while it is recognised by

Xa7 in resistant plants. This effector contains a variable number of direct repeats in its central region and indel mutations result in disparate phenotypes: from host recognition evasion to loss of virulence (Yang et al., 2005). Similar events could take place in the avirulence gene AvrA from *R. solanacearum* (Poueymiro et al., 2009). Another astonishing event of pathoadaptation was observed in the transgenic Bs2 pepper plants, which express an R-protein against AvrBs2 of *X. axonopodis* pv. *vesicatoria.* Bacterial populations recovered in wild type peppers presented full AvrBs2 effector gene while those recovered after passage through transgenic crops displayed dominant mutated forms of AvrBs2 due to the imposed strong selective pressure for effector loss (Wichmann et al., 2005). This host-driven selection is not an isolated event as it is observed in other pathogens.

Another system to increase pathogen virulence or to gain the ability to infect new host plants is the acquisition of novel effectors. Recombination events conferring new regulation properties, reactivation of pseudogenes after excision of a transposable event, bacteriophage infection and horizontal gene transfer events may all contribute to broaden bacterial effector repertoire under certain conditions (Grant et al., 2006). If the new effector confers a selective advantage it will be maintained, otherwise, will be lost (McCann and Guttman, 2008). However, the conserved targets of AvrB, AvrRpm1 and AvrRpt2 show that blocking plant defence signalling cannot be done by unlimited ways. Some host proteins could be ket targets, explaining the high redundancies of T3Es. Of course, the plant takes advantage of this situation by mimicking targets proteins to trigger defence responses.

Finally, some NB-LRRs are polymorphic within a plant population especially in the leucine rich repeat domain, and this removes the strong negative selection displayed on their effectors (Bakker et al., 2006; Hann et al., 2010). Thanks to this, recognized effectors can be maintained in bacterial populations where the arms-race can continue.

CHAPTER 4: *Ralstonia solanacearum,* a devastating plant pathogen

CHAPTER 4

RALSTONIA SOLANACEARUM, A DEVASTATING PLANT PATHOGEN

Previously known as *Pseudomonas solanacearum* or *Burkholderia solanacearum* (Yabuuchi et al., 1992), since 1995 the causal agent of bacterial wilt is presently called *Ralstonia solanacearum* (Yabuuchi et al., 1995). *Ralstonia solanacearum* is a soil-borne bacterial plant pathogen that belongs to the β-proteobacteria and infects some important crops such as tomato and potato, which leads to huge economical losses. This, together with the facts that (1) its genome sequence and many genetic tools are available, (2) it can naturally uptake DNA and (3) it can artificially infect the model plant *Arabidopsis thaliana*, make this pathogen an ideal research model.

4.1. The bacterial wilt disease

Mode of infection

Bacterial wilt is a devastating disease in tropical and subtropical crops, including potato (brown rot), tomato (southern wilt disease), tobacco, banana (moko disease), peper, peanut and eggplant (Figure 21). The bacterium infects more than 200 plant species from 50 botanical families (Allen et al., 2004).

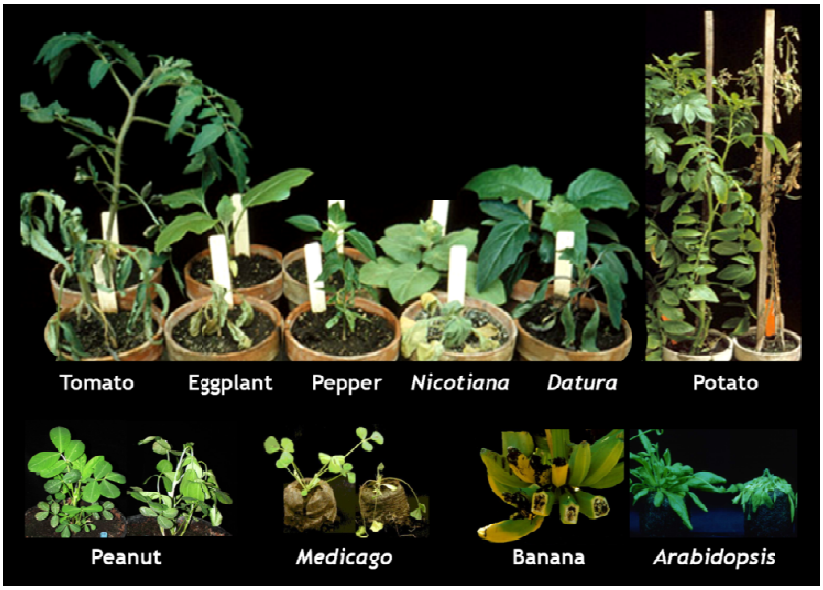


Figure 21: Bacterial wilt on different host plants.

Some of the plants that can be infected by *R. solanacearum* include banana, potato, tomato or pepper plants. Image obtained from Marc Valls.

The infection disease cycle involving *R. solanacearum* is presented in Figure 22. The pathogen can survive for long periods as a saprophytic organism in the soil or in waterflows (Morris et al., 2009). Bacterial invasion take place through wounds or lateral root emergence sites and then bacteria rapidly progress (hours) to the apoplastic space of the root cortex. Shortly afterwards, *R. solanacearum* traverses the sites that present a weak endoderm and finally reaches the proto-xylem. Secretion of cell-wall-degrading enzymes are key for the colonization of the xylem and systemic infection of the host plant after 2-3 days due to extraordinary multiplication (reaching 10^{10} cfu/ml) (Vasse et al., 1995). The huge density of bacteria and exopolysaccharide block the water flow, causing the wilting phenotype and eventually, plant death (Allen et al., 2004). The severity of the disease depends on many factors including temperature, soil moisture and the host/bacterium interplay. Propagation of the pathogen is mainly due to seed potatoes that carry latent infections and can develop the disease in favourable conditions. Therefore, strict controls to detect the presence of *R. solanacearum* in infected tubers are imperative to control the disease.

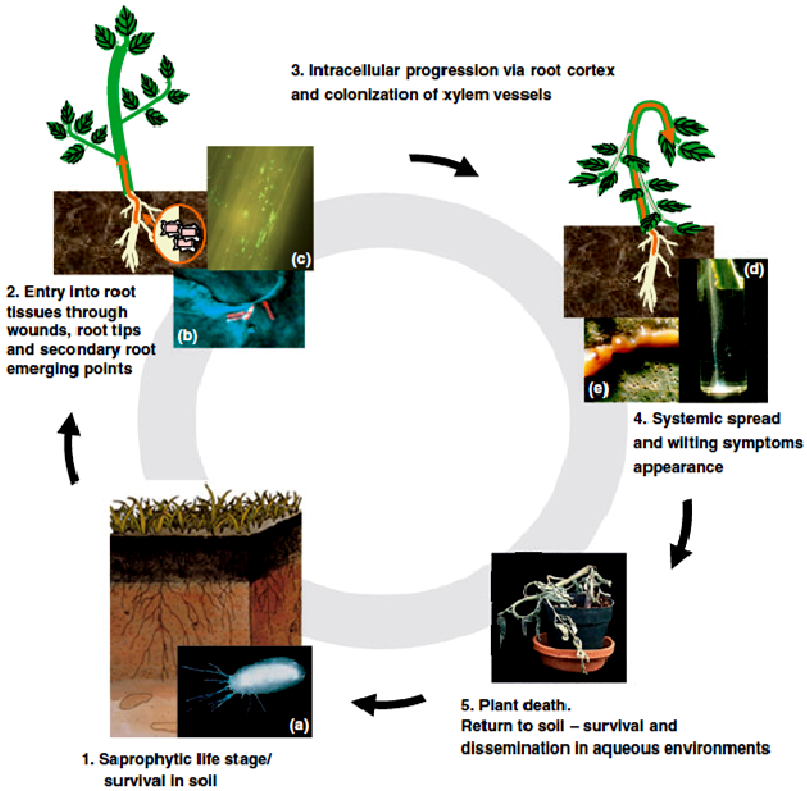


Figure 22. Infectious cycle of *R. solanacearum*.

Infection of *R. solanacearum* starts by host root penetration and it is followed by a rapid multiplication and progression until xylem is colonized. Afterwards, the bacteria multiply and block water flow, which is the cause of the wilting symptoms. (a) Transmission electron microscopy picture of GMI1000, (b) Confocal image of attached bacteria (in red) to the plant surfaces, (c) GFP-expressing bacteria on a tomato root surface, (d) bacterial fluid secreting from the plant after a stem cut, (e) bacterial exopolysaccharide EPS from infected stem. Image extracted from (Genin, 2010).

***R. solanacearum* as a species complex**

R. solanacearum is considered a species complex as it is genotypically and phenotypically very heterogeneous (Gillings and Fahy, 1994). Other members of this species complex are the *R. syzygii* and the blood disease bacterium (Taghavi et al., 1996; Fegan and Prior, 2005). Several classification systems have been proposed in the last years in an attempt to create subgroups and better classify the species complex. On a metabolic basis, strains can be divided into five biovars depending on their ability to use different sugars and alcohol carbohydrates or into five races according to the host range (Buddenhagen et al., 1962; Buddenhagen, 1986 ; Hayward, 1994). Race 1 has the widest host range, infecting mostly solanaceous plants while race 2 infects exclusively banana. Race 3 mainly infects potato and tomato, it has a lower temperature optimum and is the causative of the major potato losses reported worldwide. Strains affecting ginger and mulberry tree have been classified in the two additional race groups: races 4 and 5, respectively. A new classification approach based on sequence analysis of four genes, divided the *R. solanacearum* species into five different phylotypes (Fegan and Prior, 2005) that correlate with their geographical origin (Figure 23) (Genin, 2010).

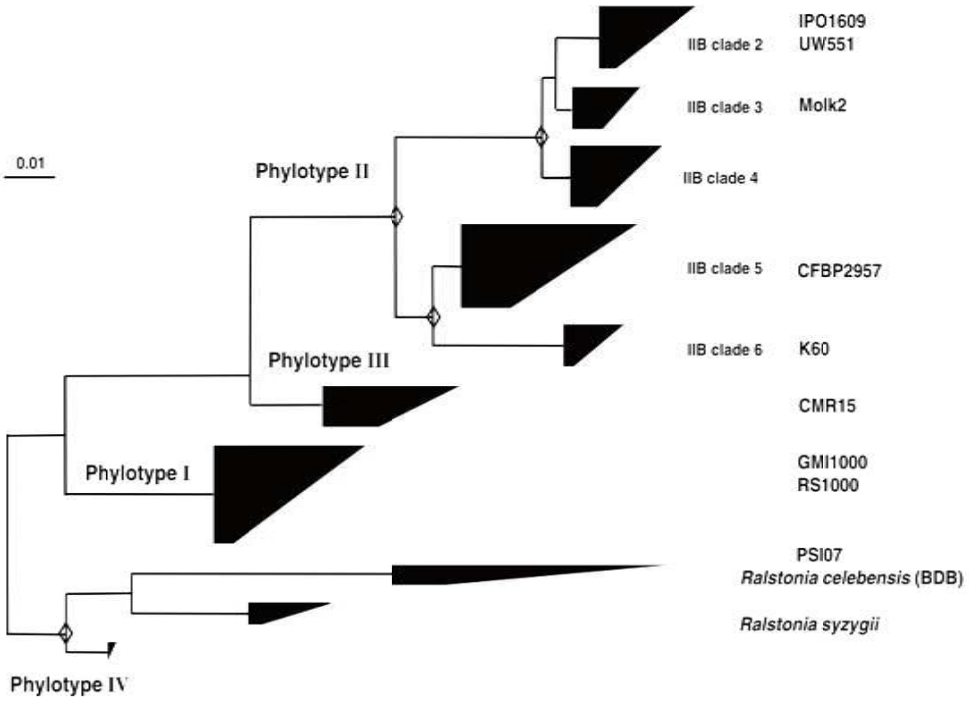


Figure 23. Classification of *R. solanacearum* species complex.

R. solanacearum species complex is classified into different phylotypes according to the endoglucanase sequence comparison. Phylotype classification correlates with geographical distribution: I (strains originating primarly from Asia) II (from America), III (from Africa and surrounding islands in the Indian Ocean) and IV (from Indonesia). *R. celebrensis* and *R. syzygii* are also part of this complex. Most of the indicated strains are those whose genome has been already sequenced. Image obtained from (Genin, 2010).

The best studied strain is GMI1000 that belongs to Phylotype I, Race I and Biovar 3. The genome sequence from GMI1000 (Salanoubat et al., 2002) was obtained in 2002, being the second bacterial plant pathogen to be completely sequenced. Afterwards, genomes of other strains representative of the different phylotypes in this species have also become available (Gabriel et al., 2006; Guidot et al., 2009; Remenant et al., 2010).

Distribution of *R. solanacearum* and impact on agriculture

The geographical distribution of *R. solanacearum* infections belonging to its three major races has been reviewed by the European Plant Protection Organization (EPPO) in 2006 (Figure 24), highlighting a recent spread of this quarantine disease to temperate regions of Europe (http://www.eppo.org/).

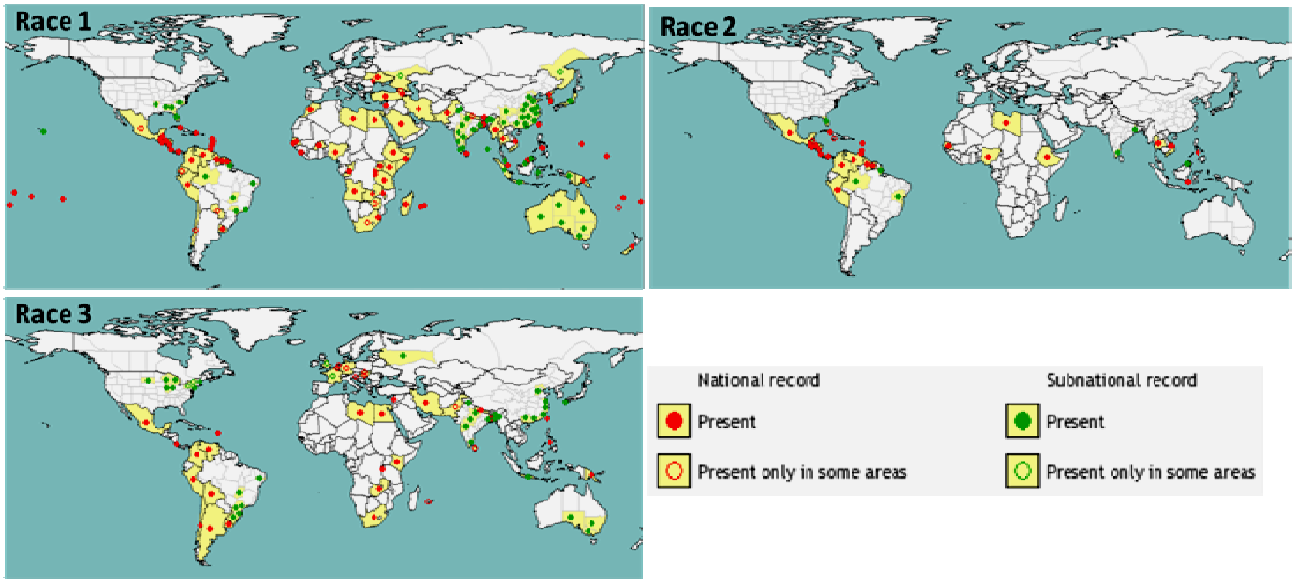


Figure 24. Worldwide geographical distribution of the three major *R. solanacearum* races.

While race 1 and 2 are quite restricted to tropical areas, or not predominant in temperate regions, race 3 have also been recorded in the colder climates of Europe. (http://www.eppo.org/)

Many reports show that race 3 has the ability to survive in waterways as has been proven in The Netherlands, Sweden, England, France and Belgium (Caruso et al., 2005). This could be explained by the presence of weed reservoirs of the bacterium like *Solanum dulcamara* or *Urtica dioica* that remain asymptomatic (Hayward, 1991). The presence of *R. solanacearum* in these plants from aquatic systems seems to have been the cause of several bacterial wilt outbreaks (Caruso et al., 2005). Several crops from temperate countries are thus at risk of bacterial wilt in the future. This is especially important for solanaceous crops, the natural host of most *R. solanacearum* strains.

Amongst solanaceous, tomato is the second most important crop worldwide and Spain is now the eighth largest tomato producer in the world and second in Europe with 3.7 million tonnes, 30-40% destined for exportation. The value generated for tomato production in Spain, which accounts for around 30% of the total value generated by vegetables, is expected to steadily increase in the future. Although no clear resistance has been detected, some cultivars such as Hawaii 7996, show increased tolerance to bacterial wilt, which is a dominant monogenic trait (Grimault et al., 1994). After wheat and rice, potato is the third most important food crop, with a world-wide production of more than 300 million tons in 2007 and its consumption is clearly expanding in developing countries which nowadays account already for more than half of the total harvest (http://www.potato2008.org). Optimization of production levels and resistance to biotic and abiotic stresses is still a challenge for potato breeding programs. *S. phureja* and other wild potato relatives have been used with limited success for introgression of partial resistance to bacterial wilt, but recently, *S. commersonnii* has emerged as a very promising source of resistance (Carputo et al., 2009).

4.2. Genome structure and virulence determinants of *R. solanacearum*

R. solanacearum harbours a bipartite genome consisting of a chromosome (3.7 Mb) and a megaplasmid (2.1 Mb) (Salanoubat et al., 2002). Although most housekeeping genes are codified in the larger replicon, some genes important for bacterial metabolism or infection are only present in the megaplasmid and this forces the bacteria to mantain both replicons. The average base composition in the *R. solanacearum* genome is GC 67% . However, some regions display not only a differential G+C percentatge but also an alternative codon usage. This feature has been attributed to tRNAs or mobile genetic elements (e.g. insertion sequences or bacteriophages) and thus it is highly plausible that these regions have been acquired by horizontal gene transfer events, playing a pivotal role in the rapid adaptation of the bacterium (Guidot et al., 2009).

Plant infection and colonization by *R. solanacearum* require the injection of so-called effector proteins into host cells through the Type III Secretion System (T3SS) (Galan and Collmer, 1999; Marlovits and Stebbins, 2009). The T3SS is the main pathogenicity determinant of *R. solanacearum* since in its absence bacteria can no longer cause disease (Boucher et al., 1987). The T3SS is encoded by the *hrp* gene cluster placed on the megaplasmid and occupies a region of 23 Kb (Salanoubat et al., 2002). However, other bacterial components and secretion systems (I, II, IV, V, two-partner secretion and tat export pathway) are also important for bacterial

fitness or adaptation to host plants (Table 5) (Brown and Allen, 2004; Poueymiro et al., 2009; Genin, 2010).

Class	Gene/product	Role in pathogenic adaptation	Ref.
T3Es	Unknown	Suppression of PTI or ETI	
	Several T3E	Contribution to bacterial fitness *in planta*	a
	Gala7	Specific host pathogenicity factor on *M. truncatula*	b
Protection enzymes	Catalases, peroxidases...	Detoxification of ROS	c
	Polyphenol oxidase	Degradation of phenolic compounds	d
	Polyamine biosynthesis		
Efflux pumps	*acrA*, *dinF*	Resistance to antimicrobials	e
Alternate metabolism	Enzymes (Pehb, Egl, metE...), transporters	Degradation pathways of host substrates, metabolic versatility	f
Surface appendages	Type IV pili	Attachment to host cell surfaces	e
	Filamentous haemagglutinin -like proteins	Potential bacterial adherence factor	f
Host attachment	Lectins Rsl and RsI-II	Potential adherence factor to plant cell wall	g
Chemotaxis		Attraction to plant roots with possible host selectivity	h
Secondary metabolites	Hdf	Contributes to bacterial fitness *in planta*	i
	Ralfuranone	Unknown; production controlled by virulence regulators	j
Phytohormones	Ethylene	Unknown; production of ethylene and auxin	k
	Auxin	controlled by the virulence regulator HrpG	
	trans-Zeatin		

Table 5. List of the main virulence determinants in *R. solanacearum*.

Several pathogenicity determinants contribute to the virulence of *R.* solanacearum. Some of them are important for attachment or motility whereas others might have a role in evading host recognition, blocking plant defences or bacterial survival in poor nutrient environments. Table modified from (Genin, 2010). a (Macho et al., 2010); b (Angot et al., 2006); c (Valls et al., 2006; Flores-Cruz and Allen, 2009); d (Hernandez-Romero et al., 2005); e (Brown et al., 2007); f (Brown and Allen, 2004; Genin and Boucher, 2004); e (Kang, 2002); f (Salanoubat et al., 2002; Brown and Allen, 2004); g (Valls et al., 2006); h (Yao and Allen, 2006); i (Macho et al., 2010); j (Delaspre et al., 2007; Schneider et al., 2009); k (Genin and Boucher, 2002; Valls et al., 2006).

Exopolysaccharide (EPS) is a secreted molecule composed of *N*-acetylgalactosamine, 2-*N*-acetyl-2-deoxy-galacturonic acid and 2-*N*-acetyl-4-N-(3-hydroxybutanoyl)-2,4,6-tri-deoxy-D-glucose (Orgambide et al., 1991). It is also considered to be a major virulence determinant in *R. solanacearum* (Denny and Baek, 1991). This molecule is well-conserved among the genetically diverse *Ralstonia* species complex and it is required for full virulence through a still unknown

mechanism (McGarvey et al., 1999). Recent experiments point out that EPS might act as an elicitor in resistant tomato plants (Milling et al., 2011).

Amongst its virulence determinants, *R. solanacearum* expresses detoxifying enzymes to neutralize plant-derived phenolic compounds or antimicrobials triggered by plant defences (Hernandez-Romero et al., 2005; Brown et al., 2007; Flores-Cruz and Allen, 2009). The apoplast is low in nutrients, so additional secreted enzymes (e.g. plant cell wall degrading enzymes) also seem to assist exploiting the plant niche to get nourishment (Brown and Allen, 2004). Another metabolite required for *R. solanacearum* pathogenicity is the 3-hydroxypalmitic acid methyl ester (3-OH-PAME) that is involved in quorum sensing, which is key to trigger EPS production at high bacterial densities and cause the xylem blockage (Flavier et al., 1997; Genin and Boucher, 2002). Attachment to plant cell surfaces is mediated by type IV pili appendage, vital for adherence, twitching motility and virulence (Kang, 2002). Putative virulence factors are the phytohormones produced by the bacterium (especially ethylene that may promote bacterial growth) (Valls et al., 2006; Macho et al., 2010) and enzymes that may protect bacteria against plant defence products such as catalases, peroxidases or polyamine biosynthesis (Hernandez-Romero et al., 2005; Valls et al., 2006; Flores-Cruz and Allen, 2009).

4.3. Regulation of the T3SS and effector predictions in *R. solanacearum*

Regulation of the T3SS in *R. solanacearum* is tightly regulated through an extremely well characterized signalling cascade shown in Figure 25.

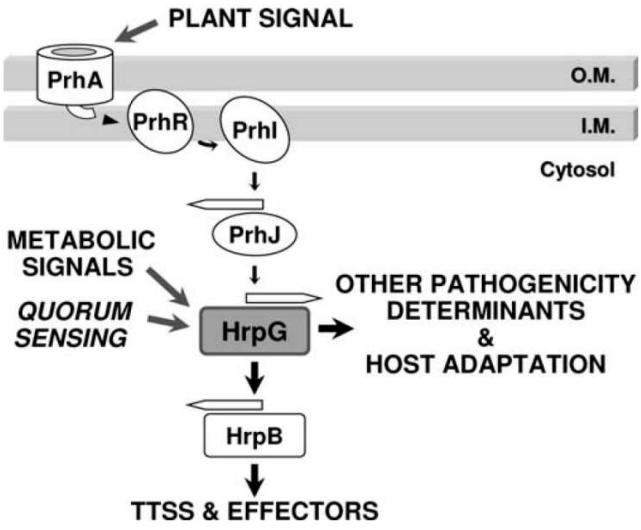


Figure 25. Simplified model of the Hrp regulation cascade in the pathogen *R. solanacearum*.

Once the plant is detected, a complex and well characterised signalling cascade takes place in order to activate T3SS and secretion of all its substrates (Valls et al., 2006). O.M.:Bacterial outer membrane; I.M.: Bacterial cytoplasmatic membrane.

Upon perception of a plant signal through the PrhA receptor, a signal transduction cascade is triggered to activate the HrpG regulator. This key transcriptional activator integrates this signal with others related to growth conditions and quorum sensing to modulate the final response (Brito et al., 1999; Genin et al., 2005; Plener et al., 2010). HrpG activates the master regulator HrpB, which belongs to the AraC transcriptional regulator family. HrpG induces as well transcription of a number of accessory pathogenicity determinants encoding protection enzymes and phytohormone biosynthesis enzymes. HrpB, in its turn, directly induces the transcription of all genes encoding the T3SS machinery as well as the Type III-secreted effectors (T3Es) and some other virulence-related genes. Homologues to HrpB and HrpG have been found in *Xanthomonas* and *Burkholderia* species (Wengelnik and Bonas, 1996; Lipscomb and Schell, 2011). Both regulators are needed for virulence since *R. solanacearum*, strains lacking them are no longer pathogenic (Vasse et al., 2000). HrpB binds specifically to hrp_{II} box found in the promoter of its target genes (Genin et al., 1992; Cunnac et al., 2004). Bioinformatical screening for putative effectors together with functional analysis combining gene expression profiles with disruption experiments permitted the identification of almost 50 putative substrates for the T3SS (Cunnac et al., 2004). However, the total number of effectors in *R. solanacearum* GMI1000 -or its homologue RS1000- is currently estimated over 70 (Mukaihara and Tamura, 2009; Poueymiro and Genin, 2009), being half of them located in the megaplasmid. In addition, almost 50% of the effectors appear to be specific from *R. solanacearum* as homologues in other species are not found. In addition, Mukaihara and colleagues demonstrated that the putative chaperone HpaB is required for most T3E in *R. solanacearum* to ensure proper translocation into plant cells (Mukaihara et al., 2010). Noteworthy, the GMI1000 effector repertoire presents various gene families that are conserved within the different strains in the *R. solanacearum* species complex. Major families are the SKWPs (6 members), the GALA family (seven members) and the AWR family (five members) (Poueymiro and Genin, 2009). The SKWPs are effector proteins that containing heat/armadillo-related repeats whose function remains still undeciphered. The GALAs are LRR proteins with a conserved GAxALA sequence and an F-box domain. These proteins were found to interact with SKP1-like protein and are thought to recruit plant proteins through the F-box domain and bring them to the SCF-type E3 ubiquitin ligase complex to interfere with host ubiquitin/proteasome pathway (Angot et al., 2006). Cumulative disruption of the seven GALA genes rendered *Ralstonia* less virulent on *Arabidpsis* and tomato. Furthermore, GALA7 was found to be a host specific effector to *Medicago truncatula.* The last effector family, AWRs, received their name due to the presence of an Ala-Trp-Arg conserved motif (Cunnac et al., 2004). A *R. solanacearum* strain devoid of AWR2 (*brg31*) and another devoid of *brg8* were the

only two single gene mutant constructions (out of 48 tested) that showed a delay on symptom development on tomato plants. All three different effector families are conserved among *R. solanacearum* strains. Other important effectors in *R. solanacearum* are AvrA and PopP1 (Carney and Denny, 1990; Lavie et al., 2002). These effectors behave as the major avirulence genes in tobacco species since strain lacking them become virulent on *N. benthamiana* plants (Poueymiro et al., 2009). The PopP2 effector (belonging to the YopJ/AvrRxv family like PopP1), has received most attention in the last years since it is one of the few cases illustrating the gene for gene model. This effector is recognized by RRS1-R R-protein which is present in some *Arabidopsis* ecotypes and acts as explained in the previous chapter (Deslandes et al., 2003; Bernoux et al., 2008; Tasset et al., 2010).

The high aggressiveness of *R. solanacearum*, its species complexity, its persistence in the field and the lack of resistant commercial varieties make bacterial wilt very difficult to control (Hong et al., 2005). Moreover, this species is one of the best characterised in terms of pathogenicity gene regulation. A better understanding of its infection mechanism –including the function of T3Es– and isolation of plant resistance/tolerance loci will probably contribute to the development of alternative systems to overcome infection in the future.

Objectives

OBJECTIVES

The objectives for this thesis are depicted below:

- **Determination of the origin and distribution of *awr* genes among pathogenic bacteria**

We aimed to evaluate the conservation of *awr* gene family among *R. solanacearum* strains and also check their presence in other organisms by using bioinformatical tools. Our results would provide valuable information about AWR evolution and putative function.

- **Functional characterisation of the AWR proteins of *R. solanacearum* GMI1000**

We decided to reveal the contribution of AWRs to *R. solanacearum* interactions with various of its host and non-host plants since their function remains still elusive. To address this, we applied gain-of-function and loss-of-function approaches both in the natural strain and in heterologous systems.

- **Identification of AWR targets in the plant host**

In order to further dissect the mechanism of AWR effector proteins once injected inside the plant cytoplasm, we searched for their targets. The nature of the plant interacting proteins was identified through a yeast two hybrid screening, providing information about the processes that are being manipulated by the plant pathogen.

Results and Discussion

CHAPTER 1: Distribution and sequence conservation of AWR effector proteins in *R. solanacearum* and other species

CHAPTER 1

DISTRIBUTION AND SEQUENCE CONSERVATION OF AWR EFFECTOR PROTEINS IN *R. SOLANACEARUM* AND OTHER SPECIES

1.1. AWRs are *bona fide* type III secreted effectors

Among all the T3E candidates in *R. solanacearum*, AWRs are one of the multigenic families present in this phytopathogen. Our interest for these genes came from a previous pathogenesis assay on tomato plants, which had shown that *awr2* was one of the very few T3Es whose single gene disruption caused a decrease in the pathogenicity, evidenced by a delay of one to two days in the establishment of disease (Cunnac et al., 2004). AWR nomenclature was attributed due to the Alanine -Tryptophan -Arginine- tryad found in a highly conserved region in their primary sequence. Strain GMI1000 contains five *awr* genes: *Rsc2139, Rsp0099, Rsp0846, Rsp0847* and *Rsp1024* that we have named *awr1, awr2, awr3, awr4* and *awr5*, respectively.

Main features of the *awr* genes and their encoded polypeptides

AWR effector proteins are large proteins composed by more than 1000 aminoacid residues. Genomic analysis of the *awr* genes in *R. solanacearum* GMI1000 showed some interesting features: *awr3* and *awr4* lay side by side and adjacent to the *hrp* gene cluster that encodes the T3SS. In addition, *awr2* and *awr5* are placed near alternative codon usage regions, revealing a possible acquisition through horizontal *gene* transfer. Four of the *awr* genes are borne by the megaplasmid whereas the fifth (*awr1)* is located on the chromosome. It had been previously proven that transcription of *awr*s (except for *awr1*) is activated by the T3SS master regulator *hrpB* (Cunnac et al., 2004) (Table 6). Excluding AWR1, these proteins are present in all the *R. solanacearum* phylotypes that have been screened for (S. Genin unpublished; Guidot et al., 2007).

Name	*Locus*	Size (aa)	*hrpB* regulation	Conservation	Injection
AWR1	Rsc2139	1063	No	Phy I	No*
AWR2	Rsp0099	1127	Yes	Phy I, II, III and IV	Yes
AWR3	Rsp0846	1242	Yes	Phy I, II, III and IV	Yes (hpx32)*
AWR4	Rsp0847	1330	Yes	Phy I, II, III and IV	Yes (hpx4)*
AWR5	Rsp1024	1240	Yes	Phy I, II, III and IV	Yes (hpx10)*

Table 6. Schematic table of the main characteristics of AWR members in GMI1000

Notice the length of the proteins and that they are *hrpB* regulated (except for *awr1*) and highly conserved in the *R. solanacearum* kingdom. Injection stands for translocation proven by cyaA reporter fusions and has been validated in GMI1000 (Cunnac et al., 2004), or in its close homolog strain RS1000 (asterisk, (Mukaihara and Tamura, 2009)). Phy: phylotype.

To reveal the degree of conservation amongst of AWRs, all their protein sequences from GMI1000 were compared through pairwaise analysis. This revealed an identity of 20-53% and a similarity of 27-62%, (Table 7). AWR3 and AWR4, which are placed contiguously in the megaplasmid, are the ones sharing the highest identity/similarity reinforcing the notion that they likely appeared by duplication. We could also distinguish another group formed by AWR1, 2 and 5 that were more similar between them than with the others.

ID./SIM. (%)	AWR2	AWR3	AWR4	AWR5
AWR1	25,9/38,3	23,9/34,6	18,9/27,1	24,7/37,1
AWR2		22,3/32,8	22,1/33,8	25,2/38,7
AWR3			52,7/62,2	23,7/33,6
AWR4				19,5/29,1

Table 7. Identities and similarities between AWRs from GMI1000 R. solanacearum.

Global pairwise analysis between all AWR members was evaluated by the EMBOSS alignment algorithm and the results of identity (ID) and similarity (SIM) are expressed in percentage.

AWRs are translocated to the culture medium

AWR2 is the only AWR effector from GMI1000 whose translocation to plant cells was previously verified using the CyaA reporter system (Cunnac et al., 2004). Strain RS1000 possesses paralogues for these five genes, which were proven to be injected into the host plant cell, except for AWR1, with the same reporter system (Mukaihara et al., 2010) (Table 6). In all cases, secretion was evaluated only for the N-terminal of the protein (first 230 aminoacids) fused to adenilate cyclase under their natural promoter (Mukaihara and Tamura, 2009). The fact that *awr1* is the only gene of the family placed on the *R. solanacearum* chromosome together with its non-regulation by *hrpB* (Table 6) encouraged us to suspect that it had a different role. However, AWR1 is predicted to be an effector (same as the other members) by the Effective (http://www.effectors.org) software that is a sequence-based

effector prediction platform. Prior to any functional characterization, we wanted first to evaluate full-size AWR protein secretion in *R. solanacearum* GMI1000 to have a direct evidence that AWRs are effector proteins.

Recently, a novel vector system pRC (gateway compatible) for stable gene integration in *R. solanacearum* was developed in our group (Monteiro et al.). Among other interesting features, this versatile vector allows the integration of any gene under any promoter in a permissive chromosome region of the pathogen. The AWR genes were cloned in Gateway entry vectors and transferred by LR site-specific recombination into pRCG-Pep, which harbours a gentamicin resistance cassette, the *eps* promoter and a hemagglutinin tag (3HA) for protein immunodetection or purification. For a vector scheme, see the annex section. The linearised plasmids were introduced into both the wt GMI1000 and the T3S-defficient (*hrcV*$^{-}$) strains by natural transformation. We then selected with gentamicin for targeted integration of awrs in the genome by double recombination. The resulting strains contained each an extra copy of an *awr* gene fused to the 3HA epitope tag and under the control of the *eps* promoter. This enabled us to produce entire AWRs under a strong promoter known to be extremely active at high bacterial densities (Garg et al., 2000) and to verify their T3-dependent secretion directly in *R. solanacearu*m.

Strains expressing AWRs were grown until late exponential phase in secretion inducing medium (minimal medium supplemented with 10 mM glutamate, 10 mM sucrose and 100 µg/ml Congo red). Culture medium was separated from the bacterial fraction, filtered and proteins were concentrated by precipitation with trichloroacetic acid. Both total bacterial extracts and concentrated proteins from the culture medium were then subjected to western blotting using an anti-HA antibody. Distinct band sizes corresponding to the different effectors could be distinguished in cell lysates of both strains (Figure 26). Although full-length AWRs were excellently produced in *R. solanacearum,* the protein was only detected in the medium from the wt strain. This demonstrated the T3S-dependency of the secretion and that cell integrity was not disturbed during the experiment.

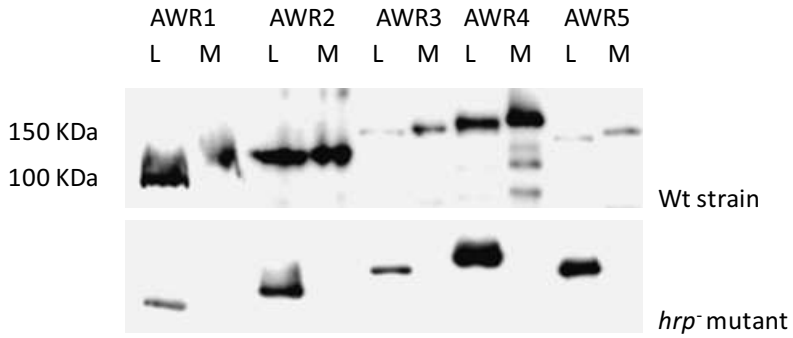


Figure 26. AWRs are translocated to the medium in *R. solanacearum* GMI1000.

All AWRs were detected in the medium fraction of the wt strain but not in the hrp mutant strain. L: bacterial lysate fraction; M: medium of bacterial culture after centrifugation, filtration and precipitation. Western blotting using an anti-HA antibody.

Our studies showed that AWR2, 3, 4 and 5 were injected to the media as described in the literature (Cunnac et al., 2004; Mukaihara and Tamura, 2009) but contrary to what was previously seen, AWR1 was also detected in the medium fraction thus proving its translocation (Figure 26). We demonstrated that this effector can be secreted in a T3-dependent manner under a strong promoter. Assays done so far relied on overexpression of *hrpB* to verify injection, and this is not a reliable system for *awr1,* whose regulation seems to be independent of this regulator. For the other AWRs, our use of the *eps* promoter avoids working with a *hrpB*-overexpressing strain to ensure high effector production levels. We have some explanations for the discrepancy of our results with those previously obtained for AWR1 with the CyaA reporter system. First, a longer N-terminal region might be required for proper secretion, second secretion levels were too low to be detected with the previous system and third, the cyaA fusion could somehow interfere with the translocation process. Other systems could be employed in order to verify AWR1 secretion. An often employed assay is the fusion of effectors to the N-terminal of others that produce an HR in a particular plant (e.g. avrRpt2 or avrBs2) lacking their secretion signal (reviewed in (Nomura and He, 2005)). In this case, an HR will only take place if the tested N-terminal region acts as a translocation signal and brings the chimeric protein to the plant cells. However, this methodology can also fail to detect an effector if expression levels are not high enough to trigger an HR or if the fusion protein is biologically inactive.

Our experiments demonstrate that full-size AWR proteins are secreted into the medium in a T3-dependent manner. Although AWR1 was considered a non-functional effector until now (Mukaihara and Tamura, 2009), our novel data shows at least its secretion to the medium. Nevertheless, its regulation seems to be independent of *hrpB* and the timing or the conditions

for expression (and thus translocation) during infection process may differ from those of the other AWR members.

1.2. AWRs are conserved in various plant and animal pathogens

We then performed a survey of all available sequences in databanks searching for AWR orthologues. Databank comparisons revealed gene and protein homologues with varying degrees of similarity in all strains of *R. solanacearum* for which genome sequences are available but also in other organisms. We confirmed that except for *awr1* (restricted to phylotype 1 strains), the other four genes in the family are present in all *R. solanacearum* strains whose genome has been entirely sequenced (Table 8). This is in agreement with comparative genomic hybridization analyses of 12 representative *R. solanacearum* strains from different phylotpes, where four AWRs were considered core effector proteins, further emphasizing their functional importance (S. Genin unpublished, Guidot et al., 2007). These conserved AWR proteins are present in phylotype I strains (GMI1000, RS1000 and some in OE1-1), II (CFBP2957, IPO1609, Molk2, and UW551), III (CMR15) and IV (PSI07 and AWR1 in *R. syzygii*). The following paralogues did not appear as a hit during the AWR GMI1000 BLAST comparisons (and thus are not included in the list on Table 9): AWR3 (RRSL_00498) and AWR4 (RRSL_00499) in UW551 or AWR2 (RSIPO_03169) and AWR4 (RSIPO_04049) in IPO1609. However, we ascertained their presence in these strains from the sequence information available at the following sites: UW551 (http://vision.biotech.ufl.edu/mycap/jsp/project/description.jsp?projectID=1) (Gabriel et al., 2006) and IPO1609 (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi) genome databases).

Table 8. Summary of all AWR-related proteins found with a BLAST search.

Representative BLAST hits for AWRs (e-value < 0.01 with a sequence coverage ≥ 30% or sequence identity ≥ 20% with higher e-values) are shown in this table (awr2_RsS sequence was obtained from S. Genin). The list contains not only proteins present in *R. solanacearum* but also in other pathogenic bacteria such as *Xanthomonas*, *Burkholderia* or *Acidovorax*. These proteins were the used for phylogenetic tree construction →

Nomenclature	Protein product	Protein size (aa)	Locus tag or gene	Acc number	Organism
awr1_RsG	AWR family protein	1063	RSc2139	NP_520260.1	Rs GMI1000
awr2_RsG	AWR protein 1	1127	RS03024/RSp0099	NP_521660.1	Rs GMI1000
awr3_RsG	AWR family protein	1242	RS05350/RSp0846	NP_522407.1	Rs GMI1000
awr4_RsG	AWR family protein	1330	RS05349/RSp0847	NP_522408.1	Rs GMI1000
awr5_RsG	AWR family protein	1146	RS02361/RSp1024	NP_522585.1	Rs GMI1000
awr2_RsF	type III effector protein AWR2	1084	RCFBP_mp10070/ripA	YP_003747289.1	Rs CFBP2957
awr3_RsF	putative type III effector AWR3	1240	RCFBP_mp20290	YP_003748108.1	Rs CFBP2957
awr5_RsF	AWR5 type III effector protein	1231	RCFBP_mp20473	YP_003748288.1	Rs CFBP2957
pawr_RsF	putative awr type III effector family protein	1337	RCFBP_mp20291	YP_003748109.1	Rs CFBP2957
awr2_RsC	type III effector protein AWR2	1130	CMR15_mp10070/ripA	CBJ39728.1	Rs CMR15
awr3_RsC	putative type III effector AWR3	1233	CMR15_mp10825	CBJ40472.1	Rs CMR15
awr5_RsC	AWR5 type III effector protein	935	CMR15_mp20106	CBJ40681.1	Rs CMR15
pawr_RsC	putative awr type III effector family protein	1273	CMR15_mp10826	CBJ40473.1	Rs CMR15
1pawr_RsI	awr type III effector family protein	731	RSIPO_03901	YP_002257594.1	Rs IPO1609
2pawr_RsI	awr type III effector family protein	507	RSIPO_03902-03903	YP_002257595.1	Rs IPO1609
awr2_RsM	type III effector protein awr1	1127	RSMK05326/ripA	YP_002253123.1	Rs Molk2
1pawr_RsM	awr-related effector (central part) protein	783	RSMK02866	YP_002254962.1	Rs Molk2
2pawr_RsM	awr type III effector family protein	1329	RSMK02862	YP_002254964.1	Rs Molk2
t3e1_RsM	type III protein effector	1213	RSMK00806	YP_002253922.1	Rs Molk2
t3e2_RsM	type III effector protein	1446	RSMK03047	YP_002255080.1	Rs Molk2
1pawr_RsO	probable awr type III effector family protein	1238	Not available	BAH04967.1	Rs OE1-1
2pawr_RsO	probable awr type III effector family protein	1329	Not available	BAH04968.1	Rs OE1-1
awr2_RsP	type III effector protein awr2	1127	RPSI07_mp0069/ripA	YP_003749083.1	Rs PSI07
awr3_RsP	type III effector, awr3	1268	RPSI07_mp0791	YP_003749765.1	Rs PSI07
awr5_RsP	awr5 type III effector protein	1171	RPSI07_mp1022	YP_003749981.1	Rs PSI07
pawr_RsP	awr type III effector family protein	1341	RPSI07_mp0792	YP_003749766.1	Rs PSI07
t3e1_RsR	type III effector protein	1127	hpx31	BAH47283.1	Rs RS1000
t3e2_RsR	type III effector protein	1238	hpx32	BAH47284.1	Rs RS1000
hyp1_RsR	hypothetical protein	1335	hpx4	BAD42384.1	Rs RS1000
hyp2_RsR	hypothetical protein	1156	hpx10	BAD42389.1	Rs RS1000
hyp3_RsR	hypothetical protein	1067	RSc2139	BAH47286.1	Rs RS1000

awr2_RsS	putative type III effector, AWR2	1126	Not available	Not available	*R. syzygii* (Rs complex)
pawr_RsU	AWR family protein	1230	RRSL_01071	ZP_00945871.1	Rs UW551
hyp1_RsU	hypothetical Protein	1127	RRSL_03418	ZP_00944088.1	Rs UW551
hyp2_RsU	hypothetical protein	1212	RRSL_00546	ZP_00946487.1	Rs UW551
hyp_AaA	hypothetical protein	1081	Acav_1072	YP_004233561	Aa subsp. *avenae* ATCC 19860
hyp1_AaC	hypothetical protein	1054	Aave_1090	YP_969460.1	Aa subsp. *citrulli* AAC00-1
hyp2_AaC	hypothetical protein	1453	Aave_2261	YP_970613.1	Aa subsp. *citrulli* AAC00-1
pawr_Bgl	putative awr type III effector family protein	1225	bglu_2g02250	YP_002907931.1	Bgl BGR1
hyp1_Bg	hypothetical protein	1202	BgramDRAFT_0549	ZP_02881740.1	Bg C4D1M
hyp2_Bg	hypothetical protein	855	BgramDRAFT_0575	ZP_02881766.1	Bg C4D1M
hyp1_B1	hypothetical protein	1194	BC1001_6040	YP_004232461	B sp. CCGE1001
pawr_B1	putative awr type III effector family protein	1148	BC1001_6065	YP_004232486.1	B sp. CCGE1001
hyp1_B3	hypothetical protein	1266	BC1003_3626	YP_003908858	B sp. CCGE1003
pawr_B3	putative awr type III effector family protein	1201	BC1003_3651	YP_003908882	B sp. CCGE1003
atp_BpN	ATP/GTP binding protein	1104	BpseN_32135	ZP_02494128.1^	Bp NCTC 13177
hyp_Bp1	hypothetical protein	1083	Bpse112_32691	ZP_02502372.1	Bp 112
hyp_Bp6	conserved hypothetical protein	1269	BURPS1655_I1016	ZP_04890375.1	Bp 1655
hyp_Br	hypothetical protein	1124	RBRH_03012	YP_004022640.1	Br HKI 454
hyp_XaC	hypothetical protein	1388	XAC2009	NP_642335.2*	Xa pv. *citri* 306
hyp_XcC	hypothetical protein	1388	XCC1975	NP_637340.2	Xc pv. *campestris* ATCC 33913
hyp_XcM	hypothetical protein	1388	XcampmN_18808	ZP_06491551.1	Xc pv. *musacearum* NCPPB4381
hyp_XcVa	hypothetical protein	1388	XcampvN_23815	ZP_06487596.1	Xc pv. *vasculorum* NCPPB702
sec_XcV	putative secreted protein	1388	XCV2059	YP_363790.1	Xc pv. *vesicatoria* 85-10
sec_XoO	putative secreted protein	1371	PXO_01041	YP_001913656.1"	Xo pv. *oryzae* PXO99A
hyp_XoOa	hypothetical protein	1388	Xoryp_11725	ZP_02243302.1	Xo pv. *oryzicola* BLS256

* Representative of AAM36871.1

" Representative of AAW75797.1, YP_001903678.1, YP_201182.6, YP_451431.1

^ Representative of ZP_01769189.1, AAK73226.1, YP_001062973.1, YP_001075919.1, YP_111398.1, YP_335571.1, ZP_02407234.1, ZP_02415741.1, ZP_02451819.1, ZP_02459987.1, ZP_02510206.1, ZP_03456824.1, ZP_03794595.1, ZP_04523242.1, ZP_04810151.1, ZP_04893214.1, ZP_04900236.1, ZP_04953541.1, ZP_04969065.1

Rs: *R. solanacearum*; Aa: *Acidovorax avenae*; Bgl: *Burkholderia glumae*; Bg: *B. graminis*; Bp: *B. pseudomallei*; Br: *B. rhizoxinica*; Xa: *Xanthomonas axonopodis*; Xc: *X. campestris*; Xo: *X. oryzae*

Significant similarities outside of the *R. solanacearum* species were only detected with protein sequences and not at the DNA level. This is not unexpected since DNA sequences among distantly related species may differ considerably whereas certain protein regions important for the function retain their sequence. Related proteins were identified by BLAST in other bacterial plant pathogens such as several sequenced *Xanthomonas* strains, a couple of *Acidovorax avenae* strains and some *Burkholderia* species (Table 8). Surprisingly, AWR homologous proteins were also found in the animal pathogen *Burkholderia pseudomallei*. Interestingly enough, amongst AWR homologs in *Xanthomonas oryzae pv. oryzae* is XopZ (named as sec_XoO here), which was recently involved in virulence and inhibition of basal defence (Song and Yang, 2010). However, this protein is quite divergent from those of *R. solanacearum* and is more related to *P. syringae* HopAS1, not present in the tree as it did not appear as a BLAST result of AWRs.

To identify the most conserved regions, all proteins that showed a significant similarity to AWRs (e-value < 0.01 with a sequence coverage ≥ 30% or sequence identity ≥ 20% with higher e-values) were aligned using the MAFFT program (Katoh et al., 2002) and edited with GBlocks (Castresana, 2000). The pairwise similarity of AWR sequences intra- or inter-species were comparable. Sequence conservation extends all along the polypeptide sequence with some highly conserved regions scattered. A domain containing the AWR tripeptide -after which the protein family was named- was especially apparent as remarkably well conserved not only in *Ralstonia* but also in *Burkholderia* species (Figure 27).

Phylogeny relationship between AWR-related proteins

To better understand the relationships between different AWRs, we constructed a phylogenetic tree based on sequence similarities using a Bayesian estimation of phylogenies (MrBAYES) (Figure 27). The tree obtained was rooted in the *Xanthomonas* phylogenetic group since AWR homologues from these γ-proteobacteria seem to be more distantly related to those in the other β-proteobacteria. Interestingly, AWRs from different strains cluster together rather than with the other members from the same strain (Figure 28).

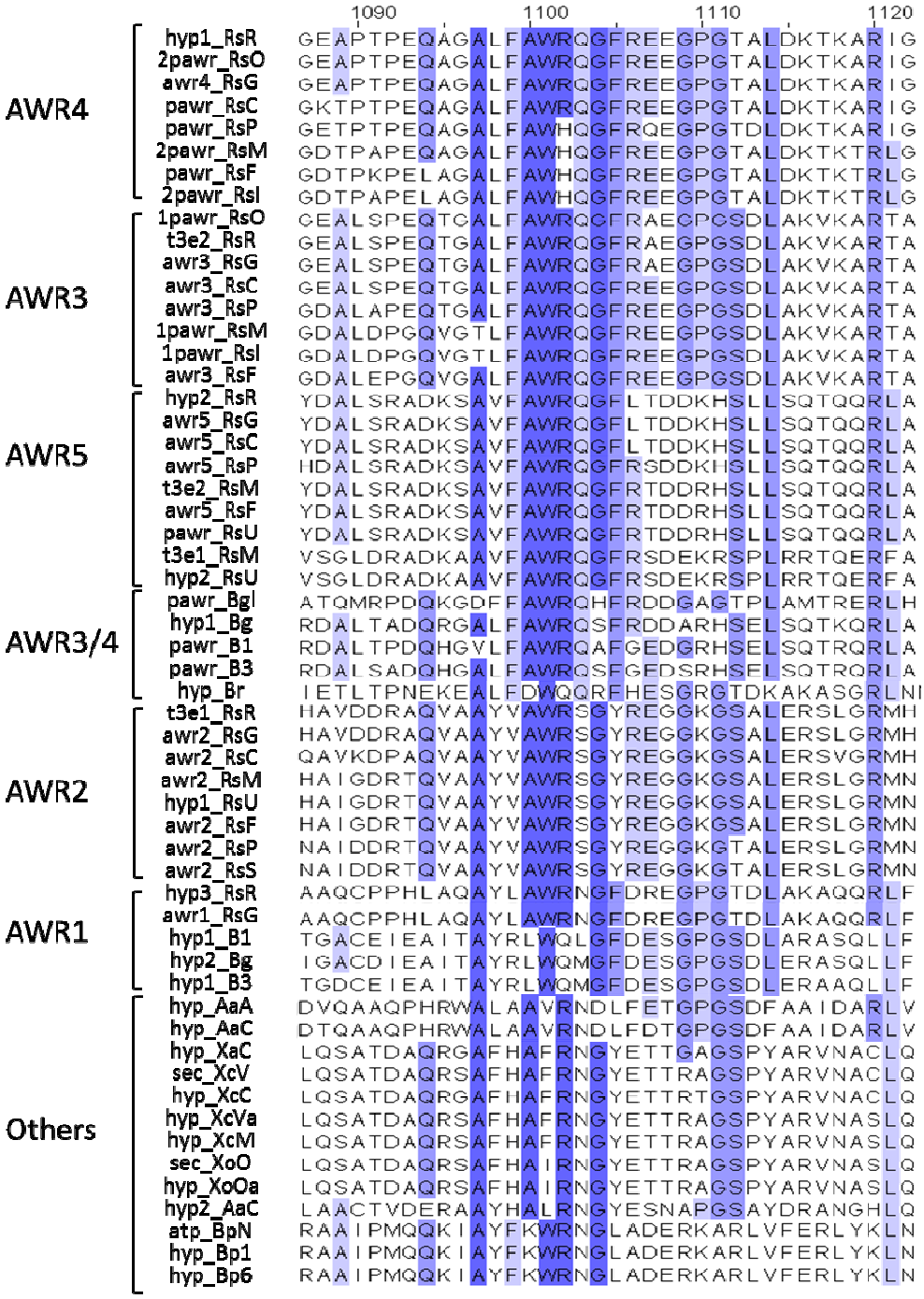


Figure 27.Sequence alignment of a conserved region in AWRs.

All identified protein sequences in databanks were aligned by MAFFT and edited with GBlocks. The blue color intensity indicates the degree of conservation, dark blue corresponding to the highest values.

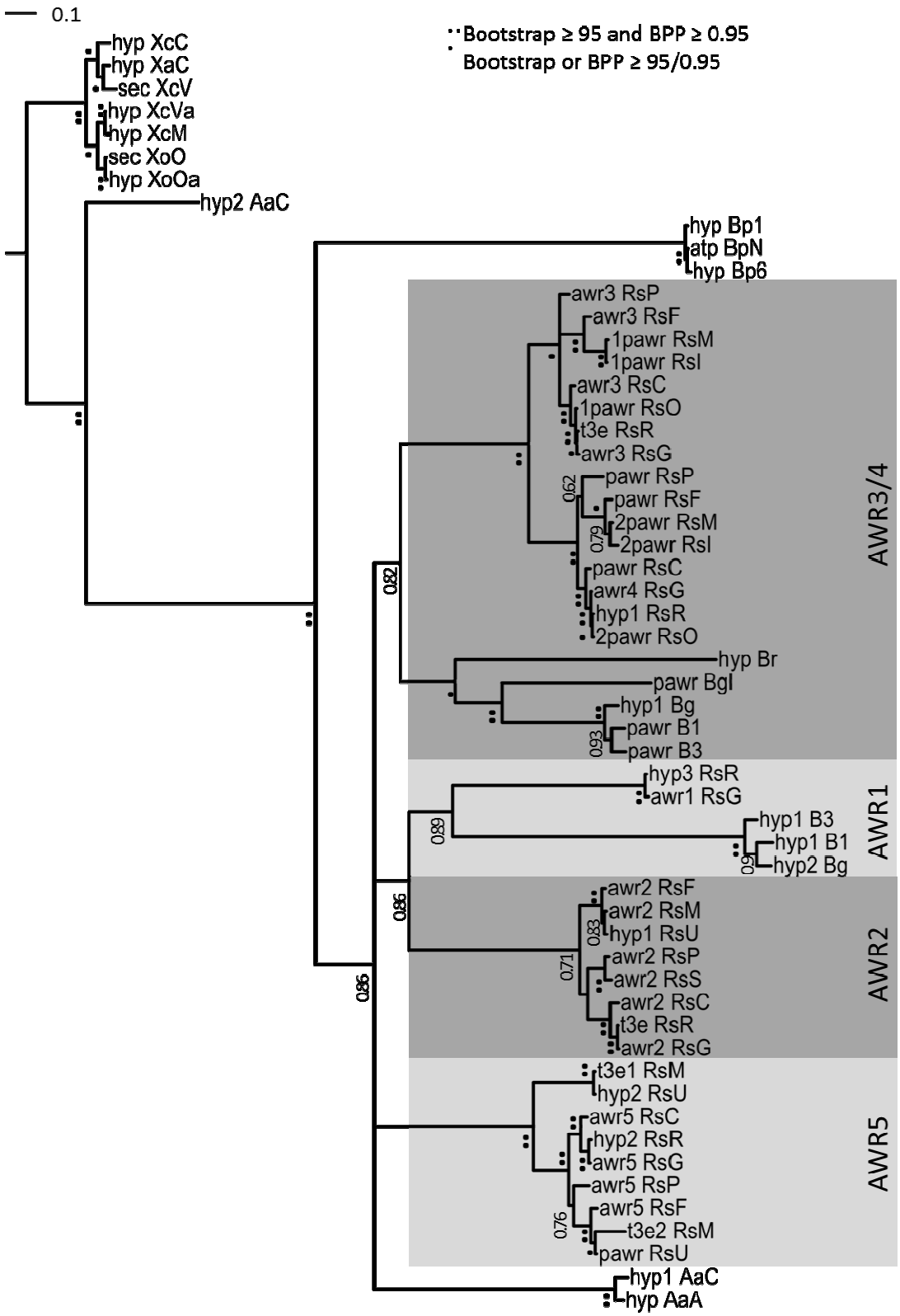


Figure 28. Rooted phylogenetic tree of the *awr* gene family.

The tree was constructed, according to the Bayesian inference, from those sequences obtained by BLAST for each GMI1000 AWR, as specified in the materials and methods. Sequences were named after acronyms of the protein annotation in the original databanks followed the species they belonged to (for more information see Table 9). Numbers in branch nodes indicate the BPP value and sequence divergence corresponds to the branch lengths.

The maximal diversification of AWRs is in *R. solanacearum* strains, whose five proteins appear as distinct branches of the tree. AWR3 and AWR4 appear as twin branches in the phylogenetic tree, which correlates with their high identity and similarity values (previous Table 8). Together with the fact that they are placed side by side in the genome, it is extremely plausible that they recently emerged from a duplication event. In addition, some plant pathogens from the *Burkholderia* genus appear to have an effector closely-related to AWR3/4 ancestor. Interestingly, another effector protein present in *Burkholderia* spp. is AWR1 whose existence was restricted so far to the *R. solanacearum* phylotype I. AWR2 appears as a twin branch for AWR1 and AWR5 is another closely-related protein of this group. Curiously, Molk2 and UW551 strains seem to harbour a second copy of the AWR5 that has been called AWR6 elsewhere (Poueymiro and Genin, 2009). This extra effector is also present in IPO1609 strain (RSIPO_01281), as verified in the *R. solanacearum* database, but not in the other phylotype II strain CFBP 2957. AWR-related proteins were also found, but less represented, in *Acidovorax*. In *A. avenae* pv. *citruli* two AWRs are found, one similar to the group of AWR1, 2 and 5 whereas the other is more related to the *Xanthomonas* group. An ancestor of AWRs is also found in the human pathogen *Burkholderia pseudomallei*. It is very plausible that the AWR present in the *Xanthomonas* generum, where the tree has been rooted, represents a common ancestor of all the family.

Based on all these observations, we propose an evolutionary model (Figure 29), in which the origin of AWR proteins is located before the speciation event between β and γ-proteobacteria (Tayeb et al., 2008; Wu and Eisen, 2008; Naum et al., 2009). From this origin, the gene family might have experienced several duplications and/or deletions in the γ-proteobacteria. The ancestor for *awr*3 and *awr*4 is also present in several *Burkholderia* species and *awr1* might have appeared as a recent duplication of *awr2*. We also propose that two recent horizontal gene transfer events took place from *R. solanacearum* towards other plant pathogens: *awr1* and the *awr2/5* ancestor.The well-known instability of the *R. solanacearum* genome (Guidot et al., 2009) and the fact that *Burkholderia* and *Acidovorax avenae* share the same soil habitat render this gene transfers very likely (Willems et al., 1992; Viallard et al., 1998; Attree and Attree, 2001; Ham et al., 2010). This evolutionary model results in 5 *awr* members in each *Ralstonia* strain, except in UW551 and Molk5, where a probably recent extra duplication in *awr5* gave rise to *awr6.* The model also explains the presence of AWRs related to the ancestral proteins.

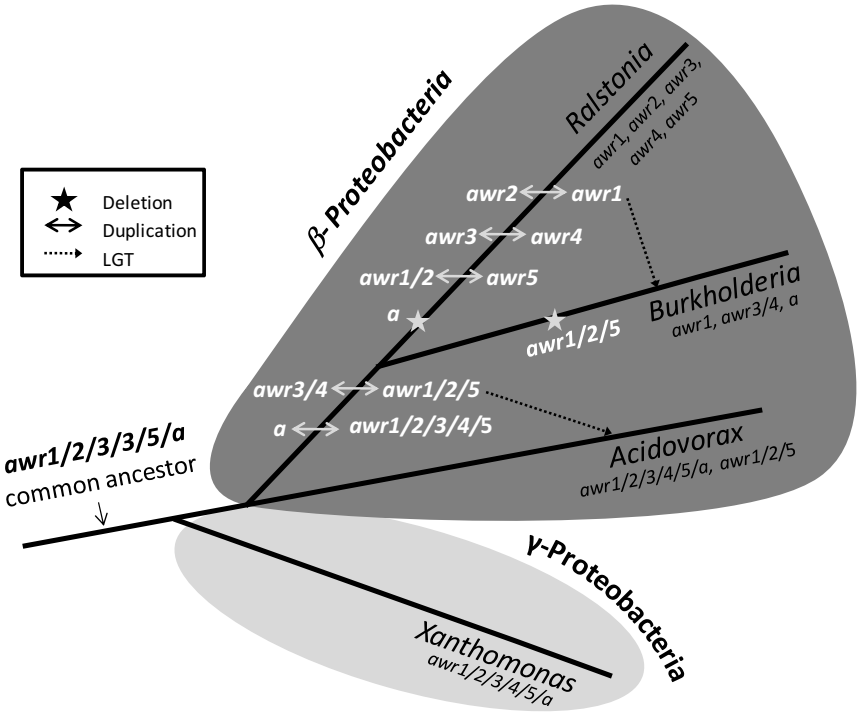


Figure 29.Model of *awr* evolution in the gram negative bacterial lineage.

The model represents the most plausible evolutionary scenario taking into account the phylogenetic relationships amongst bacteria and minimizing the number of gene duplications and losses. *Xanthomonas* and *Acidovorax* species contain the sequence considered more related to the ancestral form. Gene duplications are represented as arrows, losses by stars and putative horizontal gene transfers by discontinuous lines.

Finally, we performed BLAST comparisons of AWR protein or gene sequences to the databanks and no similarity to characterised proteins or motifs with predicted biochemical function were found. Prediction analysis for sequence motifs was performed with INTERPRO Scan software, which analyses different data sources, and no hit was detected. The same result was obtained using consensus sequences derived from the most conserved domains. 3-Dimensional structure predictions using PHYRE were also performed and rendered no statistically relevant hits for any of the AWR proteins.

In summary, *awrs* are conserved genes widespread in *R. solanacearum* and we have identified orthologues in other bacterial pathogens, although the sequence information gives no clue on their putative function.

CHAPTER 2: Fuctional characterization of AWRs *in planta*

CHAPTER 2
FUNCTIONAL CHARACTERIZATION OF AWRS *IN PLANTA*

In the previous chapter we demonstrated that AWRs are a well-conserved effector family within the *R. solanacearum* species complex and also their presence in other pathogens, including the animal pathogen *B. pseudomallei*. This highlights their importance for pathogen infection since otherwise they would have been lost or would carry missense mutations. As mentioned before, a previous study showed that disruption of a single *awr* produced a faint defect in bacterial pathogenicity. For all these reasons, we decided to evaluate the role in virulence of AWRs through gain and loss-of-function studies.

2.1 The AWR effectors jointly contribute to the pathogenicity of *R. solanacearum* GMI1000.

In order to better evaluate the combined contribution of the whole gene family in pathogenicity, we constructed a mutant of *R. solanacearum* GMI1000 devoid of all *awr* family members. To this end, we took advantage of a recently adapted methodology for precise excision of DNA sequences in the *R. solanacearum* genome that allows performing cumulative mutations (Marx and Lidstrom, 2002; Angot et al., 2006). The system is based on the use of the Cre site-specific recombinase. To subsequently delete each AWR, we conducted the following procedure: Firstly, we cloned 1kb regions flanking the ORF to be deleted surrounding the *loxP-Gm^r-loxP* cassette in plasmid pCM351. Secondly, the resulting plasmid was linearised and used to transform *R. solanacearum.* This way, the AWR coding sequence was deleted and replaced by the *loxP-Gm^r-loxP* cassette. Thirdly, the strain was transformed with the unstable plasmid pSG15 that bears the recombinase *cre*. This excised the *Gm^r* antibiotic-resistance marker by site-directed recombination, leaving a precise deletion of the AWR coding sequence. Finally, the pSG15 plasmid was eliminated by growing the strain overnight in rich medium in the absence of antibiotics. The procedure was repeated consecutively, resulting in deletion of all five *awr*s in strain Δ*awr1-5* and each of the intermediate mutants. Unexpectedly, the process turned out to be extremely tedious due to the recurrent appearance after transformation of colonies ill-pigmented or showing unusual morphologies, which made us repeat the transformation steps. These difficulties have been also encountered by other researchers (S. Genin, personal communication) and we interpret them in the light of the known genetic

instability of the bacterium due to high number of mobile elements in the genome (Salanoubat et al., 2002). Once the deletions were obtained, all strains were tested for virulence in the host plants tomato (ecotypes marmande and Hawaii 7996) and eggplant cv *zebrina*. Pathogenicity was evaluated by measuring bacterial growth in the leaves after 3 days of leaf infiltration instead of the traditional watering experiment. This novel test was chosen as it gives a quantitative output on the bacterial growth -and thus it is more reliable than the qualitative wilting test and also because it enabled us to test all the mutants at the same time, as fewer plants are needed, thus reducing the experimental variability. The system is extensively used in the *Pseudomonas* community to assay effect of T3E proteins in bacterial virulence (Sohn et al., 2007). In addition, leaf infiltration had been already validated in *R. solanacearum*, showing a high sensitivity (Macho et al., 2010). To facilitate recovery from plants and and counting of *R. solanacearum* deletion mutants, we used for the assays the intermediate strains in which the gentamicin resistance marker had not yet been excised. As controls, we used the wild type strain GMI1000 also bearing a gentamicin resistance cassette and a non-pathogenic *hrp* mutant. After inoculation of eggplant or tomato leaves, the wt strain multiplied between 3 and 4 logs inside the plant whereas the *hrp* mutant multiplied a maximum of 1.5 log (Figure 30). *R. solanacearum* grew to a similar extent in the host tomato (cv. *marmande*) and eggplant but bacterial growth was lower in the tomato cultivar *Hawaii 7996*, known to be tolerant to the disease in fields (Wang et al., 2000). In both host plants, the virulence of the quintuple *awr* mutant and all its intermediates was significantly reduced compared to the wt strain, except for the Δ*awr3,4* or Δ*awr3-5* in marmande , which is reduced but not statistically significant.

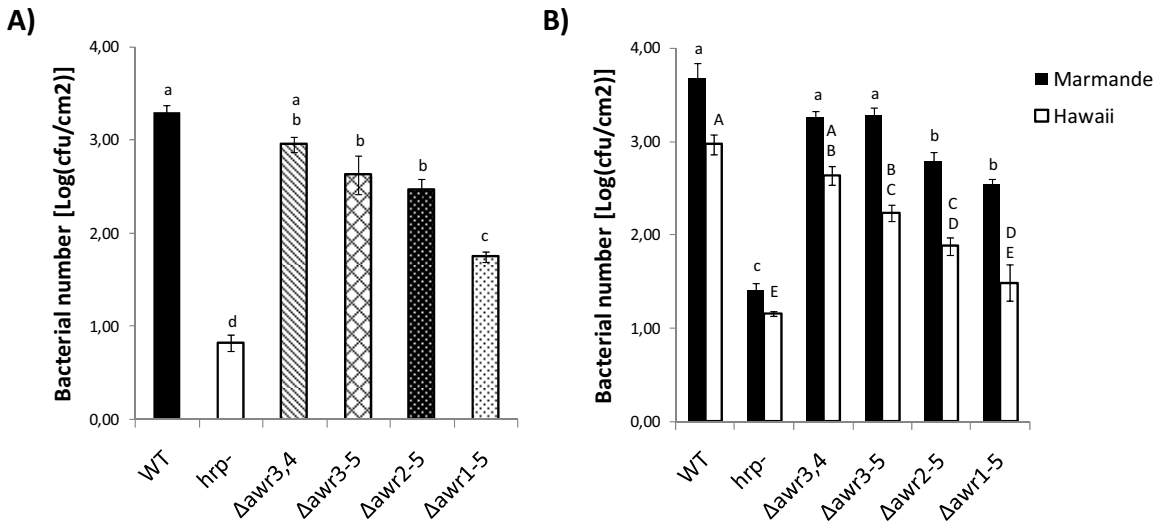


Figure 30. Bacterial growth on host plants.

R. solanacearum mutant strains as well as the control wild-type GMI1000 (wt) and its hrp-defficient counterpart (*hrpG*$^-$) were infiltrated at 10^5 cfu/ml on leaves and recovered three days later (4 days for tomato Hawaii) to monitor bacterial growth. A) *Ralstonia* growth curves in eggplant cv. zebrina. B) *Ralstonia* growth curves in tomato cv. Marmande and Hawaii. Bacterial multiplication is represented as the logarithm of recovered colony forming units per cm^2 with respect to the original inoculum. The values represent the mean of eight biological replicates and their standard errors, which were obtained in two independent assays. Statistically significant groups were calculated using a one-way analysis of variance (ANOVA) and a Tukey test ($P < 0.01$ eggplant; $P < 0.05$ tomato). A (-) sign between numbers indicates that the intermediate *awr* genes are also deleted (i.e. Δ*awr1-5* is the quintuple deletion mutant strain).

Overall, the mutant devoid of all AWRs multiplies 50-fold (log 1.5) less than the wilt type strain (Figure 30), demonstrating their role in virulence. In conclusion, AWR proteins are important for bacterial multiplication *in planta* and it seems that they exert an additive effect as each intermediate mutant tends to multiply less compared to the previous one (Figure 30).

To validate the sensitivity of our *in planta* growth tests to measure the effect of deletions, we tested the single *awr2* mutant, that had been previously proven to show a pathogenicity deffect when interrupted with pCZ367 vector (Cunnac et al., 2004). We added to the test the *Δawr1* and *Δawr5* mutants -the last to be mutated in the quintuple *R. solanacearum* mutant strain- to verify if their single absence was enough to see a significant decrease in virulence. When the effect on virulence of each AWR protein was evaluated separately, no differences were observed. Although strains lacking *awr2* grouped in a less virulent group compared to the other *awr*-depleted strains tested, their growth was not statistically different from the wt strain (Figure 31). Remains to be tested if *awr2* mutant exerts a stronger reduction in virulence when inoculated in tomato, as it was demonstrated in (Cunnac et al., 2004).

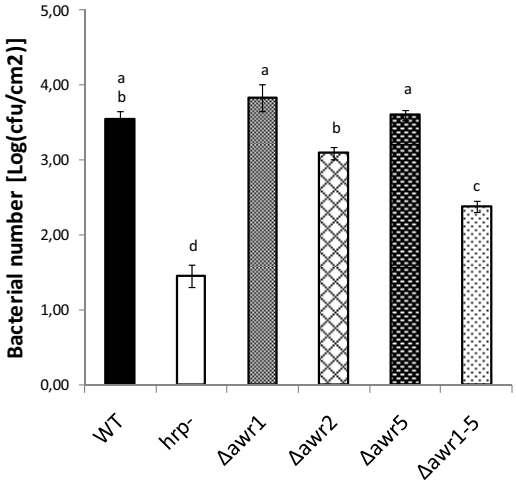


Figure 31. Pathogenicity test performed in eggplant with single-deletion *R. solanacearum* mutants.

Strain GMI1000 (wt harbouring a gentamicin cassette), its hrp-defficient counterpart (*hrpG*$^-$) and the single mutants were infiltrated at 10^5 cfu/ml on eggplant leaves and recovered from the same leaves three days later to monitor bacterial growth. Bacterial multiplication is represented as the logarithm of recovered colony forming units per cm^2 with respect to the original inocula. The values represent the mean of four biological replicates and their standard errors. Statistically significant groups were calculated using a one-way analysis of variance (ANOVA) with a Tukey test ($P < 0.05$). A (-) sign between numbers indicates that the intermediate *awr* genes are also deleted (i.e. Δ*awr1-5* is the quintuple deletion mutant strain).

These results reinforce the idea of an additive effect of T3S effectors on plant infection and colonisation. This is not surprising, as it has already been described in *R. solanacearum* and other pathogens that several effectors need to be mutated in order to visualize some effect on pathogenicity (Cunnac et al., 2004; Kvitko et al., 2009; Hann et al.). It is also interesting to note that the AWR-related effector XopZ in *X. oryzae* pv. *oryzae* has also shown to affect the capacity to cause disease (Song and Yang, 2010).

We finally wanted to test that the reduction in pathogenicity was not due to a general defect on bacterial growth caused by awr disruption. To verify that he absence of *awr* genes did not alter the growth fitness of the pathogen, we grew all strains in artificial culture media and performed growth curves starting from bacteria diluted at 0,1 OD_{600}. The results in Figure 32 confirmed that *awr* mutations did not substantially alter bacterial growth.

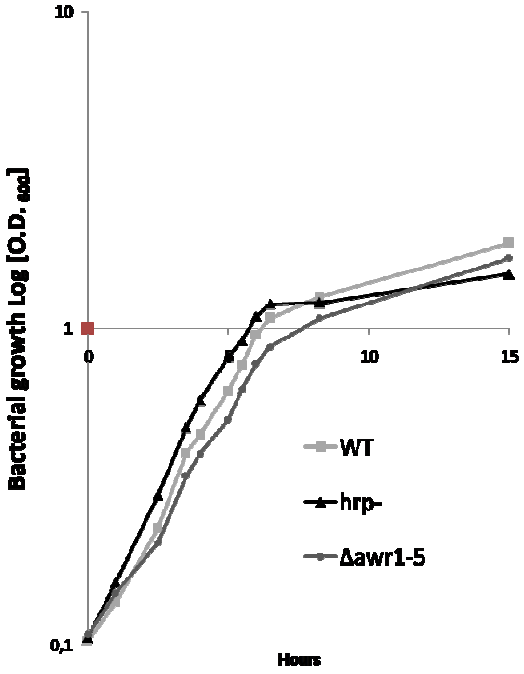


Figure 32. Bacterial growth of *R. solanacearum* mutant strains grown in rich medium.

R. solanacearum mutant strains were grown in rich media for 15 hours until late log phase and bacterial growth was monitored by checking absorbance at 600 nm. No differences on the growth slope were observed among different strains tested.

2.2. AWR effectors might be recognised in *Arabidopsis thaliana* Col-0 plants.

To complement our study on hosts plants, we next sought to analyse the effect of AWRs in the model plant *A. thaliana*.

***R. solanacearum* devoid of *awrs* multiplies faster in Col-0 plants**

The *R. solanacearum* strain devoid of four *awrs* was also infiltrated in *Arabidopsis thaliana* Col-0 leaves and bacterial growth was monitored after 3 days. Surprisingly, the absence of AWRs seemed to render the bacteria more virulent when assayed in this host (data not shown), however, multiplication of the wild type was extremely limited in Col-0, rendering the tests not very reliable. These results were thus confirmed using a classical plant wilting pathogenicity test. Indeed, both the strain lacking 4 or 5 *awr* genes showed increased virulence compared to the wild strain, advancing symptom appearance for about 2 days (Figure 33). Already 1 day after inoculation with the *Δawr1-5* strain, almost all *A. thaliana* plants showed wilting symptoms whereas only few inoculated with the wt strain started wilting.

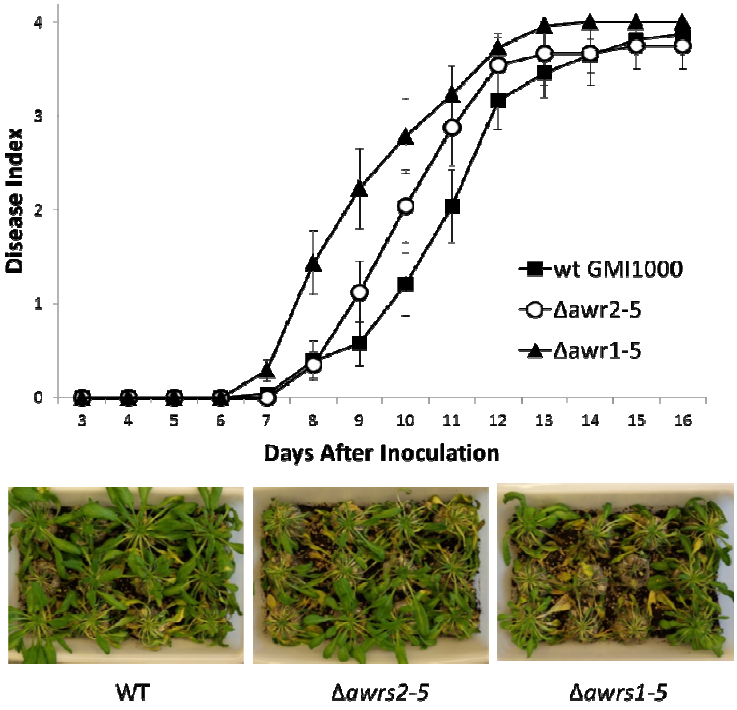


Figure 33. Pathogenicity test on Col-0 plants with the *R. solanacearum* strains devoid of AWRs.

R. solanacearum Δawr2-5 and *Δawr1-5* multiple mutant strains, together with the control strain GMI1000 (wt) were root-inoculated at 10^8cfu/ml in *A. thaliana* col-0 plants and disease progression was annotated daily according to wilting symptoms appearance: no wilting (0), 25% wilted leaves (1), 50% (2), 75% (3) and dead plant (4). Values represent mean of 12 biological replicas and their standard error. Pictures were taken at 10 days post-infection. This assay was repeated twice with similar results.

AWRs are heterologously expressed in *P syringae* strains.

In order to better characterize the effect of AWR proteins on bacterial virulence, a gain- of-function assay was performed by introducing *awr* genes in the wt *P. syringae* DC3000, an *Arabidopsis* pathogen naturally lacking them. For heterologous expression in this pathogen, we used the pEDV6 gateway-compatible vector, that enables the expression of any effector in fusion with the N-terminal of AvrRPS4 encoding the required T3-secretion signal for *Pseudomonas* (K. Sohn, unpublished; Sohn et al., 2007). Subsequently, *in planta* processing of the protein should take place due to the presence of a natural cleavage signal at the end of AvrRPS4 protein which releases the HA-tagged effector protein to be studied (Sohn et al., 2009). Some AWRs were produced with this system in both wt *P. syringae* DC3000 and the DeltaCEL mutant strain. This mutant strain lacks some important effectors for pathogenicity due to the deletion of six ORFs, what makes it less virulent than DC3000 (Badel et al., 2003; Badel et al., 2006). As this strain is less virulent, it is easier to monitor subtle effects of *Ralstonia* effectors, as some important *Pseudomonas* effectors would not mask their effect.

All AWR-expressing and the control strains used, except for the *hrp* mutant strain, were still able to trigger an HR in *N. benthamiana* plants. This proves that the derived strains contain a functional type III secretion system. To evaluate the correct expression of awr genes in *P. syringae*, we used AWR5 as a representative to check protein production. Bacterial cells were grown in *hrp*-inducing media until late log phase and the presence of AvrRPS4N-HA-AWR5 in bacterial lysates and media fraction was evaluated by western-blot (Figure 34). As it is known that the *Pseudomonas* system is much less efficient with proteins of a high molecular weight (S. Rivas, personal communication), we also used TPS, another *R. solanacearum* effector that is much smaller than AWRs (557 versus 1000-1300 aminoacids, respectively) as a control. In all cases, this effector protein was well produced in both DC3000 (3) and DeltaCEL (CEL) strains (Figure 34). It was even possible to detect it in culture media from the latter strain.

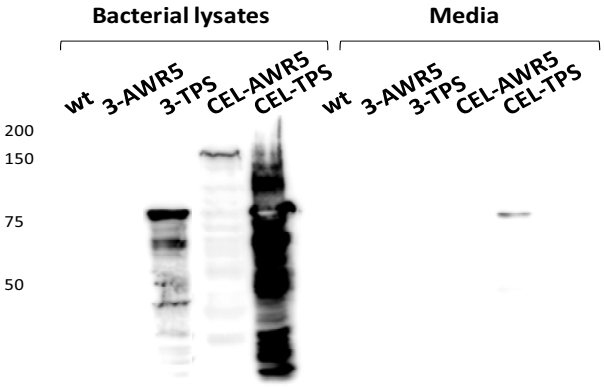


Figure 34. Production and secretion of AvrRPS4N-HA-fused *R. solanacearum* effector proteins in *P. syringae* strains.

AWR5 and the *R. solanacearum* effector TPS (used as a control) were expressed in *P. syringae* wt DC3000 strain (3) or the DeltaCEL mutant strain (CEL) and samples were subjected to an anti-HA western-blot analysis. Lysate: bacterial fraction; medium: supernatant of bacterial culture after centrifugation, filtration and precipitation.

As expected, the AWR5 effector was much less abundant and only detectable in the DeltaCEL strain, where protein production is somehow enhanced in all cases. However, we were unable to visualise secreted AWRs from any strain. Even the well-produced TPS in DC3000 cells, was undetected in the medium fraction. This shows that a huge protein production is required in order to visualise its secretion to the culture. We thus assume that AWRs are correctly translocated to the medium in spite of being undetectable by western blotting.

***P. syringae* expressing AWRs multiplies to a lesser extent than wt bacteria.**

We used AWR heterologous expression *in P. syringae* to validate the role of *R. solanacearum* effectors in another pathosystem. *awrs* were expressed by means of the pEDV6 system and bacterial solutions were inoculated on *A. thaliana* leaves (Sohn et al., 2007). *P. syringae*

DC3000 heterologously expressing different AWRs, together with the wt strain carrying an empty version of the vector and a *hrp*- strain, were inoculated at 5 x 10^5 cfu/ml to *A. thaliana* leaves and bacterial growth was evaluated after 3 days. As seen in Figure 35, expression of the effectors in the novel pathosystem caused a restriction in bacterial multiplication.

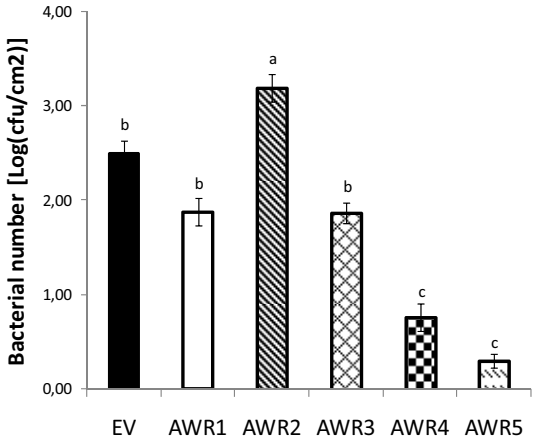


Figure 35. Bacterial growth assay in Col-0 plants with the *P. syringae* DC3000 expressing AWRs.

The strain DC3000 bearing a pEDV3 empty vector (EV), its *hrp*-defficient counterpart (*hrcC*) and the strains that heterologously express the AWRs were inoculated at 5 x 10^5 cfu/ml on leaves and recovered three days later to monitor bacterial growth. Bacterial multiplication is represented as the logarithm of recovered colony forming units per cm^2 with respect to the original inocula. The values represent the mean of four biological replicates. Statistically significant groups were calculated using a one-way analysis of variance (ANOVA) with a Tukey test ($P<0.01$). Experiment was repeated three times with similar results.

Our results show that bacterial growth was particularly reduced by AWR4 and AWR5, whereas strains expressing AWR2 exhibited increased fitness *in* planta. This correlates with the reduction in growth for the *awr2*-depleted *R. solanacearum* strain when inoculated in eggplant or in previous experiments done in tomato (Cunnac et al., 2004). These findings establish that, in addition to their role in virulence on natural hosts, some AWRs also play a role in triggering specific plant responses. Similar results for AWRs were obtained when the DC3000 DeltaCEL strain was used (data not shown). Moreover, when Col-0 plants were inoculated with wt DeltaCEL strain at 5 x 10^6 cfu/ml, disease symptoms were observed and clearly less apparent when inoculated with the AWR5-expressing strain (Figure 36).

Figure 36. DeltaCEL strain expressing AWR5 is less virulent than wt strain

The strain DeltaCEL that bears a pEDV3 empty vector (EV) and and the strain that heterologously express the AWR5 were inoculated at 5 x 10^6 cfu/ml on leaves and symptom development was observed. Pictures were taken at 4 days after inoculation.

The fact that AWR cause different responses on tomato or eggplant compared to the ones in Col-0 is not difficult to understand as *A. thaliana* is not a natural host for *R. solanacearum*. Other studies have shown disparate responses on *Arabidopsis* and tomato plants (Lin et al., 2008; Milling et al., 2011). Actually, *A. thaliana* has been an important source of plant resistances towards pathogens and its study has led to the discovery of the unique R-protein that confers resistance to *R. solanacearum* (RSS1) upon PopP2 effector detection (Tasset et al., 2010).

Effect of AWRs on callose deposition

It was known that disease symptoms of plants inoculated with the *P. syringae* DC3000 DeltaCEL strain were abolished, without affecting function of the T3SS. This decrease in virulence could facilitate the detection of putative effects of awr effectors on *A. thaliana* plants. HopM1 is one of the deleted genes in the *P. syringae* DeltaCEL strain and, due its absence, the bacterial strain can no longer suppress the plant callose deposition that is triggered upon bacterial contact as part of the plant basal defences (Badel et al., 2003; DebRoy et al., 2004). It is known that callose plays an important role in plant immunity and its quantification after pathogen challenge has emerged as a common system to quantify plant defence responses (Luna et al., 2011). We wanted to evaluate if any AWR was able to restore callose suppression in Col-0, which would indicate an interference with plant basal defences. *A. thaliana* plants were inoculated with DC3000 expressing the different *awrs* or control genes at 10^8 cfu / ml and after 12-14 hours, leaf samples were harvested, stained and visualized under the microscope in order to determine callose depositions. Water-infiltrated leaves were processed in the same way and used as a blank control. As expected, water treatment did not

induce any callose deposition while the wt DeltaCEL strain and the *hrp* mutant caused a strong production (Figure 37). Callose suppression of PstΔCEL was completely restored when bacteria were harbouring the pORF43 plasmid expressing HopM1 and its chaperone SchM as had been described (DebRoy et al., 2004). Among all *R. solanacearum* effectors tested, AWR5 triggered a high callose response and was hence functionally complementing HopM1-mediated callose suppression. This demonstrates a role of this effector in virulence on Col-0 plants when expressed in *P. syringae*. This result is conflicting with the bacterial growth experiments because if we suppose that AWR5 is being recognised by the plant cell we should expect an increase in callose deposits, not the contrary. The reduction in callose deposit due to AWR5 expression does not correlate with bacterial multiplication because *hrcC* strain is almost dying inside the Col-0 plants and it is triggering higher deposition levels. It is known that callose deposits depend on many factors such as the elicitor employed, the physiological state of the plant or environmental conditions (Luna et al., 2011). Therefore more analyses are required to decipher which are the events behind this callose suppression.

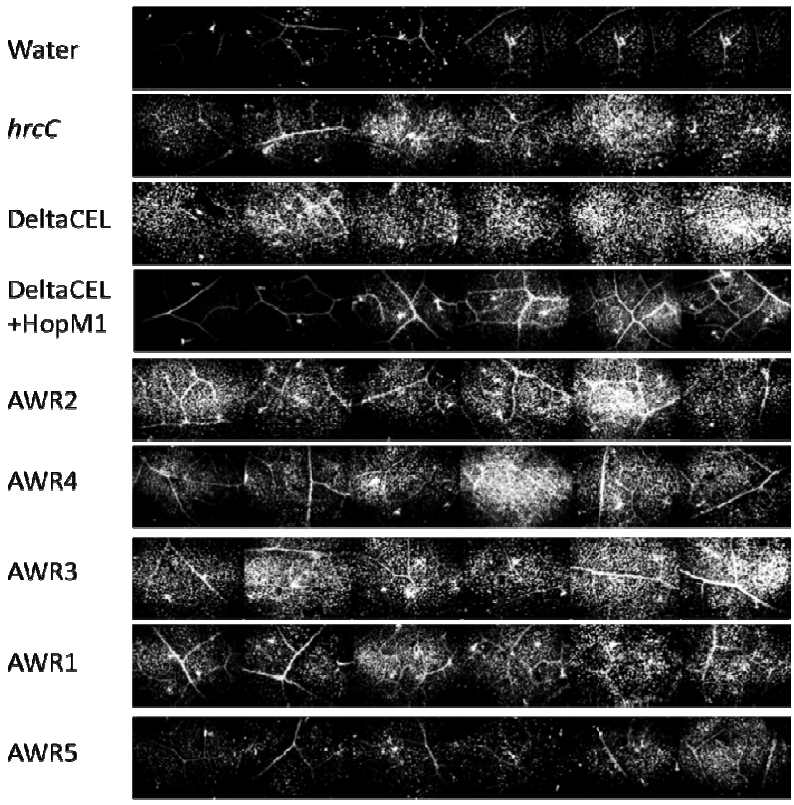


Figure 37. Callose depositions caused by inoculation of *P. syringae* on *A. thaliana*

A. thaliana Col-0 plants expressing effectors from the pORF43 plasmid were inoculated at 10^8 cfu/ml and 12 h later callose deposits were stained and visualised under a microscope (white dots). For each assayed construction, six replicates from different leaves are shown.

2.3. Transient expression of *awrs* in nonhost plants causes different levels of necrosis

Heterologous expression of bacterial effectors *in planta* and observation of the resulting phenotype provides important clues on their function but also about the plant defence through effector-triggered immunity, since there are no confounding effects coming from the activities of other secreted effectors (Wroblewski et al., 2009). As AWRs showed some level of recognition by *A. thaliana*, we decided to determine their impact on nonhost tobacco plants.

AWR expression in non-host plants triggers necrosis

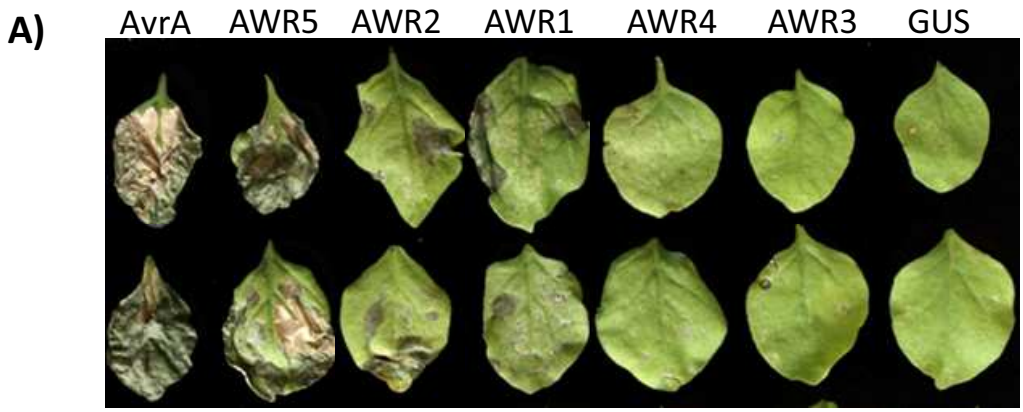
As an approach to study the function of AWR bacterial proteins and their putative recognition in some plants, we expressed these genes *in planta*. We screened for macroscopic phenotypes caused by transient *Agrobacterium*-mediated over-expression in the non-host plants *N. benthamiana, N. tabacum* and *N. glutinosa*. It was clearly apparent already at 24 hours after agroinfiltration that AWR5 caused a marked necrosis on *N. benthamiana* leaves. This phenotype involved an extensive cell death accompanied by a brown or black coloration and a later collapse of the leaf tissue (Figure 38A). The effector proteins AWR1 and AWR2 produced a similar but milder and less reproducible phenotype (Figure 38A). Leaves expressing the remaining AWRs 3 and 4 were indistinguishable from the reference leaves, expressing the beta-glucuronidase (*GUS*) gene under the same system. The rapid onset and the extent of the necrosis caused by AWR5 were reminiscent of that caused by the AvrA effector, known to lead to a hypersensitive response (HR) in *Nicotiana spp.* (Figure 38A left) (Poueymiro et al., 2009). The results shown were obtained with the effectors expressed from vector pAMPAT fused to YFPv, but same results were obtained when HA-tagged AWRs were expressed under the estradiol-inducible vector pER8 (not shown). Expression and toxicity under the inducible promoter was easier to monitor and was preferentially used in subsequent experiments.

Expression of all fusion proteins in agroinfiltrated leave samples was checked by western blot using an anti-HA antibody (Figure 38B). As shown in Figure 38B, a band corresponding to the expected full-size proteins was detected for all constructs 6-8h post-induction, minor degradation fragments being sometimes apparent. In a time-course experiment, it was found that bacterial proteins were detected *in planta* already 4h after induction; although larger amounts were produced at 24 hours post induction (not shown).

The strong impact of AWR5 expression on tobacco prompted us to investigate which domain in the protein was responsible for the phenotype. To this end, we cloned this effector in 3 gene fragments encoding the N- or C-terminal halves of the protein or the central region

overlapping them, and expressed these sequences in *N. benthamiana* and *N. tabacum.* Repeated transient expression assays for these constructs showed no visible phenotype (data not shown), revealing that the full-length protein seems to be needed for correct function or folding and thus to trigger plant responses.

To verify the phenotypes caused by AWRs and check their plant specificity we evaluated them in two other non-host tobacco species. The results are presented in a semi-quantitative scale in Figure 38C. The phenotypes caused by AWR5 and AWR2 were apparent in all plant backgrounds (if not stronger), and there was a degree of variation for AWR1 and AWR4, the former causing no phenotype on *N. glutinosa* and the latter causing a mild necrosis only in this plant (Figure 38C). AWR3 had no macroscopic effect in any case, in spite of being clearly detected by western blot (Figure 38B).



	Non-host: necrosis levels		
	N. benthamiana	*N. glutinosa*	*N. tabaccum*
AWR5	++	++	++
AWR2	+/-	+	+/-
AWR1	+/-	-	NA
AWR3	-	NA	NA
AWR4	-	+/-	-

Figure 38. Effects of AWR transient expression on *Nicotiana* plants.

A) Photograph of representative *N. benthamiana* leaves expressing AWRs or control proteins. Transient expression was performed using *Agrobacterium tumefaciens* as a vector to transfer constructs from pAMP-PAT-derived plasmids. Pictures were taken 4 days after inoculation. The experiment was repeated 4 times with the same results. B) Immunoblotting of proteins transiently produced in *N. benthamiana* under an estradiol-inducible promoter. HA-tagged proteins from total leaf extracts obtained at 6-8h post-induction were immunodetected using an anti-HA antibody. C) Summary chart of the phenotypes caused by transient expression of AWRs in *Nicotiana* plants. Average phenotypes observed from eight independent leaves are shown in a semi-quantitative scale.

The diverse necrosis caused by different AWRs is surprising taking into account the high similarity at the protein level. It is worth to mention that the AWRs displaying a stronger impact on plant physiology are those causing a major growth restriction when expressed in *P. syringae*. However, disparate effects in gain-of-function experiments *in planta* have already been described for other *R. solanacearum* effector families (Wroblewski et al., 2009). A recent report where effector candidates from *X. campestris*, *P. syringae* and *R. solanacearum* were transiently overexpressed in various plant accessions demonstrated that none of the effectors tested was able to cause an effect in all the accessions evaluated but at least one third of the effectors tested produced an effect on the leaves of one accession or more. Most apparent reactions were seen in non-host plants and ranged from chlorosis to cell death in the infiltrated area. In the same way, a plant accession was able to react to more than one effector from the different pathogens tested. Noteworthy, when avirulence effectors were assayed, they produced effects on different accessions and necrosis prevalence was higher than those that have not been described as avirulence genes (Wroblewski et al., 2009). According to the authors, cell death production could be due to high levels of effector production and hence associated with their virulence activity or due to HR event. In favour of the last hypothesis was that 80% of the necroses observed were produced by previously identified avirulence proteins (or their homologues in other species). However, recent publications discuss whether this HR might be a cause or a consequence of cascade signalling downstream of effector recognition (Coll et al., 2011). For all this, our results could be pointing out a probable AWR recognition in non-host plants, at least for AWR5.

2.4. The AWR effector AWR5 causes a hypersensitive response when transiently expressed in tobacco.

To further determine if the cell death observed after AWR5 production *in planta* corresponded to a hypersensitive response, we performed parallel experiments with this effector or the control AvrA expressed at various levels. Serial infiltrations of different concentrations of *Agrobacterium* inocula were performed on *N. tabacum* leaves, because the phenotypes were sharper in this plant and appeared earlier in time. As can be observed in Figure 39 (left image), both effectors generated lesions to the same extent (i.e. an identical inoculation threshold) and with the same timing (< 24 h). This indicates that AvrA and AWR5 equally impact the host physiology.

To further compare both phenotypes, we stained infiltrated leaves with trypan blue, which is a specific dye for dead cells that turn dark blue and has been employed to monitor incompatible

reactions between plant and pathogens (Keogh et al., 1980). Furthermore, some leaves were also stained with DAB which is a specific substance that reacts with H_20_2 deposits giving a brown coloration (Thordal-Christensen et al., 1997). Oxidative burst is an important process in plant defences and it is considered as a hallmark for pathogen recognition (Torres, 2010; Vellosillo et al., 2010). According to the literature, the results obtained confirm that AWR2 and AWR5 trigger a cell-death phenotype upon expression in *N. benthamiana* plants (Figure 39A, central image) and both effectors induce a high H_20_2 burst, even higher than the recognised effector AvrA (Figure 39A, right image). Thus, AWR2 and AWR5 in non-host plants seem to imply the recognition of these effectors by the plant surveillance system with the ensuing death of the tissue through a rapid necrosis and enhanced ROS activity, resembling a hypersensitive response (Torres, 2010).

To better ascertain the nature of the macroscopic phenotypes caused by AWRs, we sought to evaluate the expression of specific *N. benthamiana* defence- or HR- induced genes through quantitative reverse transcription-mediated real time PCR. Few years ago, *HSR203j* appeared as a good marker gene for programme cell death for incompatible interactions between *R. solanacearum* and *N. benthamiana* plants. Its expression was enhanced in GMI1000-inoculated plants (incompatible host) whereas it remained unvariable for K60-inoculated *N. benthamiana* plants (compatible reaction) (Pontier et al., 1998) and it has been previously demonstrated its role in HR production (Tronchet et al., 2001). Similarly, the *HIN1* gene expression was restricted to the inoculation area of the pathogen and was not enhanced in surrounding tissues (contrary to what was seen for the defence-related genes PR1a and PR1b) (Kanda et al., 2003; Kiba et al., 2003). Both genes are responsive to the spermine class of polyamines that are important in coping with biotic stresses (Takahashi et al., 2004; Jang et al., 2009). For instance, HIN1 protein is the homologue of the *Arabidopsis* NDR1 protein which is required for R-mediated resistances (Gopalan et al., 1996). Hence, the analysis of the timing of both gene expressions could provide hints on the nature of the interaction.

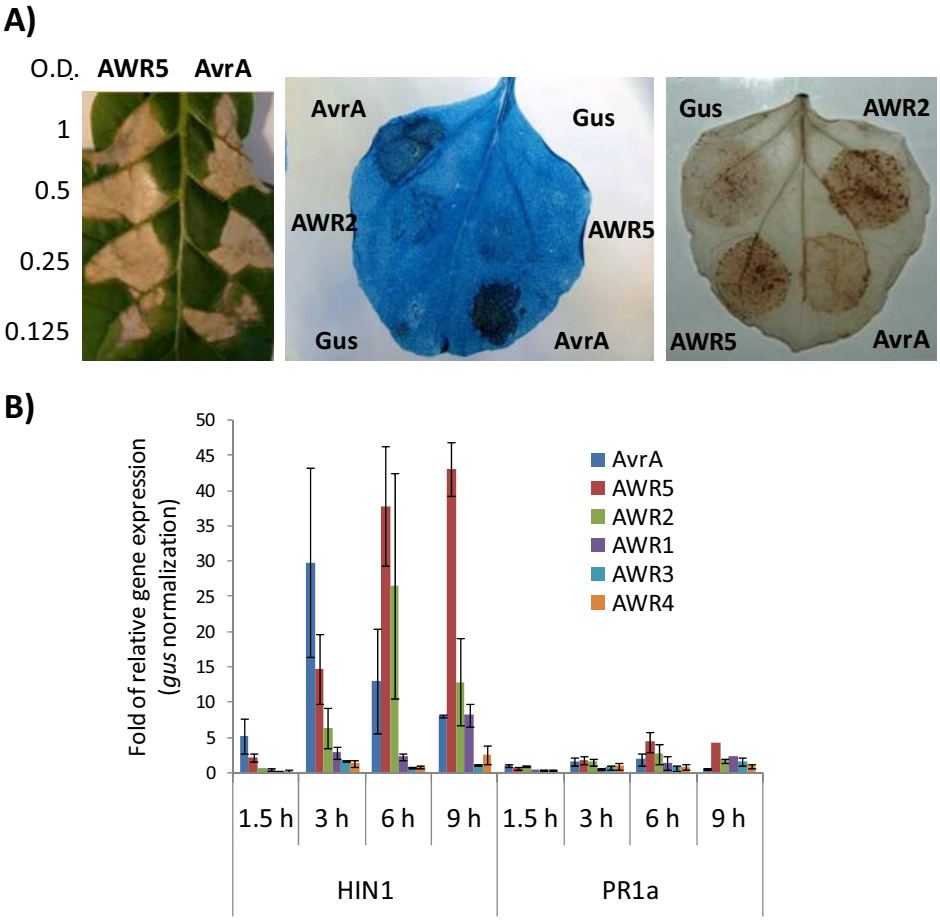


Figure 39. The cell death phenotype caused by AWR5 is comparable to a hypersensitive response

Assays performed with *Nicotiana* plants expressing AWR proteins in order to decipher if necrosis was an HR-like phenotype. A) AWR5 and AvrA were agroinfiltrated at different concentrations in *N. tabacum* (left) and phenotypes were completely comparable indicating a similar behaviour. Trypan blue staining of dead cells was performed in leaves expressing AWRs and control genes at 26 h post-induction (central) showing a bluer coloration for AWR4 and AWR5 compared to the negative GUS control. Agroinfiltrated leaves were also stained at 8 h post-induction with DAB product which is specific for H_2O_2 production. AWR4 and AWR5 induced high levels of H_2O_2 production, even higher than the AvrA control (right). All Assays were repeated with similar results. B) AWR5 triggers an early induction of specific HR plant markers. Expression of Hin1 (an HR marker) and PR1a (a defense marker) was evaluated from RNA samples extracted from *N. benthamiana* at different time points. Gene expression was normalized by the housekeeping gene tubulin and represented as fold-induction with respect to basal levels in control samples transiently expressing the *gus* gene. Values shown are the mean of two biological replicates and their standard error.

We analysed then the expression profiles of the HR-marker genes and the defence-related genes when AWRs where expressed in *N. benthamiana* plants. These experiments showed a marked increase in transcript levels of the HR-related gene *HIN1*, especially at 12h post induction (Figure 39B, left chart). Whereas some AWRs did not alter HR marker transcripts, others dramatically increased them giving an analogous response to that caused by heterologous expression of the AvrA effector known to cause an HR (Carney and Denny, 1990). AWR5 showed the strongest effect at early time points while AWR2 and AWR1 produced a less

apparent and late response which suggested a delayed plant response. This observation correlates with the phenotypes observed *in planta* as these two effectors cause a mild HR-like phenotype on tobacco. As expected, transient expression of the GUS or the AWR effectors causing no phenotype (AWR3 and AWR4) had no impact on the HR- responsive genes (Figure 39B, left chart). As a control, expression of the *Pr1a* defence gene was slightly increased again for AWR1, 2 and 5 (<2-fold changes compared to AvrA) and remain unchanged for any of the others (Figure 39B right chart). For AWR2, AWR5 and the control strains, RNA levels of other markers for HR (*HRS203j*) or disease response (*Pr1b*) were also checked, giving the same results to those obtained for *HIN1* and *Pr1a* (data not shown).

We thus interpret that the hypersensitive response only appears when an expression threshold of marker genes is reached. This threshold implies a minimum transcript level at a certain time (approx. 30-fold before 6 hours for *HIN1*), so that lower levels or even these levels but at later times are not sufficient to mount a proper programmed cell death response in the studied system. Along the same line, it has already been described that common genes are involved in both compatible and incompatible responses against pathogens, being the differential timing what brings about an outcome or the other (Tao et al., 2003). Taken together, our phenotypic and molecular analyses prove that some AWRs can trigger an HR response when overexpressed in *Nicotiana spp.,* a logical reaction considering that these species are non-host to the GMI1000 strain and the similar effects of other identified avirulence genes (which can be exemplified with AvrA) (Wroblewski et al., 2009) .

In an attempt to get clues on events that lead to the AWR-caused necrosis, the plant cell compartment they target was checked. Agroinfiltrated *N. benthamiana* leaves that transiently expressed the YFPv gene fusions were removed and observed under confocal microscopy. It can be clearly seen in the pictures presented in Figure 40 that fluorescence from all effector fusions is evenly localised in the cytoplasm and/or membrane compartment. In contrast to the YFP protein alone, which is partially retained in the cell nucleus, none of the effectors targets this compartment. Photographs were taken at short intervals post induction, as for some constructs collapse of the cell structures started to be apparent after 24-48 hours.

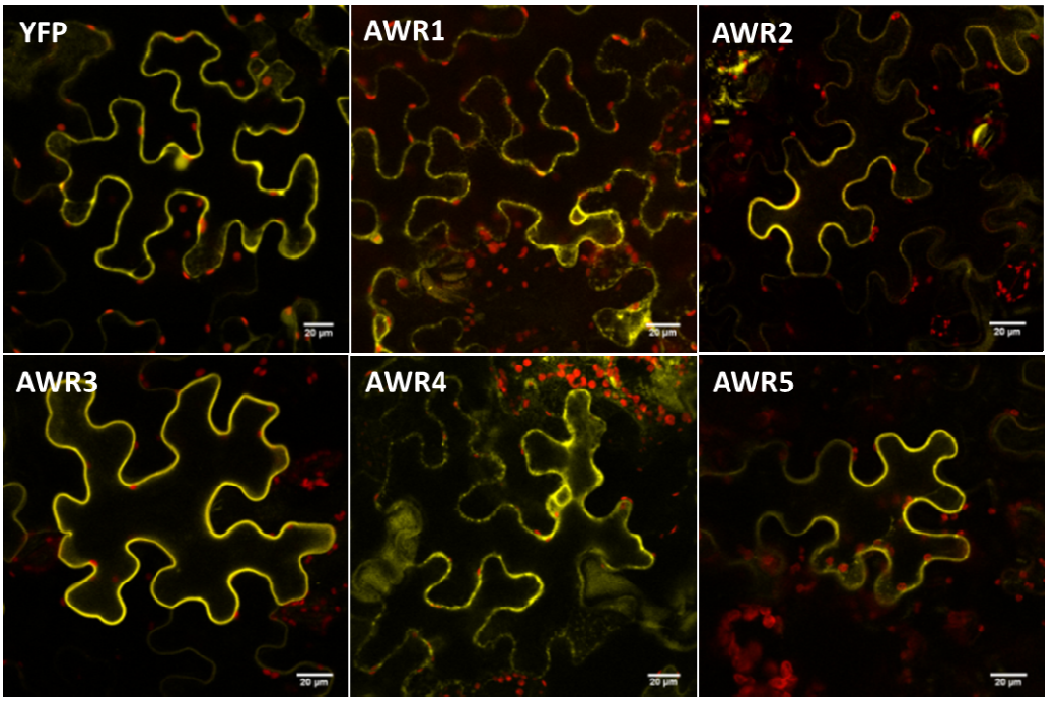


Figure 40. AWR subcellular localization in *N. benthamiana*.

Fluorescence confocal microscopy images of the 525-550 nm (AWR-YFPv) or 610-700 nm (chloroplasts) spectra were obtained after excitation with a 514 nm light. Pictures of representative cells expressing the bacterial effectors taken at 8 hours post-induction are shown. As a reference, notice the cytoplasmatic and nuclear localization of the control YFP protein. Bars correspond to 20 µm.

CHAPTER 3: AWR characterization in yeast

CHAPTER 3:
AWR CHARACTERIZATION IN *S. CEREVISIAE*

In the previous chapter, we demonstrated that AWRs are important effectors for *R. solanacearum* virulence but may also be detected somehow by the plant surveillance systems. As for most effectors, the biochemical function and/or their plant targets of AWR in both compatible and incompatible interactions still remained elusive. Increasing evidences show that effectors interfere with key cellular processes in the host, which might be conserved among eukaryotes (Yoon et al., 2003; Kramer et al., 2007; Tabuchi et al., 2009; Salomon et al., 2011). This fact together with the absence of resistance proteins in yeast, makes this model a valuable utility to study the virulence activity of a particular effector. Another advantage of the yeast *Saccharomyces cerevisiae* is its high genetic amenability, as compared to plants. Actually, in the recent years, several studies on effector function characterization in yeast have been published both for animal (Lesser and Miller, 2001; Rabin and Hauser, 2003; Yoon et al., 2003) and plant pathogens (Munkvold et al., 2008; Salomon et al., 2011).

In order to better understand the mechanism of action of AWRs, their impact on yeast growth and viability was evaluated. A yeast-two-hybrid assay was also performed in order to decipher some plant targets for AWR4. Preliminary experiments where then performed to start the validation of found plant targets.

3.1. Some AWRs display a strong toxicity in yeast cells.

Our first aim was to perform a yeast-two-hybrid (Y2H) screening to identify plant proteins intreracting with AWRs. To do so, effectors were cloned into the pGBG vector under a constitutive ADH promoter that was suitable for Y2H. We noticed that transformation of yeast cells with pGBG-*awr* had a very low efficiency if compared to the transformation with the pGBG empty vector. This fact pointed out a putative toxicity effect of AWRs in yeast cells. Furthermore, preliminary yeast-two hybrid screenings for pGBG-*awr2* did not give any result since positive clones were lost at every step of the assay. In order to evaluate the possible contribution of these effector proteins to yeast toxicity we alternatively cloned AWRs in the galactose-inducible pGAL vector. Yeast cells were grown overnight in repressing media, then serially diluted and plated either in repressing media (glucose) or inducing media (galactose) to monitor the effects of AWRs in cell growth/viability.

We compared the growth of yeast cells expressing AWRs with those harbouring an empty vector or a control *gus* gene. AWR5 and AWR2, followed by AWR1, were dramatically inhibiting the yeast growth (Figure 41). These results correlate with the HR-like phenotypes observed in the non-host plants. Since R-proteins are absent in yeast cells, the effects that we are observing here might be related to a direct effect of AWRs. Important pathways must be altered by these effectors and this causes a dramatic reduction in growth. Noteworthy, even a minimal escape of AWR5 expression in absence of induction substantially impacted the yeast growth. AWR4 did not cause any effect on yeast growth as was observed upon *in planta* expression. Regarding AWR3-expressing yeast, it seems to be affected in growth, contrary to what has been observed with transitory expression in *N. benthamiana*.

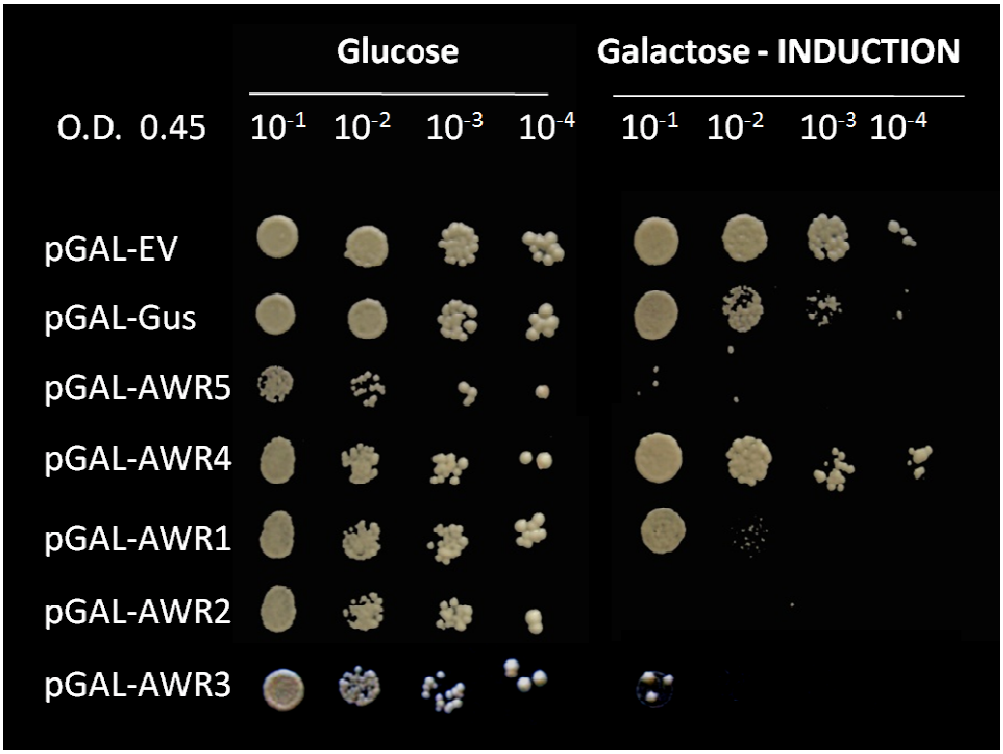


Figure 41. Expression of AWRs in yeast results in a toxicity effect at different extents.

AWRs were expressed in yeast cells under a galactose inducible promoter and serial dilutions where plated on glucose- or galactose-supplemented media to monitor cell growth.

In several attempts we tried to check protein expression in yeast cells but only the GUS control protein could be visualised. This might be unrelated to protein toxicity since AWR4 is not toxic and could not be detected either. Whether this might be due to protein size or AWR compartimentation is still unsolved. However, *awr* gene expression in yeast could be confirmed by quantitative RT-PCR (data not shown). In any case, the phenotypes observed are dramatic as 4 out of the 5 AWRs proteins are able to produce growth inhibition whereas less than 1% of the genes tested with inducible by GAL1 promoter are able to do so (Liu et al.,

1992). Actually, growth inhibition is yeast can be due to either cell death or growth arrest. This can be determined by vital staining of yeast cells (Millard et al., 1997) or by monitoring cell recovery. In the latter case, cells are grown in liquid inducing media, recovered at different time points and then plated in non-inducing media. If growth inhibition is due to cell death, the longer the cells are grown in inducing liquid media, the fewer cells will be recovered when plating. An alternative system to the sugar induction we used should be assayed to contrast the results and verify that phenotypes are independent of the carbon source employed. Implications on fermentation or respiration processes could also monitored by changing the carbon source. For instance, growth in ethanol glycerol media that forces bacterial respiration would prove if respiration is required for the toxicity phenotype. All these analyses will give some hints on AWR function. The specific effect that AWRs produce in *S. cerevisiae* is currently being characterised by other members of our team, with the hypothesis that the results may be extrapolated to the actual role of AWRs inside the host plant cells. Available mutant yeast strains and libraries will enormously facilitate the study of phenotypes derived from effector function in this system.

As AWR4 did not induce any toxicity in yeast and was well expressed in *R. solanacearum*, *hrpB*-regulated, and the first to be proven to be injected in the plant cells, this AWR was as a good candidate to seek its host protein interactors.

3.2. Defense-related plant proteins are found to interact with AWR4.

The yeast two-hybrid technique (Y2H) is a very powerful methodology to screen for protein interactions even if they are transient and weak. The assay is performed *in vivo* and in a eukaryotic organism and this is why there is a high probability that proteins are present in their native conformation, increasing the sensitivity and accuracy of the methodology (Clontech). The system is adequate for bacterial T3Es, as they are translocated to eukaryotic cells and are active in this environment. The principle behind the methodology is the transcriptional activation of certain downstream reporter genes upon binding of a transcription factor to their promoter regions (Figure 42).

AH109 is the yeast strain often employed for the Y2H and it presents auxotrophy for adenine, tryptophan, leucine and histidine. The GAL4 transcription factor is split into two: a binding domain (BD, in pGBG vector with the TRP1 selection gene) responsive for promoter binding and an activation domain (AD, in pGAD vector with the LEU2 selection marker) that will activate transcription. Baits are fused to the BD whereas preys (i.e. cDNAs to screen for) are

fused to the AD. A strain harbouring two plasmids with bait and prey genes will recover its capacity to grow in media lacking tryptophan and leucine. Only if bait and prey interact, their fused domains will be close enough to activate transcription of the reporter genes and auxotrophy for adenine and histidine will be rescued (Clontech).

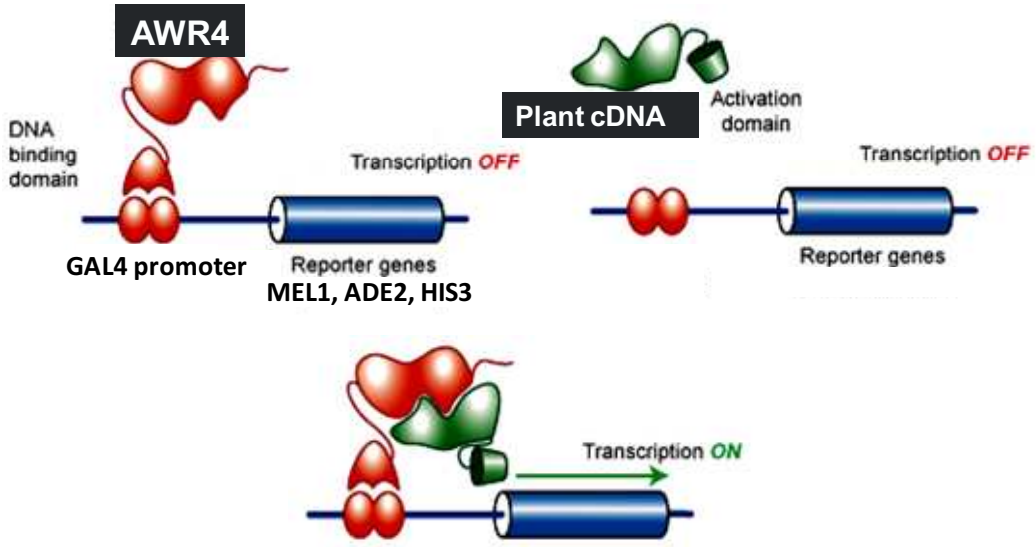


Figure 42. Concept underlying Y2H methodology.

The Y2H assay is based on the transformation of yeast cells with both BD-*awr* and AD-cDNA and the evaluation of several reporter genes. If interaction between the two proteins take place, BD and AD will be close enough to activate transcription of those reporters.

Several proteins involved in plant defence mechanisms appear to interact with AWR4

For our screening, *awr4* (bait) was cloned into the pGBG vector and an *A. thaliana* cDNA library from *R. solanacearum*-infected roots (prey) into pGAD. Vectors used for the Y2H were adapted to be gateway-compatible (L. Deslandes unpublished) and hence slightly differed from those described in the Y2H manual (Clontech) (see materials and methods for further information). A general scheme on the process from initial transformation until clone sequencing is presented in Figure 43. A total number of 1,7 million clones were screened after co-tranformation with the BD-AWR4 and AD-cDNA. Among them, around 360 candidate clones were re-streaked in the selection medium to verify interactions. X-α-GAL activity was then assayed in verified positive clones that showed a rescue for adenine and histidine auxotrophy. These positive clones (around 245) were amplified by PCR and reintroduced in the yeast strain already expressing the AWR4-BD bait. After re-transformation, almost 50 clones were re-confirmed as positive interactors and most of them were sequenced.

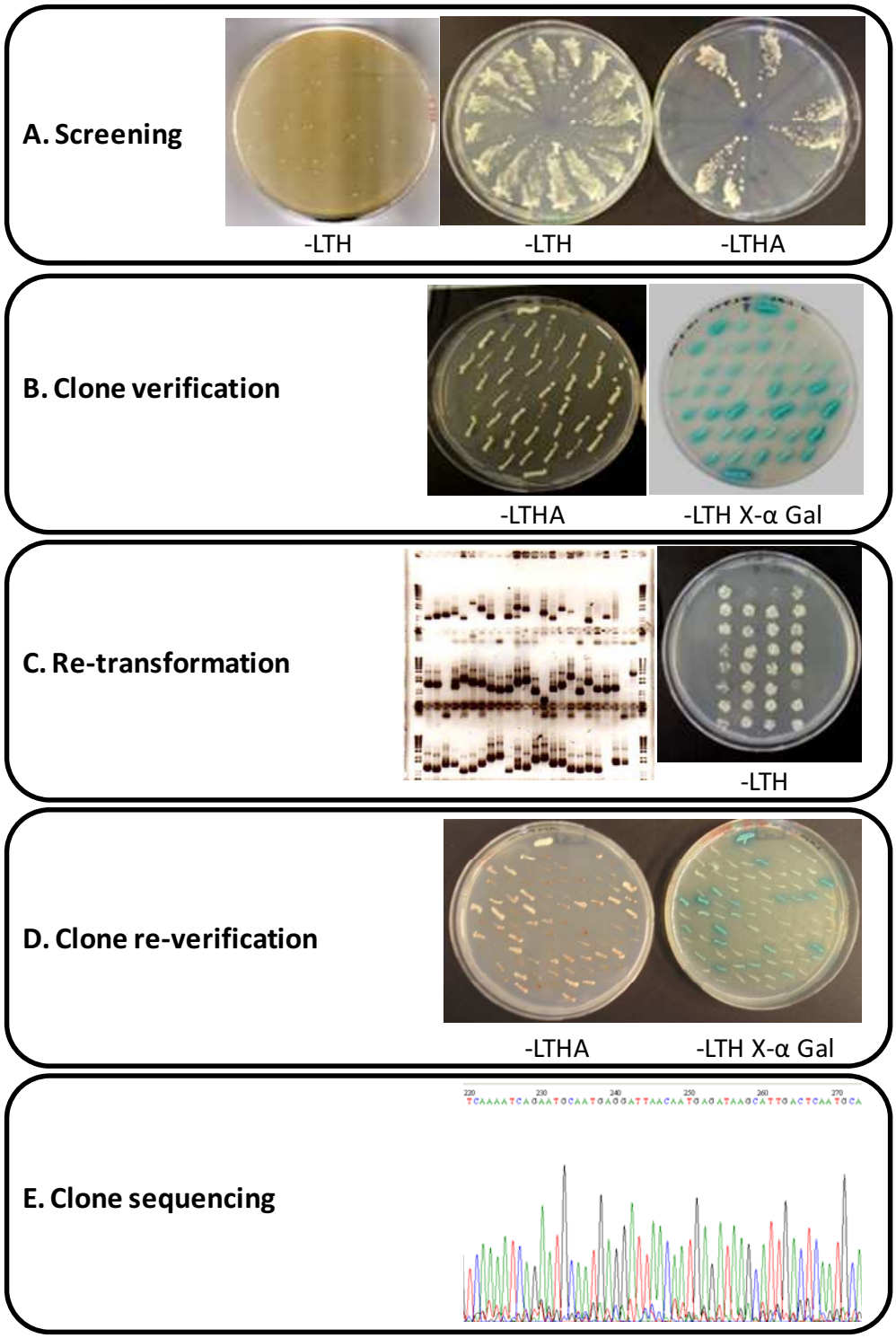


Figure 43. Diagram of the Y2H assay for AWR4.

Flowchart followed to find AWR4-interacting proteins. A) His autotrophic colonies were recovered from BD-AWR4 and AD-cDNA co-transformants in the AH109 yeast strain. Clones were re- streaked on -LTHA selective medium). B) Positive clones were verified for MEL activation with X-α-GAL. C) Verified positive clones were amplified by PCR, recombined in the empty AD vector and re-transformed in AH109 strain harbouring BD-AWR4. D) Positive clones were obtained and verified (same as A and B). E) Some 60 positive clones were sequenced in order to decipher the genes encoding interacting proteins.

Once we obtained the DNA sequence from verified clones, we carried out a BLAST search in order to identify the protein interacting with AWR4 and confirm the proper reading frame of the fished genes. The final list of confirmed AWR4-interacting proteins for is shown in Table 9.

Gene	Times found	Access. Num
Response to low sulphur 1 (LSU1)	7	NP_190527
Response to low sulphur 3 (LSU3)	1	NP_190526
Serine/threonine-protein kinase (WNK8)	3	NP_568599
V-type proton ATPase subunit B1	1	NM_106251
SNF1-related protein kinase catalytic subunit alpha (KIN10)	2	NP_850488
Mitogen-activated protein kinase (MPK6)	1	NP_181907
Phenylalanine ammonia-lyase 2 (PAL2)	2	NP_190894
Downy mildew resistance 6 protein (DMR6) / oxidoreductase	1	NP_197841
Hypersensitive-induced response protein 3 (Band 7 domain)	1	NP_566135
Annexin Arabidopsis 1	1	NP_174810
Glutaredoxin C3	1	NP_177861
Ornithine carbamoyltransferase	1	NP_177667
Putative pyruvate dehydrogenase E1 beta subunit	1	AAM65328
Pirin-like protein	1	NP_850385
ELC (UBCc and Vps23 core domains)	2	NP_566423
E3 ubiquitin-protein ligase makorin	2	NP_850540
Ubiquitin thioesterase OTU1	1	NP_175482
U3 small nucleolar RNA-associated protein 10 and NUC211 domain-containing protein	1	NP_187305
Nuclear transcription factor Y subunit C-9 NF-YC9 (Hap5C)	1	NP_172371
Transcription factor X1-like protein	1	BAB02266
Mn-specific cation diffusion facilitator transporter	1	ABP68858
Flap endonuclease-1	1	NP_850877
Octicosapeptide/Phox/Bem1p (PB1) domain-containing protein/ tetratricopeptide repeat (TPR)-containing protein	1	NP_194935
Heavy metal transport/detoxification domain-containing protein	1	NP_001031825
Arabidopsis thaliana RabGAP/TBC domain-containing protein	1	NM_115360
Uncharacterized protein	2	NP_176554
Uncharacterized protein	2	NP_200591
Uncharacterized protein	1	NP_566436
Unknown protein	1	NP_199201
Unknown protein	1	AAK25873
Putative protein	1	CAA16705

Table 9. List of AWR4- interacting proteins.

This table shows the proteins that were found to interact with AWR4, their access number in the protein databases and the number of clones recovered by the Y2H technique.

LS1, a low sulphur-responding protein is the most abundant clone found in the Y2H screening, being recovered up to seven times and represented by two different protein hits, hence probing its robustness. Not much is known about the role of these proteins, which have been recently named and described as *UP9C*-like genes. The *UP9C* gene codes for a protein with unknown function that is induced in tobacco upon sulphur starvation (Wawrzynska et al., 2010). LSU1 has been used as a marker for low sulphur state in plants (Watanabe et al., 2010) as it seems to be strongly induced by sulphur-deficit and to hydrogen peroxide, by salt stress

and -to a lesser extent- by $AgNO_3$ (Wawrzynska et al., 2010). Sulphur is an essential macronutrient present in aminoacids (e.g. cysteine and metionine), glutathione, phytochelatins, thioredoxins, chloroplast membrane lipids, some coenzymes and vitamins (Rausch and Wachter, 2005; Falk et al., 2007). Sulphur appears to be a limiting factor in the current environment and is not only required for general plant metabolism (and growth) but also for plant defence compounds (e.g. defensins, thionins, glucosinolates, phytoalexins...) as it is reviewed elsewhere (Rausch and Wachter, 2005; Falk et al., 2007). A complex reprogramming might respond to a changing sulphur supply and UP9C could participate of these events by influencing hormone actions or levels (Lewandowska et al., 2010). SLM1/EIL3 (functional homolog of EIN3, which is a transcriptional factor that controls the expression of the ethylene-responsive genes) is the regulator of the sulphur response (Maruyama-Nakashita et al., 2006). Why a bacterial effector should target these processes is obscure to us.

Up to six different clones found in the screening corresponded to protein kinases. Protein kinases play a crucial role in the regulation of many cellular processes, integrating an upstream signal and transducing it through phosphorylation to downstream elements (Hunter, 2000). Interestingly, two of the identified interactors (KIN10 and MPK6) play a role in plant defence. KIN10 was found twice and is the plant ortholog of the yeast Snf1, and therefore it is also also called Snf1-related kinase or SnRK1 (Baena-Gonzalez et al., 2007). KIN10 belongs to the CDPK-SnRK superfamily that is involved in interpreting calcium signals in *Arabidopsis* (Hrabak et al., 2003). It is a key regulator that integrates sugar and stress conditions, inducing transcription of a vast number of genes to promote catabolism and suppress anabolism (Baena-Gonzalez et al., 2007; Halford and Hey, 2009; Jossier et al., 2009). Furthermore, it is thought that the putative signalling molecule trehalose-6-P inhibits SnRK1 to promote the cell biosynthetic pathways (Zhang et al., 2009). A role in pathogenesis was first described in (Cernadas et al., 2008) where expression of SnRK was found enhanced in citrus upon infection with *X. axonopodis.* Another recently published work demonstrated that the AvrBsT effector from *Xanthomonas* interacts with SnRK1 in pepper and that this interaction is required for the AvrBs1-triggered HR (Szczesny et al., 2010). MPK6 is one of the most interesting plant targets identified for AWR4. MAPKs are involved in a highly conserved signal transduction for both biotic and abiotic stresses. As explained in previous chapters, MAPK cascade will induce a transcriptional reprogramming in the plant cell: transcription of defence proteins, oxidative burst, hormonal signalling and even conduce to SAR and HR (Zhang and Klessig, 2001; Asai et al., 2008; Rodriguez et al., 2010). As reviewed in (Shan et al., 2007), several bacterial effectors manage to interfere at some point of these MPK cascades to overcome plant defences. Among all the

MPK members, MPK3 and MPK6 play a major role. Recent studies demonstrate that MPK6 absence compromises disease resistance in *Arabidopsis* plants (Menke et al., 2004; Beckers et al., 2009). In addition, *R. solanacearum* growth was significantly increased in the tolerant tomato plant Hawaii 7996 if MPK3 was silenced (Chen et al., 2009). Furthermore, MPK6 is activated by H_2O_2 (ROS), O_3, PAMPs, JA and ethylene and sometimes in further activates these responses (Tena et al., 2001; Pitzschke and Hirt, 2006; Colcombet and Hirt, 2008; Xing et al., 2008; Pitzschke et al., 2009; Han et al., 2010) and positively regulates defence proteins such as phytoalexins (Ren et al., 2008). The HopAI1 effector protein from *P. syringae* specifically interacts with both MPK6 and MPK3 to interfere with plant defences (Zhang et al., 2007). Thus, AWR4 may share these kinases as targets of other effectors.

Besides SnrK1 and MPK6, the most representated kinase interactor in our screening was WNK8, which encodes a protein kinase with atypical catalytic residues present in most eukaryotic organisms (Verissimo and Jordan, 2001). It has been shown recently that WNK8 physically interacts with EDM2 in the nucleus. EDM2 is a protein (probably a transcriptional regulator) that is required for both floral transition and RPP7 R-protein-mediated resistance to the oomycete *Hyaloperonospora parasitica* in *A. thaliana* (Tsuchiya and Eulgem, 2010). Although, plant WNK8 is important only for the floral pathway, in mammals, some WNK proteins might regulate ion transport (Gamba, 2005). A recent experiment demonstrated the interaction of WNK8 with subunit C of the vacuolar H^+-ATPase (Hong-Hermesdorf et al., 2006), which gets phosphorylated. Interestingly, another clone found in the Y2H was the subunit B1 of the vacuolar ATPase, indicating a possible protein complex. ATPases placed in tonoplasts might be important for defence responses against environmental stresses such as cold or salt (Magnotta and Gogarten, 2002), whereas membrane ATPases are important for plant immunity (Elmore and Coaker, 2011).

Amongst the AWR4 plant targets, we consider phenylalanine ammonia-lyase 2 (PAL2) a very interesting candidate. PAL2 codes for the first enzyme in the phenylpropanoid pathway that culminates with the production of suberine and lignin precursors (Robinson, 1999). As discussed before, lignin and suberin are key for plant defences and contribute to avoid pathogen penetration and progression (Bhuiyan et al., 2009; Moura et al., 2010). PAL genes are rapidly induced upon pathogen infection and it seems logical that a bacterial effector could be devised to target this early step in the defence process.

Two other interesting clones that were found as AWR4 interactors are the DMR6 and the hypersensitive-induced response protein 3. DMR6 encodes a 2-oxoglutarate (2OG)-Fe(II)

oxygenase, whose function is still unknown, and negatively affects plant defence since downy mildew resistance is achieved when the gene is mutated (Van Damme et al., 2008). DMR6 appears to be activated upon direct contact with the pathogen *H. parasitica* in both compatible and incompatible interactions. Protein 3 belongs to the plant Band 7 proteins which are localized in lipid rafts and are somehow involved in R gene-mediated resistance since they are highly induced in plant tissues that undergo HR. In addition, this protein has been identified in the R-protein RPS2 membrane complex (Qi and Katagiri, 2009).

Other candidate AWR4-interacting proteins such as annexins and proteins involved in arginine, pyruvate or ubiquitin metabolism could also play a role in plant defence. Annexins are eukaryotic proteins that bind to membranes in a calcium-dependent manner (opening calcium channels when the ion increases in response to environmental stimuli) and probably help the plants to overcome plant stresses (Clark et al., 2010). In addition, annexin 1 is also able to regulate H_2O_2 (ROS) accumulation through its peroxidise activity. In the same way, glutaredoxin plays an important role in ROS protection and redox homeostasis (Kalinina et al., 2008). Ornithine carbamoyltransferase was also found to interact with AWR4. This key enzyme for arginine biosynthesis is the one inhibited by phaseolotoxin produced by *P.syringae* pv. *phaseolicola* (Mitchell, 1976). Other enzymes found in the screening were pyruvate dehydrogenase E1 and pirin. Pirin decides the direction of pyruvate metabolism (Orzaez et al., 2001) and is considered as an apoptosis-related protein in mammal cells, involved in seed germination and seedling development in plants (Soo et al., 2007). Several detected interacting partners were implicated in ubiquitin-related processes and it is not surprising as ubiquitination is a mechanism that controls multiple plant cellular processes including disease resistance (Devoto et al., 2003).

The results of the Y2H provided an important number of proteins important for plant defences that were found to interact with AWR4. Many were involved in biotic stresses (PAL2, MPK6, DMR6, LSU1, KIN10...) and some of them were chosen for further characterization (see next section).

Plant targets LSU1, WNK8, PAL2, KIN10 and MPK6 are specific for AWR4

Firstly, we sought to reproduce the interactions between AWR4 and some of the positive clones found in the Y2H screening interactions (LSU1, KIN10, MPK6 and PAL2). For that, plasmids harbouring BD-*awr* and AD-*interactor* fusions were co-transformed in yeast cells and their capacity to grow in selective media was evaluated. As seen in Figure 44, interaction with

AWR4 was validated in all cases, as cells were able to grow in selective media, contrary to yeast harbouring an empty pGAD vector. We next determined if interaction was specific for AWR4. For that, we checked growth in selective media for yeast strains containing interactors fused to the GAL4 AD domain together with the two other AWRs fused to the BD domain: AWR2 (with and without SD sequence) and AWR5.

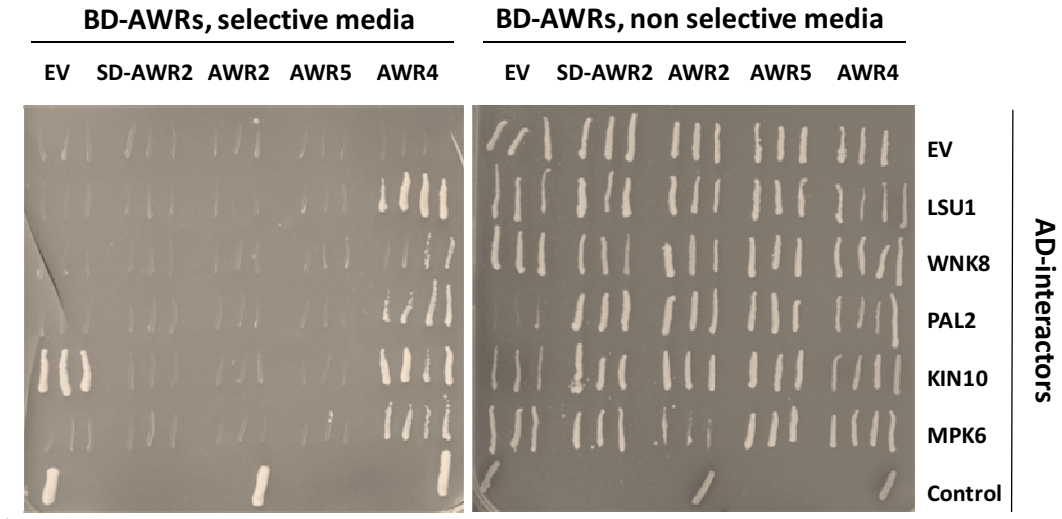


Figure 44. AWR4-interacting proteins seem specific for this member of the AWR family.

Yeast strains expressing both BD-*awr* and AD-*interactor* were grown in both non selective conditions and in selective mediun for protein interactions by Y2H. The empty pGAD vector was used as a negative control and pGBKT7-p53/pTD1-1 as a positive control for interaction.

All interacting proteins tested were unable to activate reporter gene expression without the presence of AWR4 fused to the AD. This proofs the specificity of the interactions and eliminates the possibility of growth due simply to autoactivation. KIN10 showed growth when combined with a BD empty vector in the selective medium pointing out to a possible autoactivation of this target. Paradoxically, this is not happening when it is is combined with other AWRs fused to the BD. In light of our results, the recently described interaction of KIN10 with AvrBsT (Szczesny et al., 2010) has to be interpreted with precaution. Although AWRs are all quite similar in terms of protein sequence, we were unable to visualize an interaction between AWR4 plant targets and the other AWRs tested. Although interaction with the other AWRs should be also checked (and is under way), our results suggest a functional specificity in the *awr* gene family. The use of other techniques suitable for the evaluation of protein-protein interactions will also be necessary for further confirmation of our results.

3.3. Preliminary experiments on selected AWR4 plant targets

The AWR4 interactors that were selected for further analysis were the kinases WNK8, KIN10 and MPK6, the overrepresented LSU1 clone and the PAL2 enzyme. They were chosen either because they were found several times in the Y2H assays and/or due to its relevance in biotic stresses as highlighted previously. All the experiments were performed with original clones from the Y2H which, in most cases, are only partial C-terminal gene fragments.

Interactors are well expressed in *N. benthamiana* and do not alter AvrA-mediated HR

In order to validate interactors *in vivo*, they were transferred to a suitable vector for plant expression that contained a YPF tag. First, we studied the expression levels of fusion proteins in *N. benthamiana* to determine if it was possible to work with them in this plant (they are originally from *Arabidopsis*). Interacting proteins were transiently and constitutively expressed by means of *Agrobacterium* and tissue samples were recovered after 36-48 hours of infiltration. The homogenized samples were subjected to western-blot to validate expression (Figure 45A).

Once it was checked that they were expressed at high levels, we proceeded to perform several experiments to further characterize them. According to the scientific literature, some effector interactors are involved in protecting the plant against pathogen attack. We first evaluated their effect when overexpressed *in planta* and if this was influenced by the presence of their interactor AWR4.

As explained in previous chapters, AvrA is an effector from *R. solanacearum* that triggers an HR in tobacco plants. We transiently coexpressed AvrA with each of the selected interactors to assess if they were capable of altering the avrA-mediated HR in different tobacco species. We could distinguish neither a reduction nor an increase of HR production in this model study. In addition, no differences were observed regarding the onset timing of the HR. Expression of interactor proteins alone appeared innocuous to plants since no macroscopic phenotype was observed (Figure 45B).

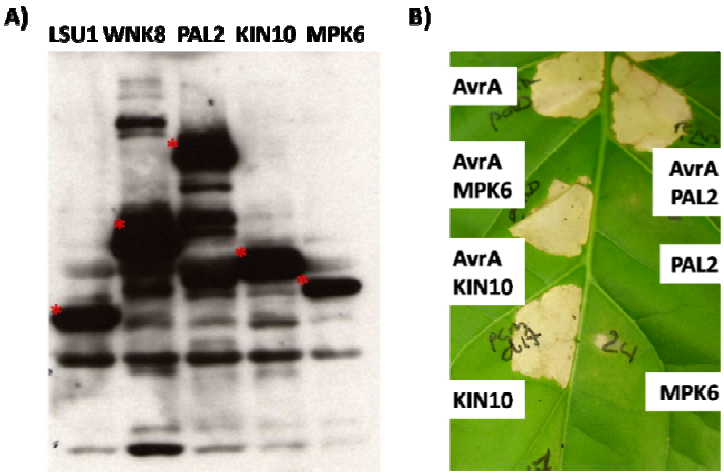


Figure 45. Expression of AWR-4 interacting proteins in tobacco plants.

A) Western-blot (α-YFP) to verify expression of AWR4 plant targets in *N. benthamiana* 48 h postinfiltration. B) Interactors do not alter avrA-mediated HR in *N. tabaccum* (picture taken 4 days postinfiltration).

AWRs promote granule appearance and increased nuclear targeting of plant interactors

We next performed subcellular localisation experiments for interactors to shed more light on their putative functions and evaluated any microscopic impact of their expression on *N. benthamiana* plants. Similar experiments were performed coexpressing interactors together with AWR4 and AWR2. Discs from *N. benthamiana* leaves expressing the selected interacting proteins under a constitutive promoter and fused to YFP were extracted and observed under the fluorescence microscope. Most of them mainly localized in the cytoplasm and/or membrane, as it was the case for LSU1, WNK8 and PAL2, whereas KIN10 and MPK6 presented a nuclear localization too (Figure 46). Thus localisation AWR4, which was cytoplasmic, overlapped with that of all its plant targets, further strengthening the feasibility of the identified interaction.

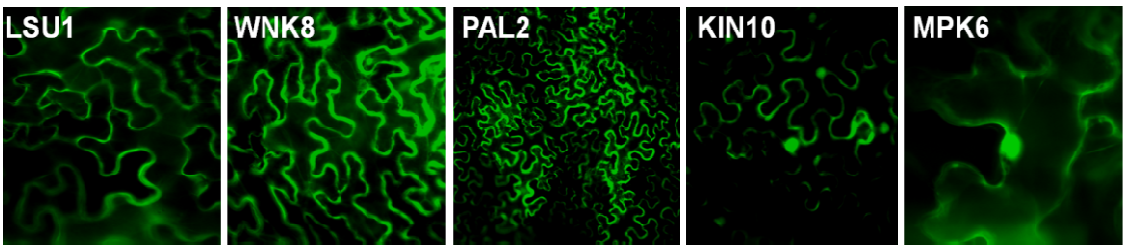


Figure 46. Localization of the YFP-fused LSU1, WNK8, PAL2, KIN10 and MPK6 AWR4-interacting proteins.

Localization is mainly cytoplasmatic for LSU1, WNK8 and PAL2, and also nuclear for KIN10 and MPK6. Images were taken with a fluorescence microscope at 48 h postinfiltration.

Once we determined the cellular compartment that was targeted by interactors, we intended to assess if AWR coexpression (estradiol inducible) changed their localization, being that an extra proof of interaction. We used both AWR2 and AWR4 to see their effects on the YFP signal *in planta*. After 8 h of *awr* induction, some changes in localization were observed, mostly for AWR2 (Figure 47). In general, there was a tendency of increasing nuclear localization upon AWR expression and the appearance of mobile spots along the cell. In particular, these phenotypes were very clear for MPK6 and PAL2 proteins when coexpressed with AWR2. Cell integrity seemed unaffected in the bright field images (not shown) and these effects had not been seen when awrs were expressed alone, all indicating that the spiking phenotype is unrelated to the described AWR toxicity. The fact that phenotypes were the same for AWR4 and AWR2 (even stronger in the latter), suggests a specific interaction with these targets even if we did not detect them in yeast.

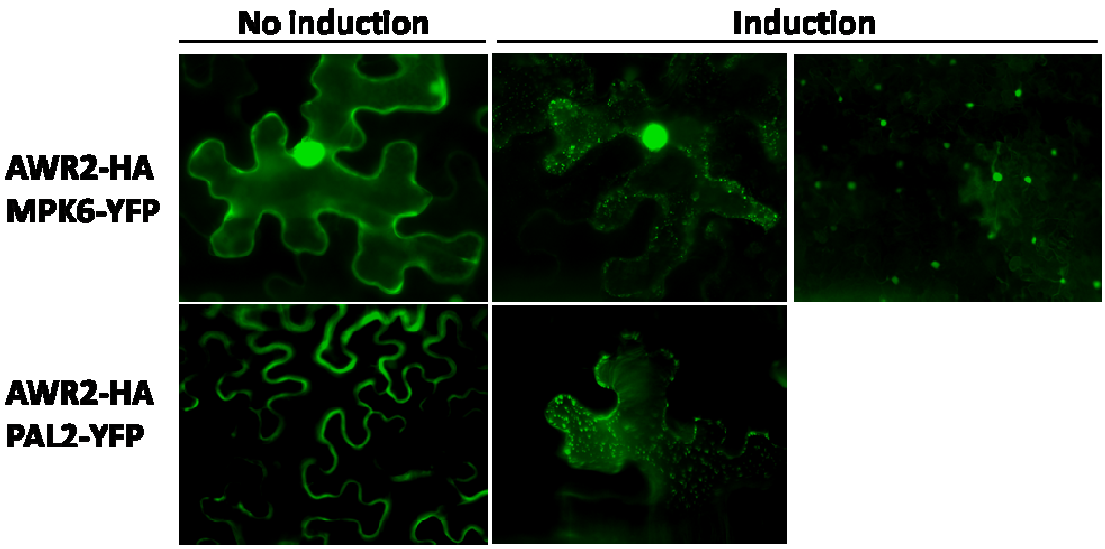


Figure 47. AWR effect on interactors localization.

Change of the localization patterns of constitutively-expressed YFP- PAL2 and MPK6 interactors upon coexpression of inducible AWR2 *in planta.* The right image in the top panel corresponds to a lower magnification to reveal fluorescent nuclei. Images were taken with a fluorescence microscope at 8 h postinduction.

AWRs do not affect stability of most interacting proteins

The effect of AWRs on interactors was also evaluated at the protein level 8 h postinduction. Stability of KIN10, MPK6, LSU1, PAL2 and WNK8 was checked in the presence or absence of AWR2 or AWR4. Expression of AWRs did not cause processing nor affect the stability of the interacting proteins (Figure 48). These results indicate that the spikes that appear in microscopic experiments upon coexpression are not due to protein degradation.

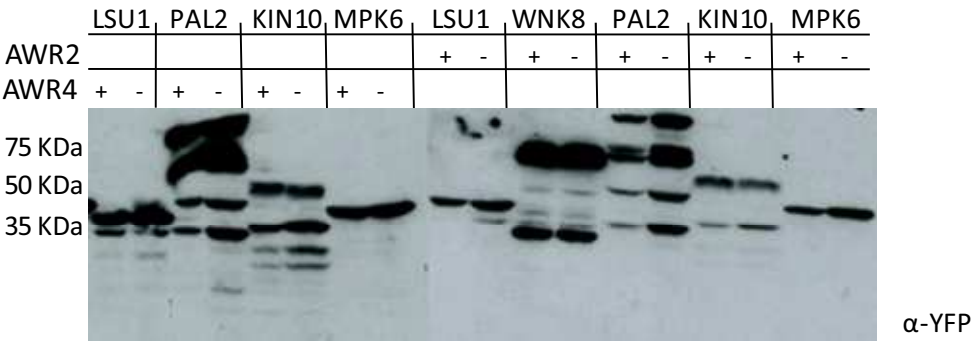


Figure 48. Evaluation of interactor stability upon AWR coexpression.

Stabilization of interacting proteins was assessed upon AWR induction. Interactors were constitutively expressed fused to YFP and AWRs under an inducible promoter fused to HA. AWR protein induction was also verified (data not shown). Samples were taken 8 h postinduction and subjected to western blotting with an anti-YFP antibody. (+) induced; (-) not induced.

3.4. Some hints on PAL2-AWR4 interaction.

Phenylalanine ammonia lyase is the first enzyme of the phenyl propanoid biosynthesis and is thus required for lignin and suberin synthesis which will reinforce cell walls and prevent pathogen penetration. Therefore, PAL seemed a very interesting plant target to further characterize.

AWR4 pulls down PAL2 in co-immunoprecipitation assays

In order to confirm the interaction between PAL2-YFP and AWR4-HA, proteins were expressed together in *N. benthamiana* plants. Samples were harvested at 24 h postinduction of AWR4 and this was pulled down with an anti-HA agarose matrix and then presence of PAL2 was detected by western-blot anti-YFP (Figure 49).

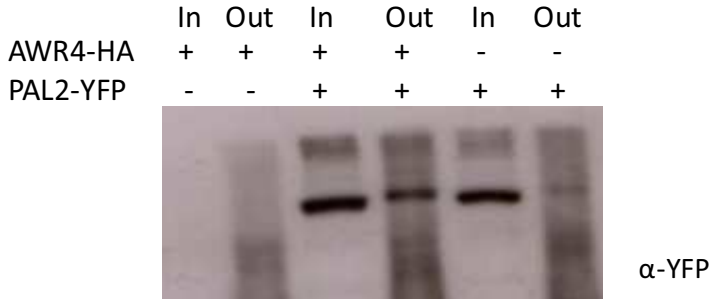


Figure 49. PAL2 is pulled down with AWR4 precipitation.

After AWR4 and PAL2 coexpression *in planta*, AWR4 was bound to resin anti-HA and eluates were subjected to WB anti-YFP for checking PAL2 presence. In: input (fraction before resin incubation), Out: output (eluate fraction after resin incubation).

From the results, we can appreciate that PAL2 appears in the immunoprecipitated fraction when AWR4 is pulled down. These results were reproduced two times out of three different replicates. However, in the third assay, it seemed that YFP-PAL2 alone showed some unspecific binding to the HA agarose.

As the experiment seemed to confirm the interaction, we better monitored AWR4 and PAL2 protein stabilities along time. For these experiments, we avoided using P19 vector in the agroinfiltrations to produce proteins to more physiological levels. In Figure 50 we can observe that protein levels for AWR4 increase with the time after induction. When we looked at the AWR-PAL2 coexpressions, PAL2 protein was slightly stabilized.

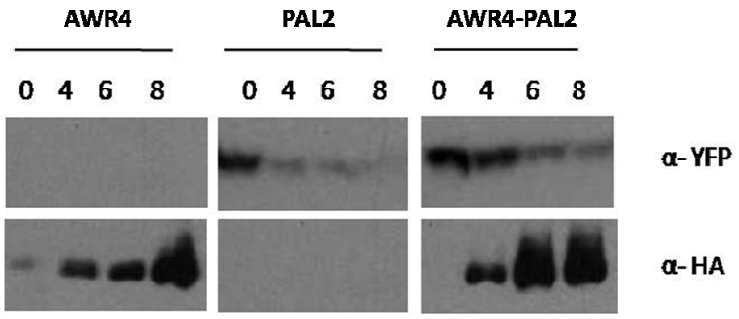


Figure 50. Evaluation of the effect of AWR4 expression on PAL2 stability.

Inducible AWR4 was fused to HA tag and constitutive PAL2 to YFP. Both proteins were expressed in *N. benthamiana* plants and their levels of expression were monitored (in absence of P19) at 0, 4, 6 and 8 hours postinduction of AWRs.

The advantage of not using P19 in the assay is that we prevent an excess of protein expression that could make difficult to visualise any interference due to the AWR. On the other hand, the disadvantage of not using this vector is that PAL2 expression overtime will decrease *per se* due to plant silencing which is no longer blocked. In any case, we did not see a major effect of AWR4 on PAL2 stability or processing.

AWRs do not interfere with PAL2 enzymatic activity

PAL2 is an enzyme that catalyzes the production of cinnamic acid from L-phenylalanine (L-phe) substrate and we sought to investigate if the interaction between AWR4 and PAL2 could have an impact on its enzymatic efficiency. First, we heterologously produced GST-fused PAL2 in BL21 *E. coli* cells and we used the supernatant to test PAL2 activity. When L-phe is not a limiting substrate, cinnamic acid apparition gives us an idea of the PAL2 activity because the product can be monitored at O.D. $_{290}$ nm. Different enzyme concentrations as well as different PAL inhibitors were tested to select the best parameters for the PAL2 assay (Figure 51). From

the enzymatic assay results we observed that detection of PAL activity could be detected using 5-15 µl of total extracts from producing *E. coli* cultures. In addition, we verified that GST did not interfere with PAL activity, which is maintained even after one cycle of freezing. Contrary to that, if PAL2 was coincubated with the competitive inhibitor 2-aminoindan-2-phosphonic acid (AIP) (Appert et al., 2003) or without the L-phe substrate, no activity was perceived.

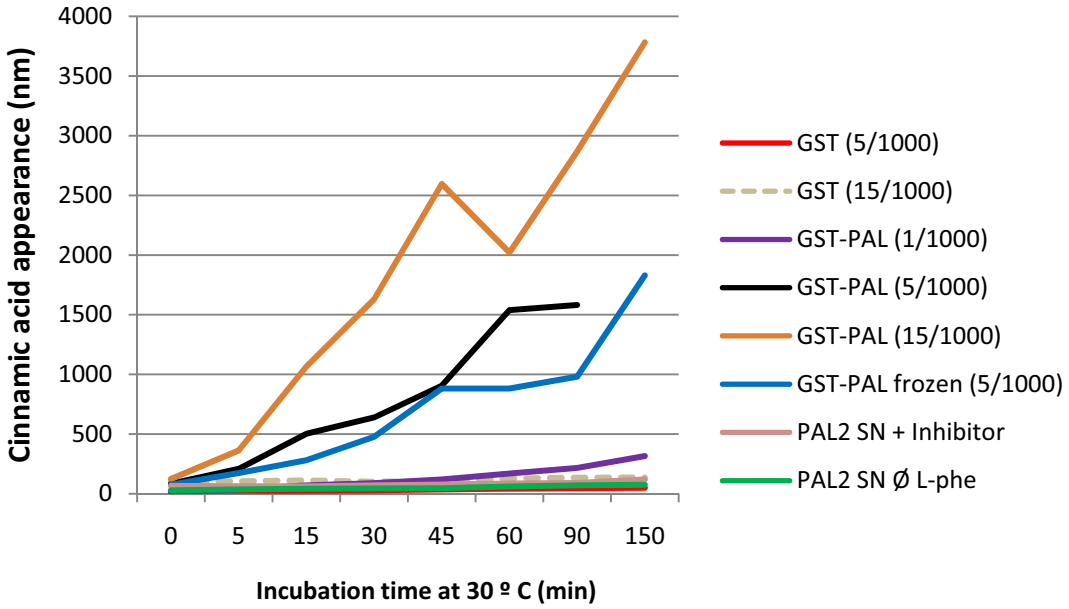


Figure 51. Optimization of PAL2 enzymatic activity.

Enzymatic activity for PAL2 was evaluated under different protein concentrations to find the best condition for subsequent assays and to proof that GST was innocuous for PAL2 activity.

Once the assay was established and optimized, we wanted to evaluate if AWRs interfered with cinnamic acid production when coincubated with the PAL2 enzyme. To do so, HA-tagged AWRs were directly produced from *R. solanacearum*, recovered from the supernatant and HA-purified. Although AWR production was verified by WB, protein concentration was not high enough to visualise it on a comassie-stained polyacrylamide gel. We did a first attempt of the assay by setting up a ratio of 15:1 (AWR-PAL2) to give the effector a stochiometric advantage. Up to three different AWRs were evaluated: AWR4, AWR2 and AWR5, and the assay was performed in previously settled conditions. The results in Figure 52 show that cinnamic acid appearance seemed not to be altered by AWR presence in these conditions.

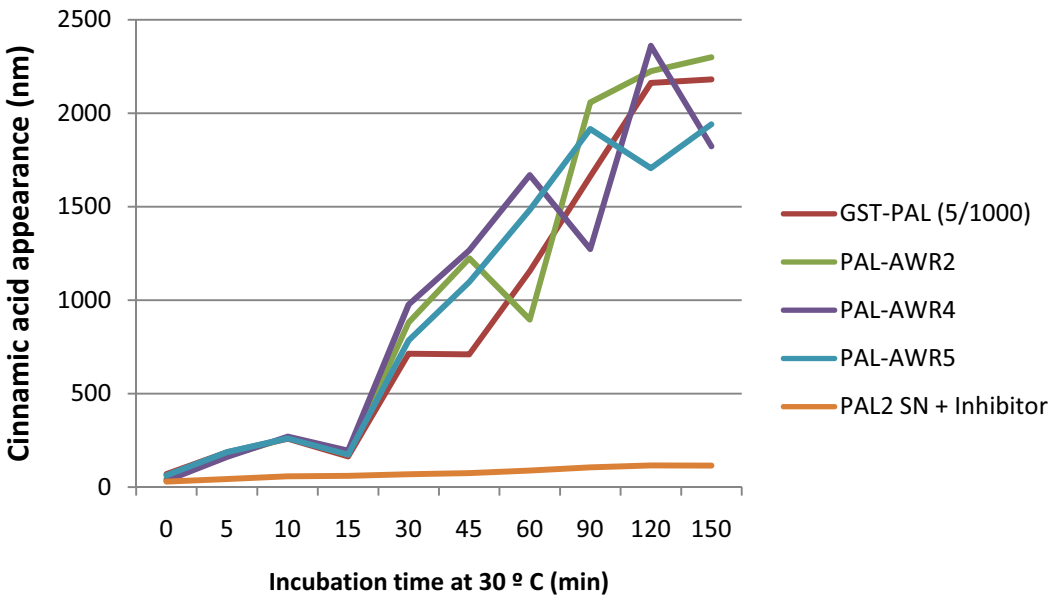


Figure 52. AWRs seem not to alter PAL2 activity.

Several AWRs were incubated with PAL2 and enzymatic activity was evaluated in order to determine if AWRs interfere somehow with its function.

Although we were not able to detect a direct interaction between AWRs and PAL2, further experiments need to be done to exclude the possibility that GST interferes with binding. We are thus willing to obtain GST-free PAL2 protein after thrombin treatment and later recover pure PAL2 protein by FPLC. In addition, different incubation times between AWR and PAL2 prior to enzymatic test should be assessed.

In conclusion, some putative AWR4 plant partners were discovered and evaluated. Main functions targeted are plant defences, suggesting that AWRs could touchdown plant immunity to successfully infect. The AWRs that are most toxic and exert a strongest phenotype *in planta* are AWR2 and AWR5 and knowing their plant targets could give hints on their role in virulence. Due to the fact that they also trigger yeast toxicity, the Y2H technique could not be employed to screen for plant interacting partners. Hence, other model systems should be assessed in order to be able to heterologously produce them and find plant targets.

CHAPTER 4: Production of AWRs

CHAPTER 4
PRODUCTION OF AWRS

The aim of this study was to study all members of the AWR effector family in *R. solanacearum*. GMI1000. Although some interactors for AWR4 had been discovered, as described in the previous chapters, the plant targets for the others were still unknown. For instance, we showed that AWR1, AWR2 and AWR4 are important effectors for both disease and resistance but their biochemical function was still unidentified. The strong toxicity of these AWRs when expressed in yeast made us search for alternative systems to the Y2H to detect interaction with plant target proteins. We thus decided to set up the production of AWR proteins fused to an epitope, with the aim of immobilising them on resin to retain interacting proteins from plant extracts.

Before starting any production, we observed that estradiol-induced pER8-AWRs were insoluble when expressed by means of *Agrobacterium* in *N. benthamiana*. In fact, only treatment with 0,1-0,5% of the detergent Triton X-100 allowed the partial solubilisation of effector proteins from plant (Figure 53).

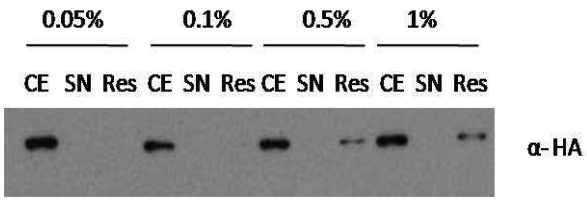


Figure 53. Solubility of AWR2 from agroinfiltrated *N. benthamiana*leaves.

AWR solubility was evaluated 4h after induction with increasing amounts of TX-100 detergent. CE: crude extract (total fraction), SN: supernatant (souble fraction), Res: detergent resuspention.

This was not surprising as AWR proteins are considerably big (more than 1000 aminoacid residues), which could lead to their precipitation in inclusion bodies because the plant cell machinery cannot cope with the heterologous protein and ensure its proper folding (Nallamsetty et al., 2005). We hence explored two alternative systems for overcoming these difficulties: the heterologous expression in *E. coli* cells and the overexpression in the native *R. solanacearum* strain.

4.1. AWRs are efficiently produced in *E. coli* and maltose binding protein enhances their solubility

E. coli has been extensively employed as a heterologous organism for protein production. We wanted to check if, contrary to the results from AWR expression *in planta*, AWRs were soluble when expressed in *E. coli* cells.

Insolubility of AWR2 when expressed with pTH19, pDEST14 and pDEST15 vectors

E. coli (BL21) production and solubility trials were initially carried out for AWR2 in pTH19, pDEST14 and pDEST15 vectors in order to determine which one performed best. All vectors tested had a T7 promoter and AWR2 was expressed as a fusion protein for pTH19 (His-tag) and pDEST15 (GST-fused) to enable its purification if required. We checked AWR2 protein production in the soluble or insoluble fraction of the cell lysates. Expression in *E. coli* BL21 was maximal when they were induced with IPTG for 3 to 4 hours at 37 ºC. The highest protein production was obtained with pDEST15, since GST might stabilize AWR2 (Figure 54). However, none of the vectors managed to ensure a soluble fraction of AWR.

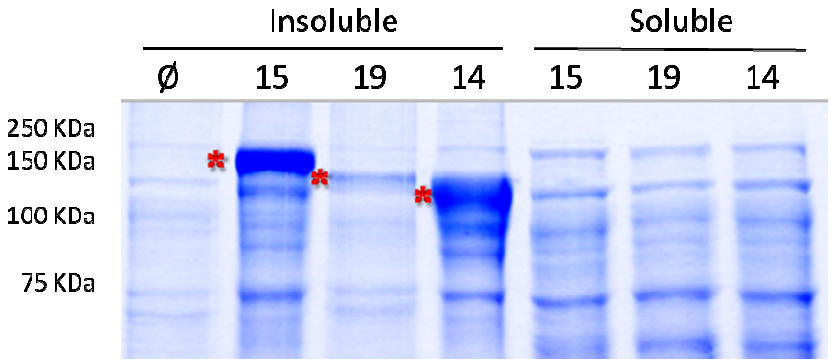


Figure 54. AWR2 is highly produced in *E.coli.*

Coomassie-stained SDS-PAGE gel that shows the insolubility of AWRs expressed in *E. coli* with different vectors. 15: pDEST15 (GST), 19: pTH19 (HIS), 14: pDEST14. Full size proteins are marked with an asterisk. Samples correspond to the soluble and insoluble fractions of total cell extracts

The maltose binding protein enhances AWR solubility

Although it had been used for long, the maltose binding protein recently appeared as an excellent fusion protein to solve solubility problems in protein production (Fox and Waugh, 2003). This 26-KDa polypeptide seems to promote the proper folding of its fused partners to avoid the precipitation of unfunctional protein as aggregates in inclusion bodies (Nallamsetty et al., 2005). We thus cloned AWRs in the gateway-compatible pDEST-6His-MBP vector. Different incubation temperatures (37, 30 and 25 ºC) and induction times (1 to 4 hours) were

assayed to optimize soluble AWR production. We succeeded in producing several AWRs fused to MBP in a soluble form when induction was performed at 25 ºC for 2.5 h (Figure 55).

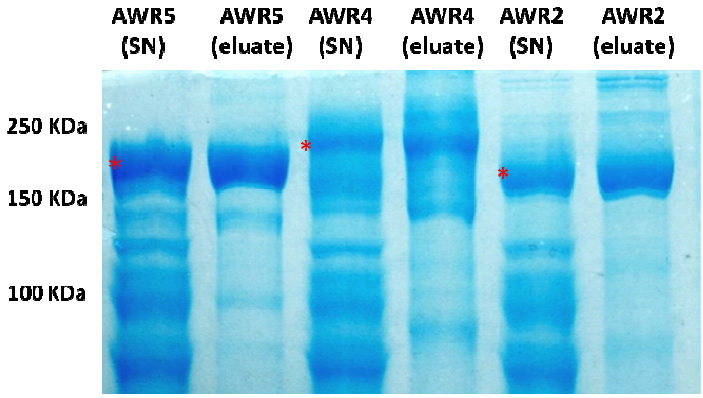


Figure 55. MBP enhances AWR production in the soluble fraction of *E. coli* cell extracts.

Comassie-stained SDS-PAGE gel of soluble (SN) and His-purified (eluate) 6His-MBP-fused AWR proteins which were heterologously expressed in BL21 *E. coli* cells. Full size proteins are marked with an asterisk.

We were also able to purify AWR proteins in *E. coil* to a quite pure preparation using a Nickel affinity resin prepacked in a column, as MBP-fusion proteins also contained the 6his tag. Presence of AWR proteins was confirmed by western-blot anti-His (data not shown). As it was difficult to ascertain the correct size of the purified bands corresponding to AWRs, we excised them from the SDS-PAGE gel, trypsin digested and analysed by MaldiTof/Tof mass spectrometry. Digested patterns were contrasted to the Mascot database that corroborated the presence of the full-length protein for all AWRs tested (scores of 450-800 and protein coverages from 30%).

4.2. AWRs can be directly purified from *R. solanacearum*

As explained in chapter 1, we recently developed a novel system to target DNA sequences to a permissive site in the GMI1000 chromosome. The system is based on a series of vectors –the pRC vectors- that, after linearisation and natural transformation integrate in the genome by double crossing-over. The system is also designed for protein production in *R. solanacearum*, as when ORFs are gateway-cloned in pRCs they generate C-terminal fusions to the HA-tag, which enables protein immunodetection and purification. Thus, we used the same constructions generated for secretion experiments (Chapter 1) to overproduce AWR effector proteins in their native strain. Overproduction was ensured by the *eps* promoter, known to be extremely active at high bacterial densities (Garg et al., 2000). This avoids using a *R. solanacearum* strain that overexpresses the *hrpB* regulator to compensate for low effector expression in the wt strain. We tested production of the AWRs 2, 4 and 5 with this system.

Contrary to the secretion studies, bacteria were grown overnight in rich medium, proteins recovered from the cell fraction after bacterial lysis and samples were subjected to a western-bot analysis (Figure 56A).

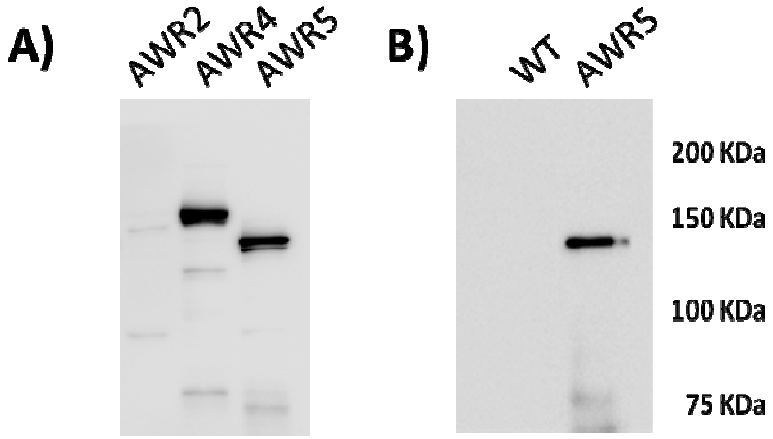


Figure 56. AWRs are can be overproduced in GMI1000 *R. solanacearum.*

A) AWRs were produced in *R. solanacearum* grown in rich medium and the SN fraction from the bacterial lysates was subjected to western blot analysis with an anti-HA antibody. B) AWR2 was overexpressed in *R. solanacearum* growing in *hrp*-inducing media and secreted protein was concentrated through pore membrane passages with centrifugation steps, followed by an HA-purification and eluate was subjected to a western-blot anti-HA (second lane).

Once a good expression was ascertained, we set up AWR purification. As AWRs are secreted via the T3SS, we decided to try their purification from the medium, to ensure that the proteins recovered were from the most native condition. To this end, we grew strain GMI1000 or the same strain expressing AWR2-HA as for secretion analyses but, to keep their natural conformation, proteins were concentrated by sequential centrifugation through a porous membrane, rather than through precipitation. After concentration, proteins were affinity-purified with an agarose resin conjugated to an anti-HA monoclonal antibody and the eluates detected by western blot with the same antibody. It can be appreciated in Figure 56B that we managed to recover and purify AWR2 from the culture medium of GMI1000 strain.

These results open new venues on effector production and purification directly from *R. solanacearum* in a soluble form. The use of the *eps* promoter should enable high levels of AWR production in the bacterium. However, in our hands, the amount of protein produced was not high enough to be directly detected by Coomassie blue staining. Thus, we are able to produce the protein from its original context, but the amounts may be too low to perform protein interaction experiments. Depending on the final conditions of the assay, the production in *E. coli* or *R. solanacearum* will be chosen.

Once AWRs can be produced and purified either from *E. coli* or *R. solanacearum*, they could be bound to an affinity column or resin and screen for plant targets by incubating it with a plant extract and then analysing the retained proteins by mass spectrometry. The advantages of *E. coli* expression are the high amounts of protein obtained and that production is very simple. Contrary to that, effectors are not produced in original bacteria and proper folding or posttranslational modifications might be a constraint. If AWRs are produced in *R. solanacearum*, we have all the machinery needed for high production of putatively active effector protein. However, in both cases we may skip a putative protein processing or modification *in planta* that could be required for effector virulence function and/or recognition. For instance, such modifications have been demonstrated for several effectors including AvrPphB, AvrRpt2 and AvrRps4 from *P. syringae* (Axtell and Staskawicz, 2003; Mackey et al., 2003; Shao et al., 2003; Sohn et al., 2009). Effector production *in planta* could circumvent this problem because AWRs display their activity in this environment. An option could be the employ of tandem affinity purification to unravel protein complexes bound to AWRs (Puig et al., 2001). Nevertheless, our preliminary experiments showed that AWRs expressed in leaves by means of *Agrobacterium* are in the insoluble fraction and this enormously difficults the evaluation of protein-protein interactions. Indeed, the detergents used for solubilisation will undoubtedly hinder protein-protein interactions. An innovative system that could also be tried is the inoculation of *R. solanacearum* expressing AWRs-HA into host plants. If soluble protein within the plant could be recovered, this would be the most physiologically sound way of deciphering AWR effector protein plant targets by pulling AWRs down and analysing the co-immunoprecipitated proteins.

Conclusions

CONCLUSIONS

Ralstonia solanacearum is a devastating pathogen that requires the Type III Secretion System to successfully infect plants (Boucher et al., 1987). It is one of the bacterial pathogens with the highest number of predicted T3E (Kay and Bonas, 2009; Cunnac et al., 2010; Mukaihara et al., 2010) but most of their functions are still unknown, contrary to other pathogens such as *P. syringae* or the xanthomonads. Among all putative effectors found in *R.* solanacearum, we have focused here on the characterization of a multigenic family called AWR.

Bioinformatic and functional screenings have been crucial for the identification of T3-secreted effector proteins. Usually, effector candidates are tested for translocation into plant cells using the CyaA reporter system (Ladant and Ullmann, 1999). This system consists in chimeric proteins between a calmodulin-dependent adenyate cyclase (cyaA) domain from *Bordetella pertussis* and the protein of interest. CyaA-dependent production of cAMP requires the presence of eukaryotic calmodulin and thus increase of cAMP values can only be detected upon translocation of effectors from the bacterial cell to the eukaryotic cytosol. The system was first used in *Y. enterocolitica* for YopE effector protein (Sory and Cornelis, 1994) and later on adapted to plant cells to screen for phytopathogen effectors (Casper-Lindley et al., 2002; Cunnac et al., 2004; Schechter et al., 2006; Mukaihara et al., 2010). Our results from full-protein secretion studies validate the previous CyaA experiments using the N-terminus of the AWRs, since we determined the T3-dependent secretion of full-size AWR2, 3, 4 and 5 proteins in GMI1000, but also they proved for the first time the secretion of the AWR1 protein.

Lately, a huge effort has been devoted to determine the core effectome that would be important for broad pathogen infection capacity from that useful in a more specific plant-host context (Hajri et al., 2009; Schulze-Lefert and Panstruga, 2010). Effectors might each be important in a given context and discovering how they are conserved in bacterial phylogenies could shed light to the host-pathogen co-evolution and give hints on their function. AWR effector proteins are highly conserved in all *R. solanacearum* isolates and also present in other plant pathogenic strains with emerging importance such as the rice pathogen *B. glumae*, other *Burkholderia* strains, *Acidovorax avenae* infecting cucurbitaceae or poaceae and some *Xanthomonas* pathovars causing disease in rice, pepper, tomato, brassicaceae, citrus tree or even causing a bacterial wilt in banana (Willems et al., 1992; Viallard et al., 1998; Mole et al., 2007; Smith et al., 2008; Ham et al., 2010). In addition, AWR homologues are also present in the mammal pathogen *B. pseudomallei,* the causal agent for melioidosis (Wuthiekanun and

Peacock, 2006), whose T3SS is similar to that of *R. solanacearum* (Rainbow et al., 2002). Interestingly, this bacterium is capable of infecting tomato plants, as presented recently (Lee et al., 2010). It would be interesting to evaluate the role of AWRs in this pathogen because, as it was reported for *P. aeruginosa* that some virulence factors are important for pathogenesis on both plant and animal hosts (Rahme et al., 1997). Some effectors, like HopAO1 from *P. syringae* have been shown to share the same function with its homologues SpvC and OspFfrom *Salmonella* and *Shigiella*, respectively (Shan et al., 2007). This broad dispersion of *awr* genes might have occurred due to a shared ecological niche that facilitated lateral gene transfer events among different bacterial strains. Indeed, *B. graminis*, *B. pseudomallei* and *Acidovorax* spp. are soil/root bacteria and they are all abundant in the rhizosphere (Willems et al., 1992; Brett et al., 1997; Viallard et al., 1998; Frederick et al., 2001). A role in virulence of these proteins has also been established with the finding that AWRs are required for full virulence of the pathogen on natural hosts. The fact that single disruption of AWRs resulted in only a very slight effect (if any) on pathogenicity, and that the effect be very apparent in the quintuple mutant indicates that this effectors act co-operatively on the pathogenicity towards host plants tomato and eggplant. The high redundancy of the effector repertoire has been interpreted as a means for the pathogen to use particular combinations for the effective colonisation of each host (Cunnac et al., 2004; Kvitko et al., 2009). Interestingly, we found out that AWR1 (last to be mutgenised in the quintuple *awr* depleted strain) has also a role in virulence as this mutant strain multiplies less than the previous one lacking this AWR. Furthermore, it was recently shown that *R. solanacearum* Psi07, which artificially acquired a genomic portion of 24.4 Kba of foreign DNA from GMI1000 donor strain, was more virulent in tomato plants and surprisingly *awr1* was one of the 20 new ORFs that included only 2 effector genes (Coupat-Goutaland et al., 2011). This further enhances the importace of studying this AWR that has been previously considered unfunctional (Mukaihara and Tamura, 2009) and also the other AWR members.

Moreover, the impact of AWR expression on *A. thaliana* (by means of *Agrobacterium* or *P. syringae*) or non-host tobacco species (*Agrobacterium*-mediated) reveal that they are recognised by the plant surveillance system. This dual role in virulence and plant recognition has already been documented for some bacterial effector proteins and been interpreted in the light of plant-pathogen co-evolution, which results in compatible (host) and incompatible (non-host) interactions (Schulze-Lefert and Panstruga, 2010). For instance, AvrBsT from *X. campestris* pv. *vesicatoria* is recognised in pepper and *N. benthamiana* plants whereas it enhances bacterial growth in tomato plants (Kim et al., 2010). The effect of AWR proteins in

non-host plants correlates with the strong yeast growth inhibition. R-proteins are absent in this model organism, therefore we can perceive the effector virulence activity that could be conserved among eukaryotes (Yoon et al., 2003; Kramer et al., 2007; Tabuchi et al., 2009; Salomon et al., 2011), rather than any recognition event. The split into three pieces of AWR5 protein did not produce any growth inhibition neither in the resistance context (non host) nor in yeast cells. However, this is not surprising since full-size protein might be required for the proper folding and function. If the isolated fragments had given a similar phenotype as the entire protein, some limiting steps working with AWRs would have been circumvented. Indeed, a reduction in size would probably diminish the protein solubility problems and facilitate their biochemical characterization. For instance, western-blottings with the entire proteins needed to be migrated for long hours and transferred overnight and still, they often gave no signal. Since we did not appreciate any phenotype on tobacco or yeast cells with the fragments, we could not determine nor distinguish the domains responsible for recognition and for disease like, as was done for the *P. syringae* AvrPtoB effector (Abramovitch et al., 2006).

From our AWR functional analysis, it can be assumed that AWRs do not act as transcription regulators, since they do not target the plant cell nuclei. We could make a first step into the dissection of their plant targets through the yeast-two-hybrid technique. A vast number of the proteins found to interact with AWR4 were related to defence processes such as lignification or MPK signalling or other important processes related with ubiquitination, metabolism regulation or phosphorylation. The knowledge of molecular mechanisms of effector function from different pathogen species is currently limited and only few virulence targets have been found, most of them involved in defence signalling. Recently, a group described that the effector HopZ from *P. syringae* promotes bacterial growth in soybean by targeting an enzyme called 2-hydroxyisoflavanone dehydratase, being one of the few cases where an enzyme target has been proven (Zhou et al., 2011). This enzyme is involved in isoflavonoid biosynthesis, as part of the phenylpropanoid pathways, which seem to play a role in basal defence. Similarly, we identified the phenylalanine ammonia-lyase enzyme as an interacting protein for AWR4 in our yeast-two-hybrid screen. PAL2 is also involved in phenylpropanoid biosynthesis, which leads to lignin production, but in an upper position since it is the first enzyme of the pathway (Naoumkina et al., 2010). Nevertheless, as effector translocation via the T3SS does not seem to be a very efficient process, it is difficult to understand how few effector molecules could manipulate host enzymes that might be present at higher concentration in the plant, so that stoichiometry would be a limiting factor. Actually, it is not surprising that different effectors

evolved to interfere with similar processes to hamper plant defences and our results could contribute to the enlargement of this group of few effectors that target host enzymes.

Dechiphering plant targets for all AWR proteins, which have a clear effect on virulence and/or recognition, will broaden not only the knowledge on bacterial wilt disease caused by *R. solanacearum* but it could also be extrapolated to diseases caused by other pathogens. As a first approach to find AWR plant partners, we managed to purify AWR proteins in both *E. coli* and *R. solanacearum* bacterial cells. This will allow dissecting the interacting complexes between AWRs and plant proteins by liquid chromatography and tandem mass spectrometry analysis. This technique lately has emerged as a very powerful tool (Sharon, 2010) to dissect the complete pathogens secretome as it was recently done for *Salmonella* (Niemann et al., 2011) but also for unravelling protein complexes (e.g. membrane receptors involved in signal transduction in response to pathogens) (Heese et al., 2007) or for determining posttranslational modifications due to enzymatic activity of a particular effector like the autoacetylation of PopP2 (Tasset et al., 2010). Tagged effectors can be overexpressed *in planta*, immunoprecipitated and eluted protein complexes can be analysed by LC-MS/MS and peptide spectrum matching (Win et al., 2011). In contrast to the Y2H methodology, this system is far more sensitive and more versatile, because it allows to go beyond one-to-one interactions and also because it offers the possibility to search protein complexes in different types of plants (eg. host and non-host) which will provide clues on the effector functionality in different contexts. In our case, we will be able to determine the protein complexes for AWRs in both *A. thaliana* Col-0 and tomato plants to understand their different outcomes in these two host environments. The phenotypes of AWR expression in plants are diverse among family members and this could solve the limiting factor of not having a catalytic mutant as a control for the assays, as a member producing no phenotype can always be used as a negative control, with the hypothesis that the absence of phenotype is due to a decreased (or absent) interaction with precise targets.

Bacterial diseases in plants are difficult to control and any infection in crop plants lead to huge economical looses. Currently, the efforts are focused on the control of bacterial spread rather than curing the plant (Ellis et al., 2008). Regarding bacterial wilt disease, there are no curative chemicals so disease integrated management is crucial. Easier practices to avoid *R. solanacearum* spread need better field practices such as tool desinfectation, crop rotation, or control of the soil type and moisture since it is important for bacterial survival. Regarding chemical control, soil pretreatment with methyl bromide (Nesmith and Jenkins, 1976) appeared to be relatively efficient but it has been banished in most countries due to human

toxicity and environmental hazard. Some antibiotics such as streptomycin could also contribute to the disease control (Tahat and Sijam, 2010) but their application is risky and impractical. Another system based in biological control has been the use of specific phages to kill *R. solanacearum* (Yamada et al., 2007; Fujiwara et al., 2011), the use of rhizobacteria for an inhibitory effect (Lemessa and Zeller, 2007) or the use of avirulent mutant strains that might compete with the wilt type one (Frey et al., 1994). An alternative way is the employment of plant resistance cultivars such as the tomato Hawaii 7996 or grafted plants that could help control the infection. Furthermore, breeding from non-commercial resistant varieties into cultivars of agronomic or commercial interest is another genetic source of resistance (Tahat and Sijam, 2010). As resistant crop varieties to *R. solanacearum* are still unavailable, studying effector functions inside non-host plant cells and/or identifying their targets can be a source of resistance genes and help with introgression or transgenesis to improve commercial varieties.

Moreover, the study of effector proteins provides a valuable knowledge of the defence mechanisms of the plant but also the infection process of the pathogen. Since some signalling cascades are shared between animal and plant pathogens, and usually altered in the same way by the effectors, better understanding of these aspects for *R. solanacearum* could be applied to the field of animal pathogenicity.

In summary, in this work we have characterised the role of the AWR effectors in *R. solanacearum* pathogenicity and characterised in detail their physiological impact on the interactions with its host and non-host plants. We have also set up the basics for the biochemical characterisation of AWR biochemical function, which is ongoing in our group.

From the three main approaches followed in this work we extract the conclusions listed below:

Determination of the origin and distribution of *awr* genes among pathogenic bacteria

1. All AWR effector proteins are type III-secreted effectors.
2. AWR effector proteins are highly conserved in the *R. solanacearum* species complex and also present in other plant and animal pathogens.
3. The AWR family is especially diversified within the *R. solanacearum* species and different duplication and horizontal gene transfer events probably contributed to this process.

Functional characterisation of the AWR proteins of *R. solanacearum* GMI1000

4. A *R. solanacearum* strain devoid of *awr* genes shows a 50 fold growth reduction times when inoculated in host plants compared to the wt strain and hence a virulence role has been demonstrated for this effector family.
5. AWR proteins might be detected in *A. thaliana* plants since the Δ*awr1-5* strain infects faster than a wt strain and *P. syringae* strains expressing some AWR proteins grow less than the wt strain when inoculated in Col-0 plants.
6. *Agrobacterium*-mediated transient expression of AWRs in the leaves of non-host *Nicotiana* spp. plants induces a necrosis effect at different extents.
7. AWR5 displays the strongest necrosis in tobacco. This reaction resembles an HR phenotype, as confirmed by tryan blue and DAB staining together with the upregulation of HR-specific maker genes. Although the AWR2 phenotype is milder on leaves, it also seems to trigger HR-like responses.
8. Expression of AWR2 and AWR5 in yeast cells causes a dramatic growth inhibition indicating a conservation of their activities among eukaryotic cells.

Identification of AWR targets in the plant host

9. Several defence-related proteins such as MPK6, Kin10, PAL2 or DMR6 were found to interact with AWR4 with a yeast-two-hybrid assay although no direct evidence could be proven.
10. AWRs seem not to interfere with PAL2 enzymatic activity or protein stabilization.
11. AWR effector proteins can be produced in a soluble formand purified in His-tagged *E. coli* cells with pDEST-MBP vector and HA-tagged in *R. solanacearum* cells with the novel pRC vector after a stable genomic integration.

Materials and Methods

Strains, plasmids, growth conditions and plant material

The plasmids and strains used in this work are described in Table 10 (below) and concentration of antibiotics used for each organism is detailed in Table 11. For more information about the vectors used in this work, see Annex section.

Plasmid or strain	Relevant characteristics	Reference
STRAINS		
Escherichia coli		
Mach 1	*ΔrecA1398 endA1 tonA Φ80ΔlacM15 ΔlacX74 hsdR(r_K^- m_K^+)*	Invitrogen
DB3.1	*F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Smr) xyl5 Δleu mtl1, Sm^r*	Invitrogen
BL21 (DE3) pLysS	*F– ompT hsdSB(rB–, mB–) gal dcm (DE3) pLysS (Cl^r)*	Invitrogen
Agrobacterium tumefaciens		
GV3103	C58 chromosomal background, pMP90RK Ti plasmid, Rif^r Gm^r Km^r	(Koncz and Schell, 1986)
Saccharomyces cerevisiae		
AH109	*MATa, trp1-901, leu2–3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3, MEL1 GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ*	(Clontech)
BY4741	*MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0*	(Brachmann et al., 1998)
Ralstonia solanacearum		
GMI1000	*R. solanacearum* strain Phylotype I	(Boucher, 1985)
GMI1000-G^r	GMI1000::trp-lacZ, G^r	This work
hrpG$^-$	GMI1000 hrpG::Tn5-B20 mutant, Km^r	(Brito et al., 1999)
hrpV$^-$	GMI1000 hrcV::W mutant, Sp^r	(Cunnac et al., 2004)
P*eps-awr1*	GMI1000 overexpressing AWR1-HA, Ap^r G^r	This work
P*eps-awr2*	GMI1000 overexpressing AWR2-HA, Ap^r G^r	This work
P*eps-awr3*	GMI1000 overexpressing AWR3-HA, Ap^r G^r	This work
P*eps-awr4*	GMI1000 overexpressing AWR4-HA, Ap^r G^r	This work
P*eps-awr5*	GMI1000 overexpressing AWR5-HA, Ap^r G^r	This work
HrcV$^-$P*eps-awr1*	*HrcV*$^-$ overexpressing AWR1-HA, Sp^r Apr G^r	This work
HrcV$^-$P*eps-awr2*	*HrcV*$^-$ overexpressing AWR2-HA, Sp^r Apr G^r	This work
HrcV$^-$P*eps-awr3*	*HrcV*$^-$ overexpressing AWR3-HA, Sp^r Apr G^r	This work
HrcV$^-$P*eps-awr4*	*HrcV*$^-$ overexpressing AWR4-HA, Sp^r Apr G^r	This work
HrcV$^-$P*eps-awr5*	*HrcV*$^-$ overexpressing AWR5-HA, Sp^r Apr G^r	This work
Δ*awr1*	GMI1000 Δ*awr1*::pCZ367, G^r	M. Valls
Δ*awr2*	GMI1000 Δ*awr2*::pCZ367, G^r	M. Valls
Δ*awr5*	GMI1000 Δ*awr5*::pCZ367, G^r	M. Valls

Δ*awr3,4*	GMI1000 Δ*awr3* and Δ*awr4* mutant, G^r	This work
Δ*awr3,4,5*	GMI1000 Δ*awr3*, Δ*awr4* and Δ*awr5* mutant, G^r	This work
Δawr2-5	GMI1000 Δ*awr3*, Δ*awr4*, Δ*awr5* and Δ*awr2* mutant, G^r	This work
Δ*awr1-5*	GMI1000 Δ*awr3*, Δ*awr4*, Δ*awr5,* Δ*awr2* and Δ*awr1* mutant, G^r	This work
Pseudomonas syringae		
DC3000	*P.syringae* pv. tomato DC3000 strain, Rif^r	(Moore et al., 1989)
3000-EV	DC3000 carrying pEDV3 (AvrRPS4N) plasmid, Rif^r G^r	(Sohn et al., 2007)
hrcC$^-$	DC3000 Δ*hrcC* (formely known as *hrpH*), Rif^r G^r	(Yuan and He, 1996)
3000-*awr1*	DC3000 expressing RPS4N-HA-AWR1, Rif^r G^r	This work
3000-*awr2*	DC3000 expressing RPS4N-HA-AWR2, Rif^r G^r	This work
3000-*awr3*	DC3000 expressing RPS4N-HA-AWR3, Rif^r G^r	This work
3000-*awr4*	DC3000 expressing RPS4N-HA-AWR4, Rif^r G^r	This work
3000-*awr5*	DC3000 expressing RPS4N-HA-AWR5, Rif^r G^r	This work
CEL-EV	DeltaCEL DC3000 carrying pEDV3 (AvrRPS4N) plasmid, Rif^r Sp^r G^r	(Badel et al., 2003)
CEL-*awr1*	DeltaCEL DC3000 expressing RPS4N-HA-AWR1, Rif^r Sp^r G^r	This work
CEL-*awr2*	DeltaCEL DC3000 expressing RPS4N-HA-AWR2, Rif^r Sp^r G^r	This work
CEL-*awr3*	DeltaCEL DC3000 expressing RPS4N-HA-AWR3, Rif^r Sp^r G^r	This work
CEL-*awr4*	DeltaCEL DC3000 expressing RPS4N-HA-AWR4, Rif^r Sp^r G^r	This work
CEL-*awr5*	DeltaCEL DC3000 expressing RPS4N-HA-AWR5, Rif^r Sp^r G^r	This work
CEL-HopM1	DeltaCEL DC3000 with pORF43 (HopM1 and SchM) complementation	(Badel et al., 2003)

PLASMIDS

Entry vectors for GWY sytem		
pDONR207-GWY	Gateway entry vector for compatible PCR products, G^r Cl^r	Invitrogen
pDONR-*awr1*	pDONR207 vector with *R. solanacearum* GMI1000 Rsc2139 (+/-STOP), G^r	This work
pDONR-*awr3*	pDONR207 vector with *R. solanacearum* GMI1000 Rsp0846 (+/-STOP), G^r	This work
pDONR-*awr5*	pENTR-SD vector with *R. solanacearum* GMI1000 Rsp1024 (+/-STOP), G^r	This work
pDONR-*avrA*	pDONR207 vector with *R. solanacearum* GMI1000 AvrA (w/o STOP), G^r	This work
pDONR-*gus*	pDONR207 vector with *beta-glucuronidase* control gene (w/o STOP), G^r	L. Deslandes
pENTR-SD/D-TOPO	TOPO entry vector with SD sequence for compatible PCR products, Km^r	Invitrogen
pENTR-SD-*awr2*	pENTR-SD vector with *R. solanacearum* GMI1000 Rsp0099 (w/o STOP), Km^r	This work
pENTR-SD-*awr4*	pENTR-SD vector with *R. solanacearum* GMI1000 SD-Rsp0847 (+/-STOP), Km^r	This work
pENTR-D-TOPO	TOPO entry vector for compatible PCR products, Km^r	Invitrogen
pENTR-*awr2*	pENTR vector with *R. solanacearum* GMI1000 Rsp0099 (STOP), Km^r	This work
Vectors for plant expression		
pAMPAT-GWY-yfp_v	Gateway destination vector with constitutive expression under 35S promoter, Ct-YFPv tag, Cb^r Cl^r, suitable for plant expression	L. Deslandes

pAMPAT-*awr1-yfp$_v$*	pAMPAT vector with *R. solanacearum* GMI1000 Rsc2139, Cb^r	This work
pAMPAT-*awr2-yfp$_v$*	pAMPAT vector with *R. solanacearum* GMI1000 SD-Rsp0099, Cb^r	This work
pAMPAT-*awr3-yfp$_v$*	pAMPAT vector with *R. solanacearum* GMI1000 Rsp0846, Cb^r	This work
pAMPAT-*awr4-yfp$_v$*	pAMPAT vector with *R. solanacearum* GMI1000 SD-Rsp0847, Cb^r	This work
pAMPAT-*awr5-yfp$_v$*	pAMPAT vector with *R. solanacearum* GMI1000 Rsp1024, Cb^r	This work
pAMPAT-*avrA-yfp$_v$*	pAMPAT vector with *R. solanacearum* GMI1000 AvrA, Cb^r	This work
pAMPAT-*gus-yfp$_v$*	pAMPAT vector with *beta-glucuronidase* control gene, Cb^r	This work
pAMPAT-*yfp*-GWY	Gateway destination vector with constitutive expression under 35S promoter, Nt-YFP tag, Cb^r Cl^r, suitable for plant expression	L. Deslandes
pAMPAT-*yfp-lsu1*	pAMPAT vector with the gene encoding AWR4-interacting proteinLSU1, Cb^r	This work
pAMPAT-*yfp-wnk8*	pAMPAT vector with the partial gene encoding AWR4-interacting proteinWNK8, Cb^r	This work
pAMPAT-*yfp-pal2*	pAMPAT vector with the partial gene encoding AWR4-interacting proteinPAL2, Cb^r	This work
pAMPAT-*yfp-kin10*	pAMPAT vector with the partial gene encoding AWR4-interacting proteinKIN10, Cb^r	This work
pAMPAT-*yfp-mpk6*	pAMPAT vector with the partial gene encoding AWR4-interacting proteinMPK6, Cb^r	This work
pER8-GWY-*HA*	Gateway destination vector with oestradion inducible expression, Sp^r Cl^r, Ct-HA tag, suitable for plant expression	L. Deslandes
pER8-*awr1-HA*	pER8 vector with *R. solanacearum* GMI1000 Rsc2139, Sp^r	This work
pER8-*awr2-HA*	pER8 vector with *R. solanacearum* GMI1000 SD-Rsp0099, Sp^r	This work
pER8-*awr3-HA*	pER8 vector with *R. solanacearum* GMI1000 Rsp0846, Sp^r	This work
pER8-*awr4-HA*	pER8 vector with *R. solanacearum* GMI1000 SD-Rsp0847, Sp^r	This work
pER8-*awr5-HA*	pER8 vector with *R. solanacearum* GMI1000 Rsp1024, Sp^r	This work
pER8-*avrA-HA*	pER8 vector with *R. solanacearum* GMI1000 AvrA, Sp^r	This work
pER8-*gus-HA*	pER8 vector with *beta-glucuronidase* control gene, Sp^r	This work
pMDC7-GWY-*yfp$_v$*-HA	Gateway destination vector with oestradion inducible expression, Ct-YFPv/HA tag, Sp^r Cl^r, suitable for plant expression	E. Washington
pMDC7-*awr2-yfp$_v$-HA*	pMDC7 vector with *R. solanacearum* GMI1000 SD-Rsp0099, Sp^r (confocal microscopy only)	This work
pMDC7-*awr3-yfp$_v$-HA*	pMDC7 vector with *R. solanacearum* GMI1000 Rsp0846, Sp^r (confocal microscopy only)	This work
pMDC7-*awr5-yfp$_v$-HA*	pMDC7 vector with *R. solanacearum* GMI1000 Rsp1024, Sp^r (confocal microscopy only)	This work
pMDC7-*gus-yfp$_v$-HA*	pMDC7 vector with *with beta-glucuronidase control gene*, Sp^r (confocal microscopy only)	This work
Vectors for *R. solanacearum* cromosome integration		
pRCG-lacZ1	Promotorless pRCG vector containing the trp'-'lacZ reporter for *Ralstonia* genome insertion Ap^r G^r	F. Monteiro
pRCG-GWY-HA	Gateway destination vector with constitutive expression under *eps* promoter, Ct-HA tag, Ap^r Cl^r G^r, suitable for *R. solanacearum* chromosome insertion	F. Monteiro
pRCG-P*ep-awr1-HA*	pRCG vector with *R. solanacearum* GMI1000 SD-Rsc2139 with Ct HA-tag, Ap^r G^r	This work
pRCG-P*ep-awr2-HA*	pRCG vector with *R. solanacearum* GMI1000 SD-Rsp0099 with Ct HA-tag, Ap^r G^r	This work
pRCG-P*ep-awr3-HA*	pRCG vector with *R. solanacearum* GMI1000 SD-Rsp0846 with Ct HA-tag, Ap^r G^r	This work

pRCG-P*ep-awr4-HA*	pRCG vector with *R. solanacearum* GMI1000 SD-Rsp0847 with Ct HA-tag, Ap^r G^r	This work
pRCG-P*ep-awr5-HA*	pRCG vector with *R. solanacearum* GMI1000 SD-Rsp1024 with Ct HA-tag, Ap^r G^r	This work
Vectors for *R. solanacearum* mutagenesis		
pCM351	Vector for specitic site mutagenesis that contains two cloning regions with a gentamicin resistance cassette in between and distal LOXP sequences, G^r	(Marx and Lidstrom, 2002)
pSG15	pLAFR6 derivative vector containing CRE recombinase for resolution of LoxP fragments, Tc^r	S. Genin
pCM351-L/R*awr1*	pCM351 vector with left (EcoRI and KpnI) and right (HpaI and SacI) 1kb flanking regions of Rsc2139, G^r	This work
pCM351-L/R*awr2*	pCM351 vector with left (EcoRI and KpnI) and right (HpaI and SacI) 1kb flanking regions of Rsp0099, G^r	This work
pCM351-L*awr4*/R*awr3*	pCM351 vector with left (EcoRI and KpnI) and right (HpaI and SacI) 1kb flanking regions of Rsp0846 and Rsp0847 respectively, G^r	This work
pCM351-L/R*awr5*	pCM351 vector with left (EcoRI and KpnI) and right (HpaI and SacI) 1kb flanking regions of Rsp1024, G^r	This work
Vectors for *P. syringae* expression		
pEDV3-*rps4*N-*HA*	Expression vector with AvrRPS4 promoter for Nt-RPS4N gene fusion, Rif^r G^r, suitable for *Pseudomonas* expression	(Sohn et al., 2007)
pEDV6-*rps4*N-*HA*-GWY	Gateway destination vector with RPS4 promoter, Nt-RPS4N-HA fusion, Rif^r G^r, suitable for *Pseudomonas* expression	K. Sohn
pEDV6-*rps4*N-*HA*-*awr*1	pEDV6 vector with *R. solanacearum* GMI1000 Rsc2139, G^r	This work
pEDV6-*rps4*N-*HA*-*awr*2	pEDV6 vector with *R. solanacearum* GMI1000 Rsp0099, G^r	This work
pEDV6-*rps4*N-*HA*-*awr*3	pEDV6 vector with *R. solanacearum* GMI1000 Rsp0846, G^r	This work
pEDV6-*rps4*N-*HA*-*awr*4	pEDV6 vector with *R. solanacearum* GMI1000 SD-Rsp0847, G^r	This work
pEDV6-*rps4*N-*HA*-*awr*5	pEDV6 vector with *R. solanacearum* GMI1000 Rsp1024, G^r	This work
Vectors for *S. cerevisiae* expression		
pGBG-GWY	Gateway destination vector with ADH1 promoter and activation domain (AD), Nt c-Myc tag, TRP1, K^r Cl^r, suitable for Y2H assays	L. Deslandes
pGBG-SD-*awr*2	pGBG vector with *R. solanacearum* GMI1000 SD-Rsp0099, K^r (witout stop codon)	This work
pGBG-*awr*2	pGBG vector with *R. solanacearum* GMI1000 Rsp0099, K^r	This work
pGBG-*awr*5	pGBG vector with *R. solanacearum* GMI1000 Rsp1024, K^r	This work
pGBG-*awr*4	pGBG vector with *R. solanacearum* GMI1000 SD-Rsp0847, K^r (witout stop codon)	This work
pGAD-GWY	Gateway destination vector with ADH1 promoter and binding domain (BD), Nt-HA tag, LEU2, Ap^r Cl^r, suitable for Y2H assays(Adapted from pGBKT7 Y2H vector)	L. Deslandes
pGAD-l*su1*	pGAD vector with *lsu1* gene isolated from the Y2H screening for AWR4, Ap^r	This work
pGAD-*pal2*	pGAD vector with the partial *pal2* gene isolated from the Y2H screening for AWR4, Ap^r	This work
pGAD-*wnk8*	pGAD vector with the partial *wnk8* gene isolated from the Y2H screening for AWR4, Ap^r	This work
pGAD-*kin10*	pGAD vector with the partial *kin10* gene isolated from the Y2H screening for AWR4, Ap^r	This work
pGAD-*mpk6*	pGAD vector with the partial *mpk6* gene isolated from the Y2H screening for AWR4, Ap^r	This work

pGBKT7-*murine p53*	Yeast vector with ADH1 promoter and binding domain (BD) with *murine p53* insert, Nt c-Myc tag, TRP1, K^r (Positive control for Y2H assay when cotransformed with pTD1-1)	Invitrogen
pTD1-1	Yeast vector with ADH1 promoter and activating domain (AD) with *SV40 large T-antigen* insert, Nt-HA tag, LEU2, Ap^r (Positive control for Y2H assay when cotransformed with pGBKT7-murine p53)	Invitrogen
pGAL-GWY-HA	Gateway destination vector with GAL promoter, Ct-HA tag, URA3, Ap^r Cl^r, suitable for yeast toxicity assays	This work
pGAL-*awr*1-HA	pGAL vector with *R. solanacearum* GMI1000 Rsc2139, Ap^r	This work
pGAL-*awr*2-HA	pGAL vector with *R. solanacearum* GMI1000 SD-Rsp0099, Ap^r	This work
pGAL-*awr*3-HA	pGAL vector with *R. solanacearum* GMI1000 Rsp0846, Ap^r	This work
pGAL-*awr*4-HA	pGAL vector with *R. solanacearum* GMI1000 SD-Rsp0847, Ap^r	This work
pGAL-*awr*5-HA	pGAL vector with *R. solanacearum* GMI1000 Rsp1024, Ap^r	This work
pGAL-*gus*	pGAL vector *beta-glucuronidase* control gene, Ap^r	This work
Vectors for *E.coli* expression		
pTH19-GWY-HA	Gateway destination vector with T7 promoter, Nt-His tag, Ap^r, Cl^r, suitable for *E. coli* production	Invitrogen
pTH19-*awr*2	pTH19 vector with *R. solanacearum* GMI1000 SD-Rsp0099, Ap^r (without stop codon)	This work
pDEST15-GWY	Gateway destination vector with T7 promoter and Nt-GST tag, Ap^r, Cl^r, suitable for *E. coli* production	Invitrogen
pDEST15-*awr*2	pDEST15 vector with *R. solanacearum* GMI1000 SD-Rsp0099, Ap^r (without stop codon)	This work
pDEST14-GST-GWY	Gateway destination vector with T7 promoter, Ap^r, Cl^r, suitable for *E. coli* production	Invitrogen
pDEST14-*awr*2	pDEST14 vector with *R. solanacearum* GMI1000 SD-Rsp0099, Ap^r (without stop codon)	This work
pDEST-His_6MBP-GWY	Gateway destination vector with Tac promoter, Nt-His_6MBP tag, Ap^r, Cl^r, suitable for *E. coli* production	(Nallamsetty et al., 2005)
pDEST-His_6MBP-*awr*2	pDEST-His_6MBP vector with *R. solanacearum* GMI1000 Rsp0099, Ap^r	This work
pDEST-His_6MBP-*awr*4	pDEST-His_6MBP vector with *R. solanacearum* GMI1000 SD-Rsp0847, Ap^r (without stop codon)	This work
pDEST-His_6MBP-*awr*5	pDEST-His_6MBP vector with *R. solanacearum* GMI1000 Rsp1024, Ap^r	This work
pGEX-2T	pGEX-2T vector with pal2 entire gene, Tac promoter, Nt-GST fusion, Ap^r, suitable for *E. coli* production	(Chen et al., 2005)
pGEX-2T-*pal2*	pGEX-2T vector with *pal2* entire gene, Ap^r	This work
Other plasmids		
pGEM-T	Vector with 3' -T overhangs to ensure PCR product ligation, LacZ selection, Ap^r	Promega
pRK2013	Helper plasmid for *E. coli* conjugation in triparental mating transformation, Km^r	K. Sohn

Table 10. List of strains and plasmids used in this work.

Antibiotic	Stock (mg/ml)	*E. coli* (µg/ml)	*Ralsto.* (µg/ml)	*Pseudom.* (µg/ml)	*Agrobact.* (µg/ml)
Ampicillin (Ap)	100	100			
Carbenicillin (Cb)	50	50			25
Chloramphenicol (Cl)	30	30			
Gentamicin (G)	10	15	10 (5 liq.)	15	25
Kanamycin (Km)	50	50	50	50	50
Rifampicin (Rif)	25	50		50	50
Spectinomycin (Sp)	10	40	40	100	50
Tetracycline (Tc)	10	10	10 (5 liq.)		

Table 11. List of the antibiotics used and the employed concentration for each bacterial strain.

Escherichia coli cells were routinely grown overnight in Luria-Bertrani (LB) medium at 37 ºC (Sambrook, 2001). *Agrobacterium tumefaciens*, *Saccharomices cerevisiae* and *Pseudomonas syringae* were normally grown overnight at 30 ºC in YEB, YPDA or L-media, respectively (Sambrook, 2001). *Ralstonia solanacearum* cells were grown in complete B medium or minimal medium (MM) supplemented with 20 mM glutamate and incubated at 28-30 ºC (Boucher et al., 1987).

The plants used for the thesis experiments were 5-week old plants *Arabidopsis thaliana* ecotype Col-0, 4-week *Nicotiana benthamiana*, 5-week *N. glutinosa*, 6-8-week *N. tabacum* cv. Bottom special, 4-5 week old *Lycopersicon esculentum* cv. *Marmande*, 4-5 week *L. esculentum* cv. *Hawaii 7996* and 5 week *Solanum melongena* cv. *Zebrina* grown in long-day light conditions (short-day light conditions for Col-0 plants) with constant temperature at 22 ºC and humidity around 60%. After agrobacterium infiltrations, plants were kept in the same conditions whereas after *Ralstonia* or *Pseudomonas* inoculations they were incubated in a chamber with constant light conditions and constant temperature at 25 ºC.

Amplifications and clonings

PCR amplifications were typically performed with the proofreading Pfx DNA polymerase (Invitrogen) in a 50 µl-mix containing 0,3 mM of each dNTP, 0,6 mM of each primer, 2 mM MgSO4, 2X Pfx amplification buffer, 2X enhancer solution, 0,2 µg of DNA and 1,25 U of Pfx DNA polymerase. Verification PCRs were routinely performed with the non-proofreading GoTaq DNA polymerase as recommended by the supplier (Promega). Amplification cycles were always performed around 5 ºC below the melting temperature of the employed primers. For all clonings, DNA fragments were electrophoresed in agarose gels in TAE containing SYBR Safe

DNA gel stain (Invitrogen), bands were excised and purified with the Expin GEL SV (GeneAll) and introduced to final vector by Gateway recombination or ligation event and transferred into *E. coli* cells. Plasmids were then recovered with the Exprep Plasmid SV kit (Geneall) for clone verification with restriction enzymes (New England) and sequencing with BigDye terminator v3.1 (Applied Biosystems) followed by further cloning if required.

TOPO cloning or BP/LR recombinational cloning with the GATEWAY system was performed according to the supplier's manual (Invitrogen) and primers used for amplifications are listed in Table 12. Some of the genes were amplified with and without STOP codon or alternatively this was inserted/deleted afterwards with the QuikChange XL-Site directed mutagenesis kit (Stratagene). Unless stated contrary in the plasmids table (Table 10), clones without stop codon were only used for expression with C-terminal fusions.

For the generation of the *R. solanacearum* deletion mutant strains by the cre-lox system we amplified the around 1 Kb-long 5' (L) and 3' (R) flanking regions of the coding sequence of interest (for primer specification see Table 13) with the Pfx polymerase as specified above and the fragment in general was first cloned into pGEM-T vector (Invitrogen) to increase cloning efficiency. Prior to overnight ligation with pGEM-T at 4 ºC 5' A-overhangs needed to be added to the PCR products as follows: 6 µl of the PCR were incubated with 1 µl 10X Reaction Buffer containing $MgCl_2$, 0,2 mM dATP and 5 U GoTaq polymerase at 70 ºC for 20 minutes. The EcoR1 and SacI intern restriction sites respectively for 2139L (GAATTC>GATTTC) and 0099R (GAGCTC>GAGCAC) were mutagenized with the QuikChange XL-Site directed mutagenesis kit as specified by the provider (Stratagene) prior to cloning to the final pCM351 vector. Afterwards, cloned fragments in pGEM-T were excised with restriction enzymes and ligated overnight into the final pCM351 digested vector using the T4 DNA ligase (New England Biolabs) as described by the provider.

Primer name	Characteristics	Sequence
p2139GwyF	Amplification of *awr1* w/o stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGTCG ATTGGCAGATCAAAGT
p2139GwyR		GGGGACCACTTTGTACAAGAAAGCTGGGTGTTCGGGA GACAGTCCCGCAAGTTCT
2139mutInSTOP_F	Introduction of stop codon in *awr1* ORF	CTTGCGGGACTGTCTCCCGAATAGCACCCAGCTTTCTTG TACAAAG
AWR3024-1	Amplification of *awr2* with stop	CACCATGAAATTCTTCTCATTCAGATCGTCCGTCC
AWR3024TOPO-4stop		TCAGAGCACCTCGCCGGCCGC
p0846GwyF	Amplification of *awr3* w/o stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGCCC AATCTCCCCCGCGTTT
p0846GwyR		GGGGACCACTTTGTACAAGAAAGCTGGGTCACCGAAT TTGAGACTATTCAATTCCCG
0846mutInSTOP_F	Introduction of stop codon in *awr3* ORF	GAATAGTCTCAAATTCGGTTAGGACCCAGCTTTCTTGTA CAAAG
p1024GWY-1	Amplification of *awr5* with stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGAGA GGAGGGACAGCCTTG
p1024GWY-2		GGGGACCACTTTGTACAAGAAAGCTGGGTACTACGCG GTCGGGTCGGCTTG
Mut1024F	Mutation of stop codon in *awr5* ORF	CAAGCCGACCCGACCGCGTTGTACCCAGCTTTCTTGTA CAAAG
1024Nt_GwyF	Amplification of N-terminal part of *awr5* w/o stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGAGA GGAGGGACAGCCTTG
1024Nt_GwyR		GGGGACCACTTTGTACAAGAAAGCTGGGTCCGACCAA TGCTCGAGGATG
1024C_GwyF	Amplification of central part of *awr5* w/o stop	GGGGACCACTTTGTACAAGAAAGCTGGGTCCAACGCG GTCGGGTCGGCTTG
1024C_GwyR		GGGGACCACTTTGTACAAGAAAGCTGGGTCCGAGGCG TAGTCCTCCCAGG
1024Ct_GwyF	Amplification of C-terminal part of *awr5* w/o stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGCTG GACGGCAATGCGATGG
1024Ct_GwyR		GGGGACCACTTTGTACAAGAAAGCTGGGTCCGCGGTC GGGTCGGCTTG
AvrA-GwyFwd	Amplification of *AvrA* w/o stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGAGA AGAATCGGCAAC
AvrA-GwyRev		GGGGACCACTTTGTACAAGAAAGCTGGGTCGCCGTCG CTATCGCTATC

- Primers used for *awr2* and *awr4 gene* amplifications without stop codon to be cloned into pENTR-SD topo vectors are not specified since clones were directly obtained from M. Valls.
- Entry clone with gus gene was obained from L. Deslandes
- Reverse primers for mutagenesis are complementary to the forward sequences given above.

Table 12. List of primers used for all the clonings with the gateway system.

The primers used to amplify *awr* genes in order to introduce them in Gateway compatible or TOPO vectors are listed in this table. The primers used for mutagenizing or introducing a stop codon in the *awr* ORFs are also specified here.

Name	Primer sequence for amplification	Restriction enzyme used
L-2139fwd	GGAATTCCAACTGCGGCGGCACAGG	*Eco* RI
L-2139rev	GGGGTACCCCTGCTCTCCGTTCCAAA	*Kpn* I (cloned EcoRI)
R-2139fwd	CGTTAACGGTGGCGGCCCCCGGTGACC	*Hpa* I
R-2139rev	GGGAGCTCGGCGGTGAAGGGCGCGGC	*Sac* I
L-0099fwd	GGAATTCCCACGTACTCGCCCGGTAG	*Eco* RI
L-0099rev	GGGGTACCCCGGCGCAACCTTGAACAG	*Kpn* I
R-0099fwd	CGTTAACGCCGCCGATGCCGCCCGGC	*Hpa* I
R-0099rev	GGGAGCTCGCCGACGGCCGCCGCGCC	*Sac* I
L-0847fwd	GGAATTCCCCTCCGCGAGAATCAGC	*Eco* RI
L-0847rev	GGGGTACCCCTTGGGGGCGGACGGGG	*Kpn* I
R-0846fwd	CGTTAACGGAACGGGCACTCCGCCTTCGC	*Hpa* I
R-0846rev	GGGAGCTCGCGCCGGTGTCGGGATCG	*Sac* I
L-1024fwd	GGAATTCCTGCGACACGGATCGATG	*Eco* RI
L-1024rev	GGGGTACCCCATTGTTATGTCAACAAC	*Kpn* I
R-1024fwd	CGTTAACGATGCAGCAAACGCGTCCGACCG	*Hpa* I
R-1024rev	GGGAGCTCGGACGCGATGGCCTGCGG	*Sac* I

Table 13. List of primers used for the cre-lox mutagenesis in *R. solanacearum.*

Forward (fwd) and reverse (rev) primers used for the amplification of the 5' or left (L) and 3' or right (R) flanking regions of *awr* genes are listed in this table.Bacterial transformation

Bacterial transformations

E. coli strain was routinely transformed by the heat shock method as described in (Sambrook, 2001). RbCl- chemically competent *E. coli* MACH-1 cells (Invitrogen) were used as recipients for all clonings except for the GATEWAY-carrying plasmids, which were always transformed in the ccdB-resistant *E. coli* gyrA462 mutant strain DB3.1 (Bernard et al., 1994) and for the protein production which was done in *E. coli* B21 (DE3) pLysS strain.

Electro-competent *A. tumefaciens* GV3103 was transformed by electroporation in 2 mm cuvettes (2.5 Kev, 25 µF, 186 Ω) with 2 µl of DNA, incubated for phenotypic expression during 3-6 h, plated, and then incubated overnight in LB with suitable antibiotics until single colonies were isolated. Presence of plasmid was corroborated by 10 ml miniprep extraction (Geneall).

The standard triparental mating procedure was used to transfer plasmids from fresh *E. coli* to fresh *P. syringae* DC3000, DeltaCEL or *P. fluorescens* strains using fresh *E. coli* helper strain pRK2013 (recipient strain: helper strain: donor strain 3:1:1). Pelleted *E. coli* strains were resuspended in a total of 100 µl and were incubated for 2 days on a membrane in agar L-plate. Grown bacteria were then recovered, resuspended in 400 µl and plated 1:10 in L-media

supplemented with antibiotics. DC3000 and ΔCEL clones were inoculated in *N. benthamiana* plants for verification of HR production.

R. solanacearum GMI1000 natural transformations were performed as described (Boucher, 1985). Bacteria were grown for 2 days in minimal medium (MM) supplemented with 2% glycerol and 100 µl of the culture mixed with 2 µg (~10 µl) of purified linear DNA. The resulting suspension was then applied to a 25 mm 0,45 µm-pore size cellulose nitrate membrane filter lawn on the surface top of B medium agar plate. After incubation at 30 ºC for 2 days, membranes were recovered and bacteria re-suspended in 500 µl of sterile distilled water. 50 µl of the re-suspended cells were plated on B-agar medium containing the appropriate antibiotics and transformants were re-streaked to verify phenotype and obtain single-colony clones.

R. solanacearum gene deletion mutagenesis

The cre-loxP system (Marx and Lidstrom, 2002) was chosen in order to sequentially delete *awr* genes from the *R. solanacearum* genome. Each pCM351 vector harbouring the 5' and 3' *awr* sequences at both sides of a gentamicin cassette was linearized and introduced in *R. solanacearum* by natural transformation.

Gentamicin-resistant clones were selected as double recombination events where the gentamicin cassette (flanked by loxP sequences) had replaced the *awr* gene. If required for subsequent mutagenesis, the gentamicin cassette was excised by the cre-recombinase, which recognises the loxP sequences, by electroporating fresh competent *R. solanacearum* cells (2 mm cuvettes, 2.5 Kv, 50 µF, 129 Ω) with 2 µl circular pSG15 (Tc^r) vector. Transformants were plated on Tc antibiotic and sensitivity to gentamicin was confirmed. Later on, bacteria were grown for 2 days at 30 ºC without antibiotics in other to lose pSG15 and sensitivity to both Tc and G antibiotics was checked. Genomic DNA of the *awr* mutants was extracted as specified in (Chen, 1993) to verify *awr* deletion by PCR and by DNA hybridization to the GMI1000 genomic microarray as described in (Guidot et al., 2007) to confirm mutations and integrity of the genome.

Pathogenesis assays _in planta_ and callose staining

Ralstonia bacterial multiplication *in planta* was measured as follows: plant leaves were hand-inoculated with fresh bacteria at 10^5 cfu/ml (tomato and eggplant) or 10^6 cfu/ml (Col-0) with a 1 ml blunt syringe. *Pseudomonas* strains were inoculated at $5x10^5$ cfu/ml in *Arabidopsis* plants or $5x10^6$ cfu/ml for recording symptom development in Col-0 plants for DeltaCEL strain. Both

bacteria were recovered in 200 µl of water at day 0 and 3 days post-inoculation (4 days for *Arabidopsis* plants inoculated with *Ralstonia* and all inoculated Hawaii tomato plants). For each strain to be tested, two biological replicates were taken at 0 days post-induction and four at 3-4 days post-induction (each with 4 discs of 5 mm diameter from independent leaves). Bacterial suspensions were serially ten-fold diluted and plated in replicas on rich B medium (*Ralstonia*) or L-media (*Pseudomonas*) plates. CFUs were counted and bacterial growth calculated as the recovered colony forming units per cm^2 with respect to the original inoculums. Results were validated with one-way ANOVA test (Tukey postanalysis test) with the GraphPad software statistics package. HR assays were performed as described (Poueymiro et al., 2009) by infiltrating solutions of 1,5 x 10^8 bacteria/ml obtained from fresh colonies on adult *Nicotiana* plants.

For watering *Ralstonia* pathogenicity test, 5-week old Arabidopsis plants in Jiffy-7 peat pellets were root-cut, incubated with Ralstonia solution at 10^8 cfu/ml for 30 minutes and transferred to chamber again. Symptom appearance was recorded independently for each plant according to a wilting scale 0-4 (0: no wilting, 1: 25% leaves wilted, 2: 50%, 3: 75%, 4: death).

P. syringae DC3000DeltaCEL constructs (10^8 cells/ml) were hand-infiltrated in 5 week old *A. thaliana* Col-0 plants and leaf samples were taken at 12 to 14 hours after inoculation for callose staining. Harvested leaf samples were cleared several times in methanol 100%, washed with water and stained with aniline blue (0.05% in phosphate buffer, pH 8.0) for 24 hours. Leaf samples stained with aniline blue were then examined with a Zeiss Axiophot microscope.

<u>*Agrobacterium*-mediated gene expression in plants</u>

Transient *agrobacterium*-mediated protein expression was performed in *N. benthamiana* for phenotype characterization of bacterial AWR proteins (also in *N. tabaccum* and *N. glutinosa*), for checking their expression on the infiltrated leaves, for confocal microscopic observation, for trypan blue and DAB assays, and also for obtaining samples for qPCR experiments. *A. tumefaciens* overnight-cultured cells were centrifuged and resuspended in 10 mM $MgCl_2$, 10 mM MES and 150 µM acetosyringone for inoculation at $O.D_{600}$. 0.5-0.8 and then incubated for 1-2 hours at room temperature. The strain harbouring the P19 gene silencing suppressor was co-infiltrated in most of the cases to maximize the yield of plant transformation events (Voinnet et al., 2003). Bacterial strains were hand-inoculated with a needle-free syringe in different plant leaves from different plants. If required, protein expression was induced by painting the leaves 36 h post-infiltration with 5 µM estradiol and some Silwet L-77. For most assays, 3 leaf discs of 6 mm were harvested at 3-24 hours post-induction or 48 hours post-

inoculation, homogenized in liquid nitrogen with the TissueLyser (Qiagen) and processed afterwards. For protein detection, protein powder was directly resuspended in 250 µl of Laemmli buffer. For protein solubility studies, protein powder was mixed with a neutral buffer A (50 mM Tris-Hcl pH 8, 150 mM NaCl, 5 mM DTT, 5 mM EDTA i 50 µM AEBSF) and after extensive centrifugation the supernatant was kept separately. The pellet fraction was resuspended in a combination of buffer A and buffer B (buffer A + 1% Triton X-100) to test different detergent concentrations (0.05, 0.1, 0.5 and 1% Triton) in subsequent centrifugation steps to evaluate protein solubilisation. For coimunoprecipitation assays, homogenised proteins from plant leaves that co-expressed both HA-tagged AWR and YFP-tagged interacting proteins were resuspended in a buffer containing 50 mM Tris-Hcl pH 7.5, 150 mM NaCl 1 mM DTT, 10% glicerol and the protease inhibitor SIGMA P9599. Samples were first incubated overnight at 4 ºC with HA antibody, then 1 hour with Protein G sepharose (Sigma), washed and resuspended in Laemmli buffer for later anti-YFP western blotting. Anti-HA WB was also performed to verify AWR presence.

For protein localization studies, disc samples were mounted in water and observed under a fluorescence microscope (AXIOPHOT2 Zeiss, 500/20 nm, 600X) for the study of AWR4-interacting proteins or under confocal microscope for AWR localization (Leica DMIRE2, 525-550 for YFPv and 610-700 for chloroplasts, 20-40X objective). The images were obtained with the Leica confocal software and processed with the Fiji programme.

In order to analyse the HR-like leaf phenotypes, trypan blue (TB) and DAB staining were performed in leaves expressing AWRs. For TB staining, *Agrobacterium*-infiltrated leaves were boiled in a 1:3 Trypan Blue-Lactophenol solution with ethanol as described elsewhere and cleared in chloral hydrate solution (Keogh et al., 1980). For DAB staining, leaves were incubated overnight at room temperature with diaminobenzidine as previously described in (Thordal-Christensen et al., 1997).

RNA obtention and quantitative RT-PCRs

Two independent biological replicas of 6 tubes containing 6 leaf-discs (6 mm) from 6 different plants expressing each AWR and control proteins were harvested at different time points after induction and they were homogenized in liquid nitrogen. RNA was extracted from the samples (NucleoSpin RNA plant, Macherey-Nagel) according to the manufacturer protocol and quantified afterwards. Around 2µg of total RNA was subjected to retrotranscription with anchored oligo-$(dT)_{18}$ primers (Transcriptor First Strand cDNA Synthesis Kit, Roche). For quantitative real time PCR, a Light Cycler 480 (Roche) with SYBR Green chemistry was used for

monitoring gene expression, in two technical replicas, for HR and defence specific markers with the PCR amplification conditions recommended from the supplier. Primer sequences for the HR marker *Hsr203J* (F: GCCATGCTGATTGGTTCATGT; R: GAGCTGTCTCCGATGAGGAAT) together with the defence marker genes PR1a (F: CCTCGTACATTCTCATGGTCAAT; R: CCATTGTTACACTGAACCCTAGC) and PR1b (F: GCCCAAAACTCTCAACAAGACTA; R: CTTAACCCTAGCACATCCAACAC) and tubulin control to normilize (F: GGAGTTTACCGAGGCTGAAAG; CCTCCTGAGCTTCCTCTTCAT) were kindly obtained from D. Roby/S.Rivas laboratory. The sequence for the other HR marker *Hin1* (F: GAGGGTCACAAGAATACTAGCAGC; R: CGCATGTAAAGCTTCACTTCCATCTC) was obtained from (Takabatake et al., 2007). The results were presented as normalization towards gene expression of the *gus* control gene that should not interfere with the HR markers assessed.

SDS-PAGE and western-blot analysis

Samples were routinely mixed 1:1 with Laemmli buffer (0.2 M DTT, 0.125 Tris-Hcl pH 7.5, 4% SDS, 20% glycerol), loaded into 1 mm-wide 7.5% acrylamide gels and typical SDS-electrophoreses were run for 1-3 hours at 120-170 V (Bio-Rad). Proteins were transferred to PVDF membranes (Amersham) overnight at 4 ºC (30 V) or 1 hour at room temperature (100 V). For HA-tag detection, membranes were incubated overnight at 4 ºC or 3-5 hours at room temperature with anti-HA rat monoclonal antibody (clone 3F10, Roche) already conjugated to HRP (diluted 1/4000). For YFP detection, membranes were incubated 1 hour at room temperature with anti-GFP rabbit polyclonal antibody (Santa Cruz) already conjugated to HRP (diluted 1/1000). Membranes were soaked in Immobilon ECL (Millipore) and tagged proteins were detected either directly with a LAS-4000 mini system (Fujifilm) or with Amersham Hypercassette films (GE healthcare) and developed with silver nitrate (Medical Film Processor FPM-100A, Fujifilm).

Yeast assays

S. cerevisiae AH109 strain was employed for the Y2H assays whereas BY4741 was used for the yeast toxicity experiments. Yeast cells were transformed by lithium acetate as described in the Yeast Protocols manual (Clontech), adapted to 96-well plates for the screenings. AWR4 was cloned into the GWY-adapted pGBKT7-BD (hereafter pGBG) and it was used as a bait to find interacting proteins against a root CDNA library, cloned into the GWY adapted pGAD-AD, from *Arabidopsis* which waschallenged or not with *R. solanacearum*. A total of 1,6 x 10^6 independent clones were screened as explained in (Clontech) but employing co-transformation instead of yeast mating. After clone verification through Ade, His and X-α-GAL reporter

checking, plasmid from interacting yeast cells was recovered, subjected to PCR amplification, recombined in suitable pGAD vector and re-transformed in yeast cells harbouring pGAD-*awr* vector to re-validate interactions. The toxicity assay was performed as explained in (Munkvold et al., 2008). Protein extraction from yeast cells was done by a mild detergent lysis procedure with the Y-PER reagent according to the manufacturer (Thermo Scientific).

E. coli protein production

E. coli BL21 cells were used for both AWR and PAL2 protein production. Small scale production studies were performed for AWRs in pDEST14/15 and pTH19 vectors. *E. coli* inocula were grown overnight in 3 ml with 1 mM IPTG at 30 ºC, centrifuged, resuspended in 1X PBS, sonicated and centrifuged again. The supernatant fraction was separated from pelleted cell lysates, mixed 1:1 with Laemmli buffer and both fractions were subjected to a SDS-PAGE stained with coomassie blue to visualize protein solubiity. For *awr* production in pDEST-MBP vector, overnight *E.coli* inocula were diluted in 50 ml of fresh LB and grown at 37 ºC until $O.D._{600}$ 0.3-0.4 when 1 mM IPTG was added and the culture was then incubated at 25 ºC for 2-3 hours. Finally, cultures were centrifuged, resuspended in 1.5 ml of 1X PBS, sonicated, centrifuged and MBP-His-AWR proteins from the supernatant fraction were purified through a HisTrap column (GE Healthcare). Protein production was analysed in a coomassie-stained gels and confirmed by mass spectrometry.

PAL enzymatic assay

In order to evaluate the enzymatic activity of the phenylalanine ammonia-lyase protein, 1 to 5 µl of PAL2 protein (unpurified) were incubated at 40 ºC in 0.2 M sodium borate buffer pH 8.8 in a total volume of 1 ml. The reaction was started by adding phenylalanine at a final concentration of 40 µM, and cinnamic acid appearance was then monitored at $O.D._{290}$ for several hours. As a control, the AIP (2-Aminoindan-2-phosphonic acid) inhibitor (Appert et al., 2003) was added at a final concentration of 40 µM if required. PAL2 activity is referred to the quantity of product obtained, since increase of 1 unit in O.D. value equals to the production of 309 nmols of cinnamic acid. For AWR-PAL2 studies, 80 µl of purified AWR proteins were added to the PAL2 reaction mixture before starting the reaction.

AWR protein secretion, production and purification in _R. solanacearum_

For secretion studies, overnight-grown *R. solanacearum* bacteria were inoculated at $2x10^8$ cells per ml in 20 ml of minimal medium containing 10 mM glutamate and 10 mM sucrose as a carbon source. Congo red was also added to the cultures, at 100 µg/ml as it is known to

promote or stabilize secretion (Bahrani et al., 1997; Gueneron et al., 2000). Bacterial cultures where then grown at 25 ºC for 12-18 hours. Bacteria were then centrifuged at 4000 x g for 10 minutes at room temperature and media fraction was separated from bacterial pellet. Cells were resuspended in 1 ml of PBS buffer, lysed by sonication and mixed 1:1 with 2X Laemmli sample loading buffer. Culture supernatants were filtered through a 0.22 µm-pore membrane to eliminate residual cells and this was confirmed by plating 200 µl into a rich agar plate. Proteins were then precipitated by adding one volume of 25% trichloroacetic acid and incubating overnight at 4ºC. Precipitated proteins were pelleted by centrifugation at 6000 x g for 30 minutes at 4 ºC, washed twice in cold 90% acetone, dried, resuspended in 100 µl PBS, mixed 1:1 with 2X Laemmli buffer and subjected to western-blot analysis. A similar procedure was followed for *P. syrinage* secretion studies with a different *hrp*-inducing medium (50 mM KH_2PO_4 pH5.7 (KOH), 7.6 mM $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$, 1.7 mM NaCl) and supplemented with 10 mM Fructose instead.

To test purification of native proteins from the medium, 50 ml cultures grown as before were used, all steps were done at 4 ºC and 1X complete mini protease inhibitor cocktail (Roche) was added to the supernatant recovered from the culture centrifugation (40 ml). Proteins from the culture media were concentrated by filtration through Amicon Ultra 50K filters (15 ml, Millipore) at 4000 x g for a maximum of 2 hours until a final volume of approximately 500 µl. This concentrated fraction was then incubated overnight with 50 µl of an agarose resin coated with a monoclonal anti-HA antibody (Sigma). The resin was washed 4 times with PBS, directly resuspended in 50 µl of 2X Laemmli buffer and subjected to anti-HA western-blot analysis.

For bacterial production, a *R. solanacearum* overnight preinoculum was used (4 ml) to inoculate 50 ml of rich medium, which was then grown for around 7 hours at 25 ºC until $O.D._{600}$ 0.8-1. All steps were carried out at 4 ºC and 1X complete mini protease inhibitor cocktail (Roche) was added at all steps. The bacterial pellets were resuspended in 1.5 ml PBS buffer, lysed by sonication and supernatants overnight with 100 µl of the anti-HA agarose resin (Sigma) and then eluted with 100 µl of HA peptide (100 µg/ml) for 30 minutes. Samples were then mixed with 1:1 and subjected to anti-HA western-blot analysis.

<u>*In silico* analysis of AWR gene family</u>

To obtain similarity and identity values between AWR proteins and their representative homologues in other species we used the EMBOSS Needle online software that performs a global alignment of two given sequences (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

DNA and protein BLAST were performed separately for all AWRs sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi, non-redundant protein sequences) to find related members in other *Ralstonia* strains or other bacterial species. Protein sequences that showed similarity to AWRs (e-value < 0.01 with a sequence coverage ≥ 30% or sequence identity ≥ 20% with higher e-values) were aligned using the MAFFT program (http://mafft.cbrc.jp/alignment/server/, E-INS-i parameter suitable for sequences with multiple conserved domains and long gaps) and edited with GBlocks, allowing smaller final blocks, smaller gap position and less strict flanking positions (http://molevol.cmima.csic.es/castresana/Gblocks_server.html).

Phylogenetic analysis was performed with the MrBayes program which is based in the Bayesian inference method, with stringency convergency < 0.01 and results were contrasted with PhyML (http://www.atgc-montpellier.fr/phyml/) based in Maximum-Likelihood estimation (100 trials performed). All protein sequences used for tree constructions and their access number are described in Table 8.

InterPro scan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) was used as an integrated database for protein “signatures” prediction of AWRs. The PHYRE database was used for 3-Dimensional structure predictions (http://www.sbg.bio.ic.ac.uk/~phyre/).

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Resum en català

COM ATAQUEN ELS PATÒGENS?

Els bacteris són els éssers vius més abundants del planeta i, tot i que la majoria d'ells no són nocius, alguns poden causar malalties que afecten no només a la població humana sinó també a animals i plantes. Des de la gran fam de la patata que va tenir lloc a Irlanda o des dels postulats de Koch (s.XIX), el camp de la patologia ha experimentat un auge important. A partir d'aleshores, la malaltia va ser estudiada principalment sota el prisma de la fisiologia mentre que l'era de la genètica molecular va arrencar amb força a mitjans del s.XX i fins a principis del s.XXI. Durant aquesta època, es va formular la teoria de Flor (o teoria del gen a gen) que explicava la necessitat d'un gen bacterià d'avirulència (avr) i un gen de resistència a la planta (R) per tal que la planta pugués ser resistent a la malaltia. Els posteriors avenços en la biologia molecular van permetre l'estudi de les malalties mitjançant experiments de guany i pèrdua de funció. Més endavant, l'era de la genòmica es va establir amb força i ha perdurat fins l'actualitat on s'han seqüenciat més de 2000 genomes de patògens bacterians, fet que ha permès fer notables progressos en el camp de la fitopatologia.

Els patògens de les plantes es troben distribuïts en tots els regnes dels éssers vius, no obstant, la majoria d'ells pertanyen al grup dels bacteris gramnegatius i principalment al subgrup dels proteobacteris. Mentre que els patògens de plantes del gènere *Ralstonia*, *Burkholderia* i *Acidovorax* pertanyen als β-Proteobacteris, els *Xanthomonas* i els *Pseudomonas* pertanyen als γ-Proteobacteris. Els bacteris representen un greu impacte econòmic en l'agricultura ja que poden causar clorosi a les fulles, necrosi als tubercles o fins i tot la mort de la planta.

Per tal que un patogen infecti exitosament una planta, cal l'expressió de factors de virulència de forma altament coordinada com ara: exopolisacàrids, hormones i toxines, molècules d'adhesió o secreció d'altres molècules beneficioses per a la colonització i progressió del bacteri. El contacte del bacteri amb les cèl·lules vegetals és una peça clau en l'establiment de la malaltia. Per això, algunes proteïnes de membrana juguen un paper molt important en la detecció de l'hoste, com podria ser el cas de PrhA a *R. solanacearum* o bé proteïnes tipus adhesina presents a *Xanthomonas* i *Xylella* que són importants per l'adhesió en els vasos del xilema. Amb una funcionalitat diferent, les proteïnes de la membrana externa TolC d'Erwinia amylovora també poden intervenir a forjar la resistència a les fitoalexines provinents de la planta.

El balanç hormonal és vital per al benestar de les plantes i alguns bacteris tenen com a objectiu desestabilitzar-lo. Exemples notables són la producció d'auxines per *Pseudomonas syringae* pv *savastanoi* o de citoquinines per *A. tumefaciens*. A més, alguns patovars de *P. syringae* produeixen la coronatina, un anàleg de l'àcid jasmònic, que també interfereix amb el balanç d'hormones vegetals. A part de les hormones, les fitotoxines produïdes pels bacteris es difonen lliurement als teixits de les cèl lules vegetals i produeixen un efecte nociu, com en el cas de faseolotoxina produïda per *P. syringae* pv. *phaseolicola* que produeix una clorosi sistèmica a les mongetes. Per altra banda, la producció d'enormes quantitats d'exopolisacàrids, descrites a *Xanthomonas* i *Ralstonia*, serien també importants per a la virulència ja que, entre altres, podrien protegir el bacteri dels atacs de la planta. Un mecanisme important per als bacteris és el *quorum sensing* que permet controlar la densitat cel·lular mitjançant la secreció de molècules com ara l'homoserina lactona i així ajustar la regulació del procés d'infecció.

La secreció és un mecanisme important en els bacteris gramnegatius, fet que es veu reflexat en els sis sistemes diferents de secreció que s'hi han descrit fins ara. Una característica notable és que fins a tres sistemes (T3SS, T4SS i T6SS) tenen com a objectiu translocar ADN i/o proteïnes bacterianes a les cèl·lules eucariotes. Els sistemes de secreció I i II estan implicats en la secreció d'enzims extracel·lulars, proteases, lipases i les toxines; la secreció tipus III consta d'una xeringa macromolecular que transloca proteïnes directament en les cèl·lules hoste; el tipus de secreció IV consisteix en una estructura de tipus pilós important per la fixació en algunes superfícies, per la conjugació bacteriana o per la transferència del plasmidi Ti a *Agrobacterium*; la secreció de tipus VI també seria important per la virulència encara que el seu rol no estaria clar.

El sistema de secreció de tipus III es basa en la injecció d'unes proteïnes efectores al citoplasma de la cèl·lula vegetal i és el determinant de patogènia més important dels bacteris ja que en la seva absència són incapaços de produir la malaltia. Es tracta d'un sistema de secreció altament conservat en què més de 25 proteïnes són necessàries pel seu muntatge, set de les quals estan altament conservades en els patògens. Existeixen 7 famílies diferents de T3SS: Clamidials, Hrp1 (*E. amylovora* o *P. syrinage*), Hrp2 (*R. solanacearum*, *B. pseudomallei*o *X. campestris*), SPI-1 (*B. pseudomallei*, *S. flexneri* o *S. enterica*), SPI-2 (*E. coli* EPEC, *Y. pestis* o *S. enterica*), Rizobium i el tipus YSC (*Y. pestis* o *P . aeruginosa*). En general, el tipus de Hrp1 i HRP2 són els més prevalents en els patògens de plantes, mentre que els YSC, SPI-1 i SPI-2 són més representatius des patògens animals. Els gens codificants per al T3SS s'agrupen en un clúster *hrp* (resposta hipersensible i patogenicitat) que està altament regulat per tal d'induir

l'ensamblatge del T3SS només en cas de ser necessari i així evitar una despesa energètica innecessària. L'aparell de secreció es compon de dues parts principals: el cos basal caracteritzat per dues anelles que s'ancoren a la membrana interna i externa del bacteri, i un apèndix extern que contactarà amb la cèl·lula hoste. Mentre que el basal és una estructura conservada, i semblant al sistema flagel·lar, l'apèndix varia entre patògens: els animals presenten una estrutura en agulla mentre que els vegetals tenen una estructura pilosa més allargada que fa possible travessar la paret vegetal. A més a més, altres proteïnes accessòries contribueixen al correcte funcionament de l'injectisoma: els translocons i les xaperones.

Atès que les proteïnes efectores de tipus III (T3Es), que són translocades directament al citoplasma de l'hoste, són codificades per gens situats en regions amb un percentatge ric en GC i caracteritzades per l'ús alternatiu de codons fa pensar que s'hagin adquirit per transferència horitzontal. Contràriament a les vies de secreció dependents de Sec, els efectors no presenten cap pèptid senyal escindible sinó la naturalesa aminoacídica de la seva regió N-terminal seria clau per la seva secreció. La mida del repertori d'efectors depèn de cada bacteri, fins i tot de cada soca, però es poden arribar a injectar des de 20 fins a més de 70 proteïnes. Últimament, s'han descrit força redundàncies en el repertori d'efectors d'un determinat patogen en el sentit que diverses eliminacions de proteïnes efectores són necessàries per veure una disminució en la virulència. Aquest fet es podria explicar com un ús modular dels efectors: mentre que un grup particular d'efectors pot ser útil per un determinat context planta-patogen, altres subconjunts podrien ser clau en un altre context. La funció principal dels efectors és alterar les defences innates de la planta i així poder-la manipular al seu benefici.

COM ES DEFENSEN LES PLANTES?

Les plantes són organismes sèssils que es troben contínuament en contacte amb una enorme varietat d'agents patògens. No obstant això, i contràriament al que havia pensat durant molt de temps, les plantes presenten un ampli armament per combatre qualsevol incursió nociva. Les estratègies de la planta es poden agrupar en passives o actives. Les defenses passives són inherents a la planta, preexistents al contacte amb el patògen, mentre que les actives s'indueixen un cop s'ha detectat la seva presència. Per exemple, l'estructura física de la superfície de la planta, la secreció de substàncies antimicrobianes o la presència de cutícula o engruiximents com la suberització, dificultaran l'entrada dels bacteris. D'altra banda, molècules conservades dels patògens, com ara la flagel·lina, la quitina o el factor d'elongació Tu poden ser detectades específicament per receptors PRR de la planta que traslladaran la senyal per induir les respostes de defensa. Aquesta senyalitació inclourà un increment en els fluxes d'ions, una explosió oxidativa, la fosforilació de proteïnes i la síntesi de les hormones relacionades amb la defensa com l'etilè, àcid jasmònic i àcid salicílic. Aquest procés també es coneix com a immunitat induïda per PAMPs (PTI) i generalment no és específic de patogen. Sembla ser que existeixen diferents tipus de receptors membranals que detectarien específicament diferents elicitors bacterians però la cascada de respostes seria compartida. Tan bon punt la planta entra en contacte amb el bacteri, es produeix un canvi conformacional en el receptor que permet el reclutament i/o alliberament d'altres molècules que participaran del procés de senyalització. Immediatament després, la membrana sofreix una despolarització, producció d'espècies reactives d'oxigen i senyalització via MAPK (MPK3 I MPK6) per tal d'activar factors de transcripció WRKY i així induir l'expressió de gens necessaris per fer front a l'atac. L'àcid salicílic o l'òxid nítric també poden intervindre en aquest procés i els gens PR relacionats amb la defensa també s'activaran i induiran els depòsits de cal·losa o la lignificació.

Alguns patògens són capaços de superar aquesta primera barrera de defensa i fer possible la colonització de la planta. La injecció dels T3Es és fonamental per interferir amb les defenses de la planta, sobretot a nivell de PTI, i convertir-la en susceptible a la infecció. Al seu torn, la planta ha desenvolupat un mecanisme de detecció extremadament específic dels efectors bacterians mitjançant les proteïnes de resistència R. Aquest tipus de defensa d'hoste és específica de soca o raça i també se l'anomena immunitat induïda pels efectors (ETI). La senyalització que la segueix és similar a la presentada en la PTI, però, en generalment més ràpida, més forta i més prolongada. La ETI sovint condueix a una mort celul·lar programada anomenda resposta hipersensible (HR) que té com a finalitat restringir el creixement dels

bacteris. El reconeixement de l'efector per part de la planta pot ser mitjançant una interacció directa (model de Flor, com seria el cas de la parella PopP2-RRS1) o majoritàriament de forma indirecta gràcies a la detecció de la seva activitat (model de guarda o de l'esquer). Quan ambdues proteïnes (efectora i R) coincideixin en una interacció planta-patogen, es passarà d'una interacció compatible on es desenvolupa la malaltia a una interacció no compatible en què la planta esdevé resistent. Les proteïnes R (també conegudes com a NB-LRRs) es troben localitzades principalment al citoplasma i funcionen com a sentinelles que s'activen en detectar l'efector. Alguns NB-LRRs tenen una senyal de localització nuclear i per tant es creu que viatgen fins allà per activar tot un rang de defenses que inclouen la producció de ROS, NO, cascades MAPK, l'acumulació de SA i la reprogramació de la transcripció. A més a més, gràcies a les proteïnes PR i a l'àcid salicílic, l'estat d'aguait serà retransmès a zones distals de la planta mitjançant el procés de resistència sistèmica adquirida (SAR). Aquesta reacció proporciona una resistència d'ampli espectre a tota la planta. La mort cel·lular programada o resposta hipersensible (HR), comparteix similituds amb el procés d'apoptosi descrit en animals i apareix al final del procés de senyalització.

CARRERA ARMAMENTÍSTICA ENTRE LA PLANTA I EL PATOGEN

Entendre el procés d'infecció bacteriana de les plantes requereix tant la comprensió dels mecanismes de defensa d'aquesta com dels mecanismes d'atac del patogen. La relació entre les plantes i els patògens és més dinàmica del que s'havia suposat fins fa poc temps. Com ja hem vist anteriorment, els bacteris injecten unes proteïnes efectores al citoplasma de les plantes amb la funció d'interferir en els diferents nivells de defensa de la planta i així inhibir la PTI. Això permetrà doncs la colonització de la planta per part del bacteri i l'establiment de la malaltia. D'altra banda, algunes proteïnes vegetals han adquirit la capacitat per detectar, directa o indirectament i de forma específica, els efectors bacterians i així evitar la infecció. No obstant això, altres efectors poden ser entrenats per bloquejar les respostes ETI i ser capaç de multiplicar-se dins de l'hoste. Depenent de les fonts de resistència de la planta i del conjunt d'efectors que presenti el bacteri, ens trobarem amb un escenari favorable a la malaltia o bé amb la resistència per part de la planta.

Com ja s'ha esmentat anteriorment, la principal funció dels efectors és bloquejar les defenses de la planta. Per fer-ho, pertorben procesos cel·lulars tals com la ubiquitinització, la transcripció de l'hoste, el balanç hormonal, l'estat de fosforilació de les proteïnes, etc. Si bé alguns efectors interfereixen directament en la immunitat de la planta, altres ho fan per via indirecta. Fins ara, els efectors que han estat més ben caracteritzats són els de *P syringae* com per exemple AvrPto i AvrPtoB que descacoplen la senyalització de la PTI. Els efectors poden alterar també les defenses de les plantes d'una forma indirecta mitjançant el desequilibri hormonal. Alguns bacteris secreten fitohormones com ara la coronatina (*P. syringae*) o bé altres efectors com avrPtoB o HopI1 que poden modular les vies de l'àcid absísic o del salicílic. La interferència amb el trànsit vesicular, amb la degradació proteica o amb els patrons de transcripció pot representar també un clar avantatge pel procés d'infecció i colonització.

En les plantes, no existeix cap entitat cel·lular encarregada de la defensa sinó que cada cèl lula té la seva pròpia capacitat de reaccionar davant d'un patogen i de respondre de forma sistèmica i així informar de la presència de l'invasor per evitar la penetració. Un avenç important en el camp de la fitopatologia va ser el model de zig-zag que fou introduït per Jones i Dangl el 2006 per explicar la interacció entre plantes i patògens d'una forma dinàmica. El model explica com els bacteris són detectats en un principi per la planta conduint a la PTI, que posteriorment és abolida per algun efector que al llarg de l'evolució podrá arribar a ser

detectat per les proteïnes R de la planta, cosa que desencadena l'ETI fins que un altre efector sorgeixi per impedir-ho, i així successivament. Així doncs, existeix una pressió contínua sobre els efectors i sobre les proteïnes R en què les seves freqüències en la població dependran de la seva presència o absència en una determinada interacció. Les mutacions d'efectors juntament amb l'adquisició de nous pot suposar un avantatge a l'hora d'evitar el reconeixement de les plantes o també per adquirir la capacitat d'infectar nous hostes. Un millor coneixement sobre el procés d'infecció bacterià i sobre els determinants de virulència implicats podria millorar el maneig actual de la malaltia o bé per fer millora de plantes.

RALSTONIA SOLANACEARUM, UN FITOPATOGEN DEVASTADOR

Ralstonia solanacearum és un patogen del sòl que pertany al grup dels β-proteobacteris i és l'agent causal del marciment bacterià. El patogen presenta un ampli rang d'hoste ja que infecta més de 200 espècies de plantes que inclouen la patata, el bananer, la tomaquera o la pebrotera, fet que provoca enormes pèrdues econòmiques. L'elevada agressivitat de *R. solanacearum*, la seva complexitat intraespecífica, la seva persistència en el camp i la manca de varietats comercials resistents al marciment bacterià en fa molt difícil el control. Així doncs, una millor comprensió del seu mecanisme d'infecció juntament amb l'aïllament de loci de resistència o tolerància podrien contribuir al desenvolupament de sistemes alternatius per superar la infecció en el futur.

El patogen pot sobreviure durant llargs períodes de temps com un organisme sapròfit del sòl o en cabals d'aigua però pot penetrar en una planta hoste en un moment determinat i arribar fins al xilema on es multiplicarà en abundància i permetrà una infeccio sistèmica del vegetal. L'enorme densitat bacteriana i la secreció de grans quantitats d'exopolisacàrid bloqueja el trànsit hídric a la planta i això provoca el seu marciment.

R. solanacearum és considerat un complex d'espècies ja que agrupa soques genotípicament i fenotípicament molt heterogènies. Diversos sistemes de classificació han estat proposats en els últims anys en un intent per classificar millor aquest complex: segons les característiques bioquímiques de la soca (cinc biovars), segons el rang d'hoste (cinc races) i segons els anàlisis filogenètics que concorden amb la distribució geogràfica (quatre filotips). La raça 1 és la que presenta un rang d'hostes més ampli i la raça 3 és específica de patata i, contràriament a les altres, capaç d'infectar en climes atemperats fet que suposa un perill actual per Europa. La classificació per filotips és la següent: I (soques procedents principalment d'Àsia), II (d'Amèrica), III (de l'Àfrica i les illes properes a l'Oceà Índic) i IV (d'Indonèsia). Els bacteris *R. celebrensis* i *R. syzygii* també formen part d'aquest complex. La millor soca estudiada és la GMI1000 que pertany a filotip I, raça 1 i biovar 3. La seqüència del genoma de GMI1000 es va obtenir l'any 2002, sent el segon fitopatogen completament seqüenciat. Posteriorment, els genomes d'altres soques han esdevingut també disponibles.

R. solanacearum alberga un genoma bipartit que consisteix en un cromosoma (3,7 Mb) i un megaplasmidi (2.1 Mb) que conté alguns gens importants pel metabolisme o pel procés

d'infecció i això obliga els bacteris a mantenir els dos replicons. Tot i que el bacteri presenta un alt percentatge de GC, algunes regions de genoma presenten un percentatge diferencial i un ús alternatiu de codons. Aquesta característica s'ha atribuït als tRNAs o elements genètics mòbils (per exemple, seqüències d'inserció o de bacteriòfags) i per tant és molt possible que aquestes regions hagin estat adquirides per transferència horitzontal i puguin jugar un paper fonamental en la ràpida adaptació del bacteri. Com en altres patogens, el T3SS és el principal determinant de patogenicitat a *R. solanacearum*, ja que en la seva absència els bacteris no poden causar la malaltia. El T3SS està codificat en el clúster *hrp* que s'expandeix en una regió de 23 Kb al megaplasmidi. Malgrat això, altres sistemes de secreció (I, II, IV, V, dos associats i la secreció de la via d'exportació tat) i factors de virulencia extres (exopolisacàrid, proteïnes d'adhesia, sideròfors…) són també importants pel *fitness* bacterià o per a l'adaptació als hostes.

La regulació del tipus III a *R. solanacearum*, és a través d'una via de senyalització molt ben caracteritzada a partir de la percepció d'una senyal de la planta detectada pel receptor PrhA. Posteriorment, una cascada de transducció de senyals s'activa per activar el regulador *hrpG* que activarà a la vegada al regulador *hrpB*. HrpB, al seu torn, indueix directament la transcripció dels gens que codifiquen per la maquinària del T3SS, així com també els T3Es i altres gens relacionats amb la virulència. La cerca bioinformàtica juntament amb un anàlisi funcional del genoma ha permès la identificació de més de 70 proteïnes efectores a *R. solanacearum*. Cal esmentar que hi trobem diferents famílies conservades d'efectors: SKWPs (6 membres), família GALA (set membres) i família AWR (cinc membres). Les SKWPs són proteïnes efectores que contenen unes repeticions armadillo amb funció encara desconeguda. La familia GALA consta d'unes proteïnes LRR amb una seqüència conservada GAxALA i un domini F-box. La funció d'aquests efectors seria interferir en els procesos de l'ubiquitinització i degradació dependent del proteasoma. La darrera familia s'anomena AWR a causa del motiu conservat Ala-Trp-Arg. Tant els membres GALA com els AWRs se'ls ha atribuït un paper en la virulència. Altres efectors importants presents a *R. solanacearum* són Avra i PopP1 que són detectats per *N. benthamiana*, o PopP2 que il·lustra un dels pocs casos en què un gen d'avirulència i una proteïna R interactuen de forma directa.

OBJECTIUS

Els objectius plantejats per aquesta tesi es detallen a continuació:

- **Determinar l'origen i la distribució dels gens *awr* entre els bacteris patogènics**

Ens vàrem proposar avaluar la conservació de la família gènica *awr* entre les soques de *R. solanacearum* i també comprobar la seva presència en altres organismes mitjançant l'ús d'eines bioinformàtiques. Els nostres resultats proporcionarien una informació valuosa sobre l'evolució de les AWRs i la seva possible funció.

- **Caracterització functional de les proteins AWR de la soca GMI1000 de *R. solanacearum***

Es va decidir esbrinar la contribució dels AWRs en les interaccions de *R. solanacearum* amb les seves plantes hoste i no hoste. Per tal de dur a terme això, vam aplicar aproximacions de guany i pèrdua de funció tant en la soca natural com en sistemes heteròlegs.

- **Identificació de les proteïnes diana dels efectors AWR dins la cèl·lula vegetal**

Per tal de desentrellar el mecanisme dels AWRs un cop injectats al citoplasma de la cèl·lula vegetal, vam decidir iniciar la cerca de les seves dianes a la planta. Les proteïnes que interaccionen amb els AWRs van ser identificades mitjançant l'assaig del doble híbrid en llevat, fet que va proporcionar informació sobre els processos que són manipulats pel fitopatogen.

DISTRIBUCIÓ I CONSERVACIÓ DE LES PROTEÏNES EFECTORES AWR A *R. SOLANACEARUM* I A ALTRES ESPÈCIES

D'entre tots els efectors candidats a *R. solanacearum*, els AWRs són una de les famílies multigèniques presents en aquest fitopatogen. El nostre interès per aquests gens prové d'un assaig de patogènia en plantes de tomàquet on es va demostrar que *awr2* era un dels pocs efectors que en ser mutats provocaven una disminució de la patogenecitat. La nomenclatura AWR es va atribuir arran de la tríada conservada alanina-triptòfan-arginina present en la seqüència peptídica de la soca GMI1000. La família està composta de cinc gens: *Rsc2139*, *Rsp0099*, *Rsp0846*, *Rsp0847* i *Rsp1024* que hem anomenat *awr1*, *awr2*, *awr3* , *awr4* i *awr5*, respectivament. Excepte *awr1*, aquests gens estan situats en el megaplasmidi en zones d'ús alternatiu de codons i percentatge GC diferencial, fet que fa sospitar sobre la seva adquisició via transferència horitzontal. Els gens *awr3* i *awr4* es troben adjacents al clúster *hrp*. Aquests gens *awr* (excepte *awr1*) es troben regulats per *hrpB* que és el regulador clau del T3SS i codifiquen per proteïnes de més de 1000 aminoàcids. Exceptuant AWR1, la secreció de les proteïnes AWR ha estat validada mitjançant l'assaig reporter cyaA a la soca GMI1000 o en la seva homòloga RS1000. Després d'un alineament aparellat de les AWRs, sabem que presenten una identitat proteica del 20-53% i una similitud del 27-62%.

La secreció de la proteïna AWR2 ha estat l'única que s'ha provat fins al moment a la soca GMI1000. Els altres efectors AWR3, 4 i 5 es van validar a la soca RS1000. La proteïna AWR1 va aparèixer en tots els casos com a no secretada pel tipus III, tot i ser predita com a proteïna efectora, i això fa pensar que sigui un gen no funcional. En tots els casos es va avaluar només la seqüència N-terminal dels efectors i per això nosaltres ens vàrem proposar determinar la secreció dels efectors AWR sencers a la soca GMI1000. Recentment, un nou sistema de vector per a la integració gènica a *R. solanacearum* s'ha desenvolupat al nostre grup. Així doncs, els gens *awr* van ser clonats en aquest vector i posteriorment introduïts al patogen salvatge i a la soca mutant *hrp* sota el promotor fort *eps* i l'epítop HA per a la seva immunodetecció.

Per tal d'avaluar la secreció de les proteïnes AWR, les soques de *R. solanacearum* es van créixer fins al final de la fase exponencial en medi inductor dels gens *hrp* i després d'una centrifugació, el medi es va filtrar i precipitar amb àcid tricloacètic per tal de concentrar les

proteïnes secretades al medi. Tant la fracció de lisats bacterians com la de concentrats proteics del medi van ser analitzats per *western-blot* amb un anticòs anti-HA. Els efectors AWR2, 3, 4 i 5 van ser injectats al medi en la soca salvatge GMI1000 però no en la soca mutant que és deficient pel sistema de secreció de tipus III. Per tant, aquest nou vector ens ha permès validar la seva secreció dependent del T3. Sorprenentment, assajos preliminars apuntarien que l'efector AWR1 seria també secretat al medi. La raó per la qual la seva secreció no havia pogut ser detectada fins ara pot ser a causa de les limitacions del estudis mitjançant el sistema cyaA.

Un cop es va demostrar la naturalesa d'efectors per part dels AWRs, es va procedir a realitzar una cerca de proteïnes homòlogues en base a la seva seqüència peptídica mitjançant comparacions per BLAST. Exceptuant el gen *awr1,* es va confirmar que els AWRs són presents en totes les soques de *R. solanacearum* (el genoma de les quals ha estat seqüenciat). A més a més, similituds significatives als *awrs* es van detectar també en algunes espècies d'altres patògens vegetals com per exemple *Xanthomonas*, *Acidovorax* o *Burkholderia* o fins i tot al patogen animal *Burkholderia pseudomallei*.

Per identificar les regions més conservades dels AWRs, totes les proteïnes que van presentar una similitud significativa en la seqüència (E-value < 0,01 i cobertura ≥ 30% o una identitat de seqüència ≥ 20%) es van alinear i editar. La conservació aminoacídica es distribueix al llarg de l'aliniament de les proteïnes i presenta algunes zones altament conservades de forma dispersa, entre les quals es troba el domini AWR. Per entendre millor les relacions entre els diferents AWRs, hem construït un arbre filogenètic basat en similituds de seqüència i arrelat en el grup *Xanthomonas* ja que els γ-Proteobacteris es troben filogenèticament més allunyats als altres bacteris. Curiosament, cada membre AWR s'assembla més als seus ortòlegs d'altres soques que als altres membres paràlegs dins la mateixa soca. Els efectors AWR3 i AWR4 apareixen com dues branques bessones en l'arbre filogenètic, fet que es correlaciona amb la seva elevada similitud aminoacídica i per tant és molt plausible que hagin sofert un esdeveniment de duplicació recent. A més, alguns fitopatògens del gènere *Burkholderia* semblen tenir un efector estretament relacionat amb l'avantpassat del AWR3/4 i un homòleg a AWR1, l'existència del qual es limitava fins ara a *R. solanacearum* de filotip I. AWR2 apareix com una branca bessona de AWR1 i AWR5 és una altre aproteïna efectora estretament relacionada amb aquest grup. Un avantpassat més llunyà de les proteïnes AWR també es troba en el patogen humà *Burkholderia pseudomallei*.

En base a l'arbre filogenètic construït que il·lustra la conservació i l'origen de les proteïnes efectores AWR, més el panorama evolutiu dels proteobacteris descrit en la literatura científica,

proposem un model evolutiu on situem l'origen de les proteïnes AWR abans de l'esdeveniment d'especiació entre β i γ-proteobacteris. A partir d'aquest origen, la família gènica *awr* hauria sofert diverses duplicacions i/o delecions juntament amb diferents esdeveniments de transferència horitzontal gènica que culminaria amb el panorama present de les proteïnes efectores AWR.

Finalment, es va fer voler determinar si els AWRs tenien algun domini estructural conservat que fos característic d'alguna proteïna amb funció ja caracteritzada però totes les cerques van produir resultats estadísticament no significatius.

CARACTERITZACIÓ FUNCIONAL DE LA FAMÍLIA AWR *IN PLANTA*

En el capítol anterior hem demostrat que els AWRs són una familia d'efectors dins del complexe d'espècies *R. solanacearum* i que es troben també presents en altres agents patògens, inclòs el patogen animal *B. pseudomallei*. Això posa en relleu la seva importància per la infecció ja que en cas contrari s'haurien perdut o durien mutacions que haurien trencat la seva pauta de lectura i per tant serien no funcionals. Tal i com s'ha esmentat anteriorment, un estudi va mostrar que la deleció de l'efector *awr2* produïa una disminució de la patogenicitat bacteriana de *R. solanacearum*. Per totes aquestes raons, vam decidir avaluar el paper en la virulència dels AWRs a través d'estudis de guany o pèrdua de funció.

Per tal d'avaluar millor la contribució conjunta de tota la família gènica en la patogenicitat bacteriana, es va construir un mutant múltiple de la soca GMI1000 *R. solanacearum* desproveïda de tots els membres de la familia. Per dur-ho a terme, es va utilizar una metodologia que es basa en la substitució del gen d'interès per un gen de resistència a gentamicina flanquejat per les seqüències loxP. Posteriorment, la transformació del bacteri amb un vector que duu la recombinasa *cre* provocarà l'escissió del gen de resistència, permetent així la realització de mutacions successives. Malgrat que aquesta metodologia és extremadament útil, el procés has estat dificultós a causa de l'elevada inestabilitat genòmica de *R. solanacearum*.

Una vegada que les supressions es van obtenir, es va avaluar la virulència del mutant múltiple i dels intermediaris a tomaquera (marmande i Hawaii 7996) i alberginiera (zebrina). La patogenicitat es va avaluar mesurant el creixement bacterià al cap de 3 dies després de la seva infiltració a les fulles de la planta. La soca GMI1000 salvatge que du un gen de resistència a gentamicina va ser la ultilitzada com a soca control. Mentre la soca GMI1000 es va multiplicar entre 3 i 4 log dins la planta, el mutant *hrp* només ho va fer un 1.5 log. En general, el creixement bacterià va ser lleugerament inferior per totes les soques en el tomàquet tolerant a la infecció Hawaii 7996. En totes les plantes hoste vam poder visualitzar una reducció significativa del mutant quíntuple pels *awr* i totes les construccions intermèdies. En general, els mutants mancats de totes les proteïnes AWRs es multipliquen fins a 50 vegades vegades menys dins la planta, fet que demostra el seu paper en la virulència. Es va poder observar també una disminució progressiva en la virulència a mesura que els *awrs* s'anaven mutant. La virulència del mutant simple *awr2* va ser també avaluada mitjançant aquesta metodologia

d'infecció i vam obtenir resultats comparables als descrits en la literatura mitjançant la infecció per reg. No obstant, la resta de delecions simples a la soca GMI1000 no van produir cap diferència estadísticament significativa en la virulència respecte la soca salvatge. Aquests resultats reforcen la idea d'un efecte additiu dels efectors de tipus III que, de fet, ja ha estat descrit en altres patògens. Els efectes sobre la virulència bacteriana estaven lligats a l'absència dels gens *awr* ja que l'eficàcia biològica bacteriana continuava sent la mateixa, tal i com es va avaluar amb un creixment bacterià en medi ric.

Contràriament als resultats obtinguts en tomàquet i albergínia, el mutant múltiple per les *awrs* semblaria que es multipliqués més en *A. thaliana* Col-0 que la soca salvatge. Aquests resultats van ser confirmats amb l'assaig de patogenicitat per reg on es va veure que la soca *Δawr1-5* produïa un avançament d'uns dos dies en l'aparició dels símptomes respecte la soca salvatge. Paral·lelament, per tal de caracteritzar millor l'efecte de les proteïnes de AWR en la virulència bacteriana, un assaig de guany de funció es va dur a terme mitjançant l'expressió heteròloga dels gens *awr* a *P. syringae* DC3000, un patogen natural d' *Arabidopsis*. Prèviament, es va comprovar la correcta expressió dels efectors de *R. solanacearum* i es va demostrar així que aquests efectors podien ser injectats a través del sistema de secreció de tipus III de *P. syringae*.

La soca *P. syringae* DC3000 que expressa de forma heteròloga diferents AWRs, juntament amb la soca wt control i el mutant *hrp* es va inocular a les fulles d'*A. thaliana* i el creixement bacterià es va avaluar al cap d'uns dies. Els nostres resultats mostren que el creixement bacterià es va reduir notablement quan s'expressaven els efectors AWR4 i AWR5, mentre que l'expressió de l'efector AWR2 afavoria una major creixement de *P. syringae* a *Arabidopsis.* Aquests resultats estableixen que, a més del seu paper en la virulència en l'hoste natural, alguns AWRs també podrien ser reconeguts per part de la planta. Aquests resultats es van reproduir també amb la soca hipovirulent a DeltaCEL.

El fet que els efectors AWRs causin diferents reaccions en tomaquera o en alberginiera comparat amb les de Col-0, no és difícil d'entendre ja que *A. thaliana* no és un hoste natural de *R. solanacearum* i a més a més altres estudis han mostrat respostes dispars en aquestes plantes. En realitat, *A. thaliana* ha estat una font important de resistències als patògens i el seu estudi ha portat al descobriment de l'única proteïna R que confereix resistència a *R. solanacearum*.

Per altra banda, volíem avaluar si els AWRs suprimien la PTI a *A. thaliana* i per això es van observar els depòsits de cal·losa induïts per la infecció amb la soca DeltaCEL. Aquesta soca està mancada de l'efector HopM1 que suprimeix els depòsits de cal·losa de la planta i per tant

indueix uns valors normals de depòsits de cal·losa a la planta. Entre tots els efectors de *R. solanacearum* que es varen provar, la proteïna AWR5 va reduir de forma considerable els depòsits de cal·losa en la planta. Aquest resultat entra en conflicte amb els experiments de creixement dels bacteris dins la planta. Per tant, caldran més anàlisis per desxifrar quins són els fets darrere d'aquesta supressió de cal·losa.

L'expressió heteròloga dels efectors *in planta* i l'observació del fenotip resultant proporciona importants pistes sobre la seva funció ja que es troben en un context on no hi ha altres efectors que puguin emmascarar la seva activitat. Atès que els AWRs podrien ser reconeguts en algunes plantes, vam decidir determinar el seu impacte en les plantes no hoste *N. benthamiana*. La seva expressió transitòria mitjançant *Agrobacterium* va provocar una reacció de necrosi a diferents nivells depenent de la AWR expressada. El major grau de necrosi va ser causat per la proteïna AWR5, seguit de la proteïna AWR2, mentre que les AWRs 3 i 4 eren indistingibles de les fulles on s' expressava el gen control *gus*. L'expressió de totes les proteïnes de fusió amb HA va ser verificada per la tècnica del *western-blot* utilitzant un anticòs anti-HA. Els fenotips causats per AWR5 i AWR2 eren també evidents en inocular altres plantes de tabac com ara *N. glutinosa* o *N. tabacum.*

L'efector AWR5 és el que provocava una major necrosi en plantes del tabac i, curiosament, era també el que produïa una reducció més dràstica del creixement de *P. syringae in planta*. Juntament amb el fet que la seva forta necrosi s'assembli a la provocada per avrA (efector que produeix una mort cel·lular programada o resposta hipersensible) va fer sospitar que aquest efector també fos reconegut en les plantes del tabac, induint una resposta hipersensible (HR). Per tal de conformar-ho, es van realitzar inoculacions a les fulles del tabac a diferents concentracions *d'Agrobacterium* per avaluar el comportament d'AvrA i AWR5 i es va provar que la planta reaccionava de forma similar amb els dos efectors. Mitjançant la tinció amb blau tripan (marcador de cèl·lules mortes) i de DAB (marcador de producció d'H_2O_2) vam acabar de concloure que AWR5, i AWR2 més suaument, causaven una resposta de tipus HR. Això va ser confirmat *a posteriori* mitjançant unes PCR en temps real sobre unes mostres de planta que expressaven els diferents de la família AWR, on es va veure que els gens específics d'HR *Hin1* i *Hsr203j* estaven sobreexpressats quan les proteïnes AWR5 i AWR2 hi eren presents. Segons els nostres resultats, l'increment en l'expressió dels gens marcadors HR correlaciona perfectament amb els fenotips observats en les plantes.

En un intent d'obtenir pistes sobre els esdeveniments que condueixen a la necrosi causada pels AWRs, vam determinar el compartiment cel·lular al qual es dirigien aquest efectors. Per

això es van mirar discs de fulles que expressaven de forma transitòria els AWR sota el microscopi confocal. Tots els efectors es localitzaven al citoplasma cel·lular o a la membrana. Això deixa enrere la possibilitat que els AWRs siguin factors transcripcionals ja que en cap moment es dirigeixen al nucli cel·lular.

CARACTERITZACIÓ DELS EFECTORS AWR A *S. CEREVISIAE*

En el capítol anterior, hem demostrat que els AWRs són efectors importants per la virulència de *R. solanacearum* però també poden ser detectats d'alguna manera pels sistemes de vigilància de les plantes. Com per a molts altres efectors, la funció bioquímica i/o les seves proteïnes diana en la cèl·lula vegetal segueixen sense resoldre's. El llevat ha esdevingut els últims anys un bon model per estudiar la funció dels efectors ja que molts mecanismes cel·lulars estan conservats en els eucariotes. A més a més, les proteïnes de resistència no es troben presents en aquest context i això facilita l'estudi de la funció dels efectors.

En expressar els AWR en cèl·lules de llevat, mitjançant un sistema induïble per galactosa, ens vam adonar que els llevats presentaven una inhibició del creixement. Aquesta inhibició era molt dràstica per l'efector AWR5 i la seguia l'efector AWR2. De nou, aquests dos efectors tornen a ser els que provoquen un fenotip més important. A causa de la gran toxicitat en llevat o per les grans dimensions de les proteïnes AWR, va ser impossible determinar la seva expressió mitjançant *western-blot*. Una caracterització més profunda d'aquests fenotips, per exemple determinar si es tracta de mort cel·lular o relentiment del creixement cel·lular, contribuirà a tenir un millor coneixement de la seva funció.

La tècnica del doble híbrid en llevat és molt potent per trobar proteïnes que interactuïn amb els AWRs. Per això es va escollir l'efector AWR4, ja que era el menys tòxic i per tant l'únic amb el qual es podia treballar sense problemes, per trobar proteïnes interactores a partir d'un crivellatge amb una genoteca d'arrels d'*A. thaliana* que havia estat infectada o no amb *R. solanacearum.* Més d'un milió i mig de clons van ser crivellats i els positius pels gens reporters d'auxotrofies i X-α-GAL avaluats van ser amplificats per PCR i retransformats en el llevat. Després d'una segona ronda de confirmacions, una cinquantena de clons positius es van seqüenciar per poder determinar quins eren els gens codificants mitjançant una cerca de similitud de seqüència per BLAST.

D'entre totes les proteïnes interactores identificades, LSU1, MPK6, AKIN10, PAL2 o DMR6 ens van semblar prou interessants ja que estan involucrades en les defenses de la planta. La LSU1 és una proteïna que respon a baixos nivells de sulfur i fou trobada fins a 7 vegades. El sofre sembla ser un factor limitant en el medi natural i és necessari tant pel metabolisme general de les plantes com per la síntesi de compostos necessaris per la defensa (defensines,

glucosinolats, fitoalexines ...). Diferents quinases van aparèixer també com a interactors de l'AWR4, éssent la MPK6 la més important en termes de defenses ja que forma part de la cascada de senyalització per estrés biòtic. KIN10 està involuclada en la integració de la regulació del metabolisme de sucres i estrés a la planta. Un altre interactor prometedor és la fenilalanina amoni-liasa 2 (PAL2) que codifica pel primer enzim de la via dels fenilpropanoides que culmina amb la producció dels precursors de la suberina i la lignina que són importants per a les defenses. La proteïna DMR6 codifica per la 2-oxoglutarat (2OG)-Fe (II) oxigenasa i s'ha relacionat amb el mecanisme de resistència al patogen *H. parasitica.* Altres proteïnes d'interès són les annexines, la quinasa WNK8 o l'enzim ornitina carbamoiltransferasa. Tot i l'elevada similitud entre les proteïnes AWRs, els interactors LSU1, WNK8, PAL2, KIN10 i MPK6 interaccionaven de forma específica amb la proteïna AWR4 i no amb els altres membres de la família AWR. Tanmateix, caldria utilitzar tècniques alternatives per confirmar aquestes interaccions proteïna-proteïna.

En un intent de caracteritzar les interaccions entre l'AWR4 i les proteïnes LSU1, WNK8, PAL2, KIN10 i MPK6, es van realitzar experiments d'estabilitat o d'interferència amb la resposta hipersensible causada per l'efector avrA en *N. benthamiana*. En cap cas es va veure que aquests interactors poguessin accelerar o alentir la mort provocada per avrA. Tampoc vam poder veure que l'efector AWR4 provoqués una estabilització proteica dels seus efectors ni una inducció de la degradació ni un canvi de localització. Pel que fa a PAL2, no es va poder determinar si els AWRs interferien d'alguna manera en la seva activitat enzimàtica.

PRODUCCIÓ DE LES PROTEÏNES AWR

Les proteïnes AWR són una família important d'efectors a *R. solanacerarum*: hem demostrat que AWR1, AWR2 i AWR4 són importants tant per la virulència com per la resistència. No obstant, la seva funció encara no ha estat descoberta. La forta toxicitat que produeixen alguns AWRs en el llevat ens ha forçat a posar a punt sistemes alternatius a fi de cercar les seves dianes a la planta.

Inicialment, es va veure que les proteïnes expressades transitòriament a *N. benthamiana* fusionades a un epítop HA no podien ser obtingudes de forma soluble. Posteriorment, es va decidir avaluar la seva solubilitat a *E. coli* sota l'expressió de diferents vectors amb fusió a GST, His o sense epítop de fusió. Es va aconseguir produir l'efector AWR2 en grans quantitats però en cap dels casos es va poder obtenir proteïna soluble. Probablement, les seves dimensions proteiques poden dificultar la seva producció i podrien precipitar en cossos d'inclusió a causa d'una saturació de la maquinària cel lular. En vistes que aquests vectors no eren adients per una correcta solubilitat proteica, vam utilitzar el vector pDEST-MBP per expressar quimèricament les AWRs amb la proteïna d'unió a maltosa (MBP). A partir d'aquest moment, i gràcies a una inducció de la producció a baixa temperatura (al voltant dels 25 ºC) hem pogut detectar els AWRs en les fraccions solubles dels lisats bacterians. Mitjançant aquest sistema, els AWRs es van purificar i analitzar per espectrometria de masses que va verificar la seva presència i integritat.

Després de veure la dificultat per expressar les AWRs de forma heteròloga a *E. coli,* vam pensar que la seva expressió directament a *R. solanacearum* seria més fàcil. Així doncs, vam utilitzar les mateixes soques de *Ralstonia* utilitzades per demostrar la secreció dels AWRs i les vam créixer en medi ric fins al final de la fase exponencial. Després, les proteïnes es van precipitar i la seva producció es va analitzar mitjançant un *western-blot*. Totes les proteïnes van ser produïdes, gràcies a l'elevada expressió del promotor *eps* i poden ser purificades mitjançant la seva unió selectiva a una resina anti-HA. Un cop hem aconseguit produir de forma soluble les AWRs, tan en *E. coli* com en *R. solanacearum*¸ ja només cal utilitzar-les fixades en una columna per buscar proteïnes interactores de diferents plantes d'interès.

CONCLUSIONS

Ralstonia solanacearum és un patogen devastador que requereix el sistema de secreció de tipus III per infectar exitosament les plantes. Tot i ser un dels patògens amb el nombre més elevat d'efectors, les funcions de la majoria estan encara per determinar. D'entre tots els efectors presents a *R. solanacearum*, nosaltres ens hem centrat en la caracterització d'una de les seves famílies multigèniques: la família AWR. Estudis bioinformàtics i funcionals han estat crucials per a la identificació dels efectors. Els nostres resultats demostren la secreció de les proteïnes AWR senceres i així validen els experiments realitzats anteriorment amb el fragment N-terminal mitjançant el sistema cyaA. També vàrem demostrar per primera vegada la secreció de la proteïna AWR1.

Les proteïnes AWR tenen un paper important en la virulència de *R. solanacearum* ja que un patogen mancat dels cinc gens *awr* es multiplica fins a 50 vegades menys dins de les plantes hoste. Per altra banda, els mutants *awr* simples només van exhibir un efecte molt lleu sobre la patogenicitat i això apunta cap a un efecte cooperatiu entre els efectors. Aquesta redundància sovint s'associa a un sistema per utilitzar combinacions diferents d'efectors per a la colonització d'un hoste en particular. Curiosament, hem observat també que *awr*1 (l'últim en ser mutagenitzat en el mutant quíntuple) també té un paper en la virulència ja que aquesta soca mutant es multiplica encara menys que l'anterior (soca que conté *awr1*). A més, recentment s'ha vist que una soca de *R. solanacearum* Psi07 que ha adquirit artificialment una porció de 24.4 Kb del genoma de la soca GMI1000 és més virulenta en tomaquera i, sorprenentment, *awr1* va ser una de les 20 noves ORFs de les quals només dos gens efectors hi estaven representats. Això recalca la importància d'estudiar aquesta proteïna AWR que anteriorment ha estat classificada com a no funcional, però també els altres membres de la família.

A més, l'impacte de l'expressió dels AWRs en *A. thaliana* (a través d'*Agrobacterium* o de *P. syringae*) o en plantes no hoste com el tabac (mediada per *Agrobacterium*) revelen que aquests efectors són reconeguts pel sistema de vigilància de la planta. Aquest doble paper en la virulència i el reconeixement de la planta ja ha estat documentat per altres efectors bacterians i s'ha interpretat com una coevolució entre planta i patogen que s'acaba traduint en interaccions compatibles (hoste) o incompatibles (no hoste). L'efecte de les proteïnes AWR en plantes no hostes es correlaciona amb la inhibició del creixement en llevats que expressen les proteïnes. No obstant això, en aquest organisme model les proteïnes R estan absents i per tant

estem percebent l'activitat de virulència dels efectors AWR que podria estar perfectament conservada entre els organismes eucariotes. Cada efector pot ser important per un context determinat i descobrir com es conserven en les filogènies dels bacteris podria donar pistes sobre la seva importància. Les proteïnes efectores AWR estan altament conservades en totes les soques de *R. solanacearum* però també es troben presents en altres soques fitopatogèniques. A més, els homòlegs AWR també estan presents en el patogen humà *B. pseudomallei*, l'agent causal de melioïdosi. L'àmplia dispersió de gens *awr* podria venir donada pel fet de compartir un nínxol ecològic que facilitaria la transferència lateral de gens entre les diferents soques patogèniques.

A partir de la nostra anàlisi funcional de les proteïnes AWR, es pot suposar que aquestes proteïnes no actuen com a reguladors de la transcripció ja que no es dirigeixen al nucli de les cèl·lules vegetals. Per tal de fer un primer abordatge sobre la funció dels AWRs, es va optar per un crivellatge en llevat mitjançant la tècnica del doble híbrid. Un gran nombre de proteïnes que vàrem trobar com a interactors de la AWR4 estaven relacionats amb processos de defensa, com ara lignificació o senyalització MPK o altres processos importants com la ubiquitinació, regulació del metabolisme cel·lular o interferència amb l'estat de fosforil·lació proteica.

Els AWRs tenen un efecte clar sobre la virulència i el reconeixement en les plantes, per tant, el descobriment de les molècules amb qui interactuen dins la cèl·lula vegetal permetrà una ampliació no només del coneixement actual sobre la malaltia del marciment bacterià sinó que també podria ser extrapolat a les malalties causades pels altres agents patògens. Així doncs, vam aconseguir produir proteïnes AWR pures en *E. coli* i en *R. solanacearum,* cosa que permetrà diseccionar els complexes d'interacció entre les AWRs i les proteïnes vegetals per immunoprecipitació i posterior anàlisi mitjançant la tècnica d'espectrometria de masses. L'estudi de les funcions dels efectors o la identificació de les seves proteïna diana dins la cèl·lula vegetal poden ser una font de nous gens de resistència a les malalties a partir de la introgressió en varietats comercials.

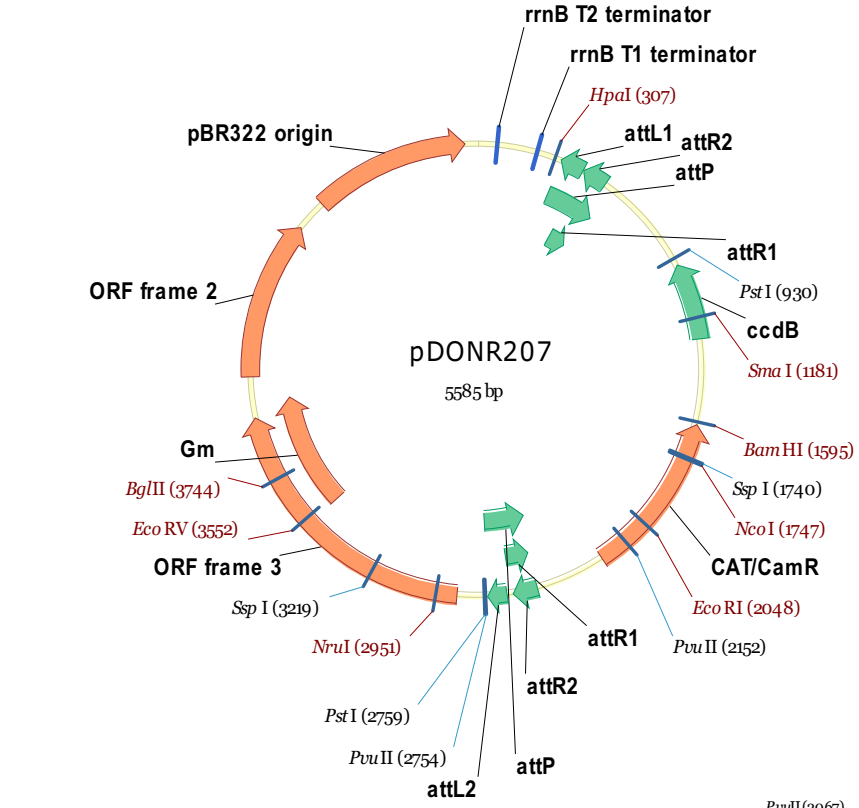
En resum, a partir d'aquest treball s'extreu el següent:

1. Totes les proteïnes efectores AWR són efectors secretats pel tipus III. .
2. Les proteïnes efectores AWR estan molt conservades en el complex d'espècies de *R. solanacearum* i també són presents en altres patògens animals i vegetals.

3. La família AWR està especialment diversificada dins de l'espècie *R. solanacearum.* Probablement, la duplicació i la transferència horitzontal de gens va contribuir a aquest procés.
4. Una soca de *R. solanacearum* desproveïda dels gens *awr* presenta un creixement 50 vegades menor quan s'inocula una planta hoste en comparació amb la soca salvatge.
5. Les proteïnes AWR podrien ser detectades ja que la soca de Δ*awr1-5* infecta més ràpid les plantes d'*A. thaliana* que la soca salvatge i les soques de *P. syringae* que expressen alguns dels efectors AWR créixen menys que la soca salvatge quan són inoculats en plantes Col-0.
6. L'expressió transitòria dels AWRs en les fulles de les plantes no hoste *Nicotiana* spp. indueix diferents graus de necrosi.
7. La proteïna AWR5 ocasiona la necrosi més forta en tabac. Aquesta reacció s'assembla a un fenotip de resposta hipersensible (HR), tal i com es confirma amb la tinció per trypan blue i DAB, juntament amb la sobreexpressió de gens marcadors específics de la HR. Encara que el fenotip de AWR2 és més suau en les fulles, també sembla desencadenar respostes de tipus HR.
8. L'expressió de AWR2 i AWR5 en cèl·lules de llevat provoca una inhibició espectacular del seu creixement, fet que indica una conservació de les seves activitats entre les cèl·lules eucariotes.
9. Diverses proteïnes relacionades amb la defensa com MPK6, Kin10, PAL2 o DMR6 van aparèixer com a interactors per a l'efector AWR4 mitjançant un crivellatge de doble híbrid tot i que encara no s'ha pogut detectar de forma directa.
10. Els AWRs sembla que no interefereixin en l'activitat enzimàtica de la PAL2 o en la seva estabilització.
11. Les proteïnes efectores AWR es poden produir de forma soluble i purificar en cèl lules d'*E. coli* amb el vector pDEST-MBP (epítop His) i en cèl lules de *R. solanacearum* (epítop HA) amb el nou vector pRC.

Annex

VECTOR MAPS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rrnB T1 terminator
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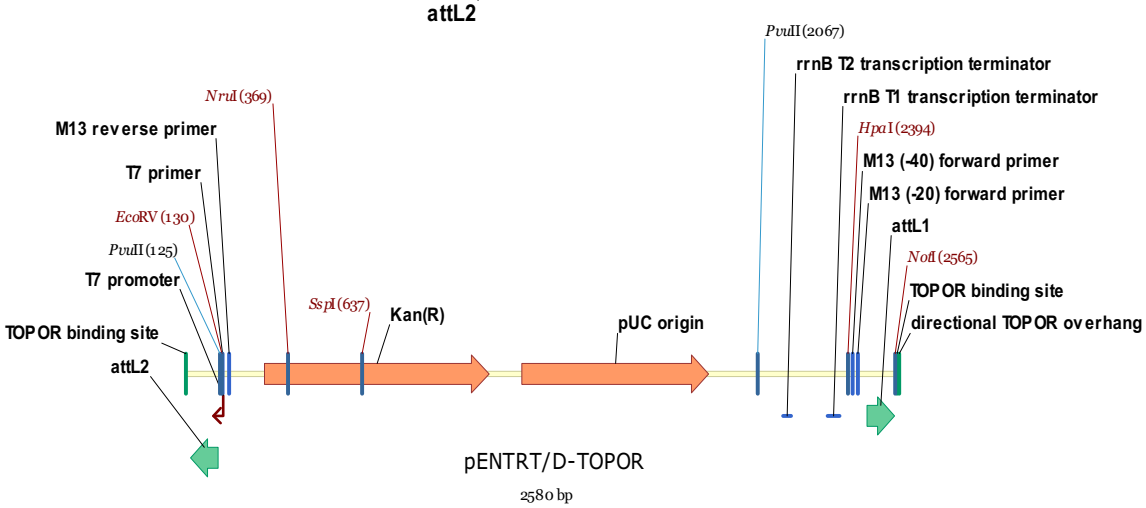
attL1
attR2
attP
attR1
ORF frame 2
PstI (930)
ccdB
pDONR207
5585 bp
SmaI (1181)
BamHI (1595)
Gm
BglII (3744)
SspI (1740)
NcoI (1747)
EcoRV (3552)
CAT/CamR
ORF frame 3
EcoRI (2048)
SspI (3219)
attR1
NruI (2951)
PvuII (2152)
attR2
PstI (2759)
PvuII (2754)
attP
attL2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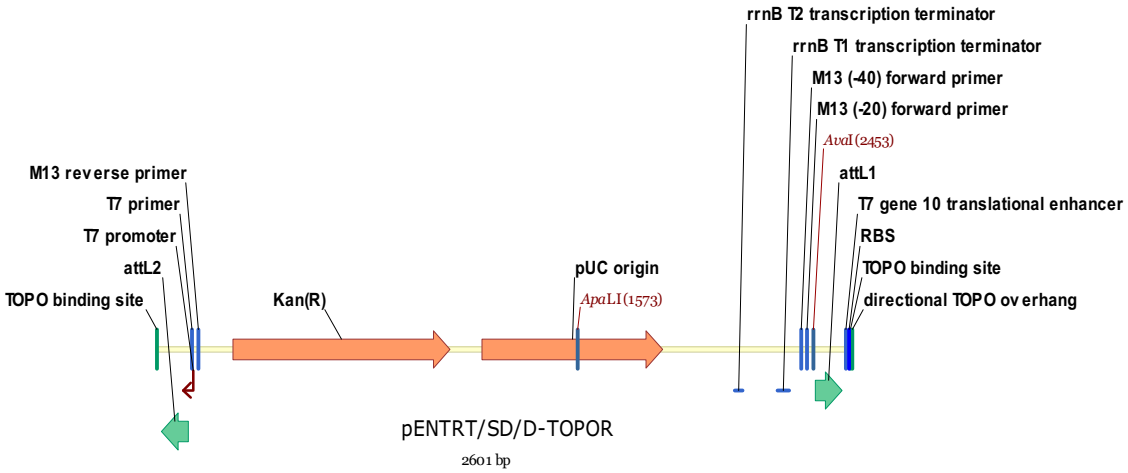
rrnB T2 transcription terminator
NruI (369)
rrnB T1 transcription terminator
M13 reverse primer
HpaI (2394)
T7 primer
M13 (-40) forward primer
EcoRV (130)
M13 (-20) forward primer
PvuII (125)
attL1
T7 promoter
NotI (2565)
SspI (637)
TOPOR binding site
Kan(R)
TOPOR binding site
pUC origin
directional TOPOR overhang
attL2
pENTRT/D-TOPOR
2580 bp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rrnB T1 transcription terminator
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attL1
T7 primer
T7 gene 10 translational enhancer
T7 promoter
RBS
attL2
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TOPO binding site
TOPO binding site
Kan(R)
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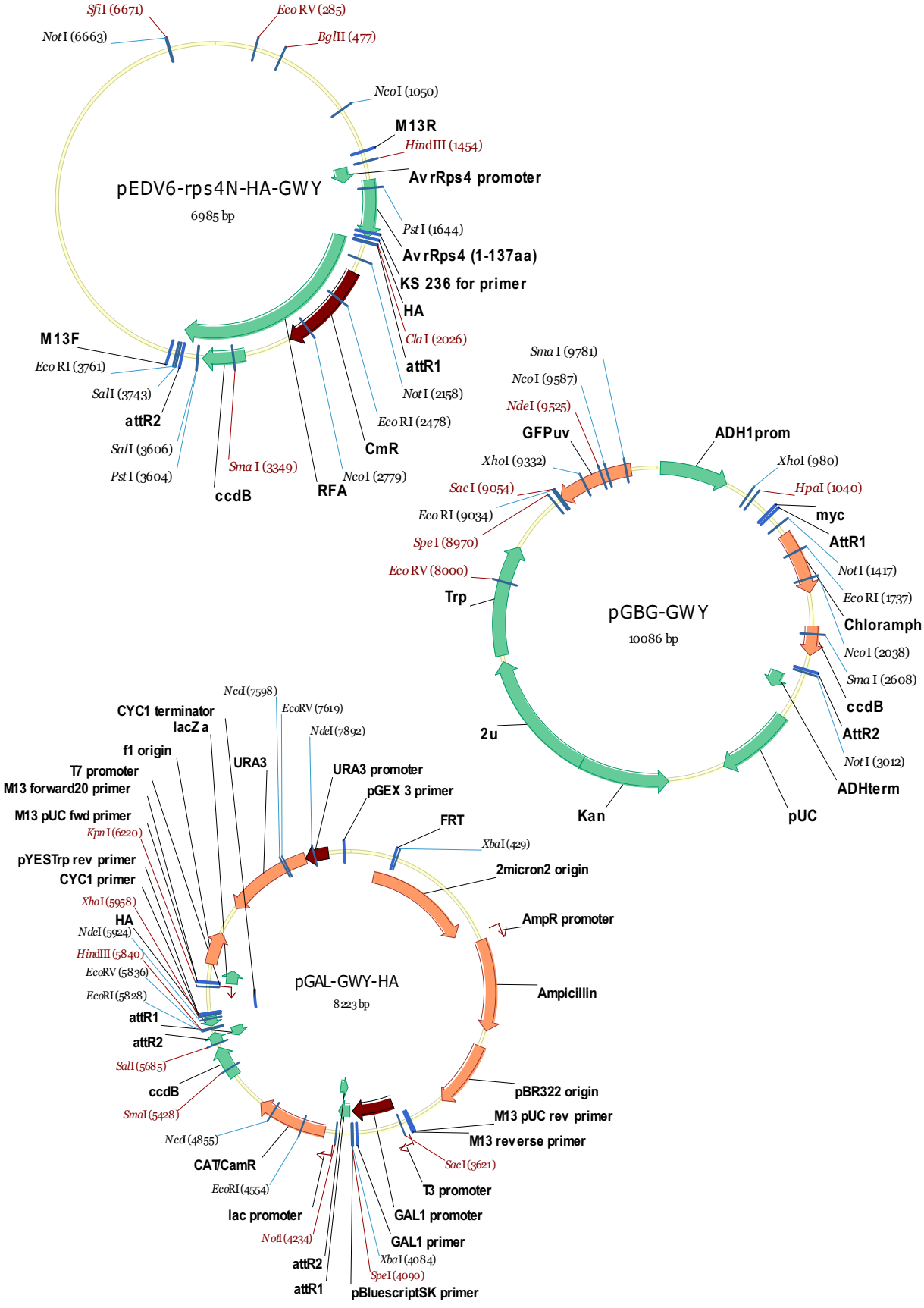
directional TOPO overhang
pENTRT/SD/D-TOPOR
2601 bp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NotI (6663)
BglII (477)
NcoI (1050)
M13R
HindIII (1454)
AvrRps4 promoter
PstI (1644)
AvrRps4 (1-137aa)
KS 236 for primer
HA
ClaI (2026)
attR1
NotI (2158)
EcoRI (2478)
CmR
NcoI (2779)
RFA
SmaI (3349)
ccdB
PstI (3604)
SalI (3606)
attR2
SalI (3743)
EcoRI (3761)
M13F
pGBG-GWY
10086 bp
SmaI (9781)
NcoI (9587)
NdeI (9525)
GFPuv
XhoI (9332)
SacI (9054)
EcoRI (9034)
SpeI (8970)
EcoRV (8000)
Trp
2u
Kan
pUC
ADHterm
NotI (3012)
AttR2
ccdB
SmaI (2608)
NcoI (2038)
Chloramph
EcoRI (1737)
NotI (1417)
AttR1
myc
HpaI (1040)
XhoI (980)
ADH1prom
pGAL-GWY-HA
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EcoRV (7619)
NdeI (7892)
CYC1 terminator
lacZa
f1 origin
T7 promoter
M13 forward20 primer
M13 pUC fwd primer
KpnI (6220)
pYESTrp rev primer
CYC1 primer
XhoI (5958)
HA
NdeI (5924)
HindIII (5840)
EcoRV (5836)
EcoRI (5828)
attR1
attR2
SalI (5685)
ccdB
SmaI (5428)
NcoI (4855)
CAT/CamR
EcoRI (4554)
lac promoter
NotI (4234)
attR2
attR1
pBluescriptSK primer
SpeI (4090)
XbaI (4084)
GAL1 primer
GAL1 promoter
T3 promoter
SacI (3621)
M13 reverse primer
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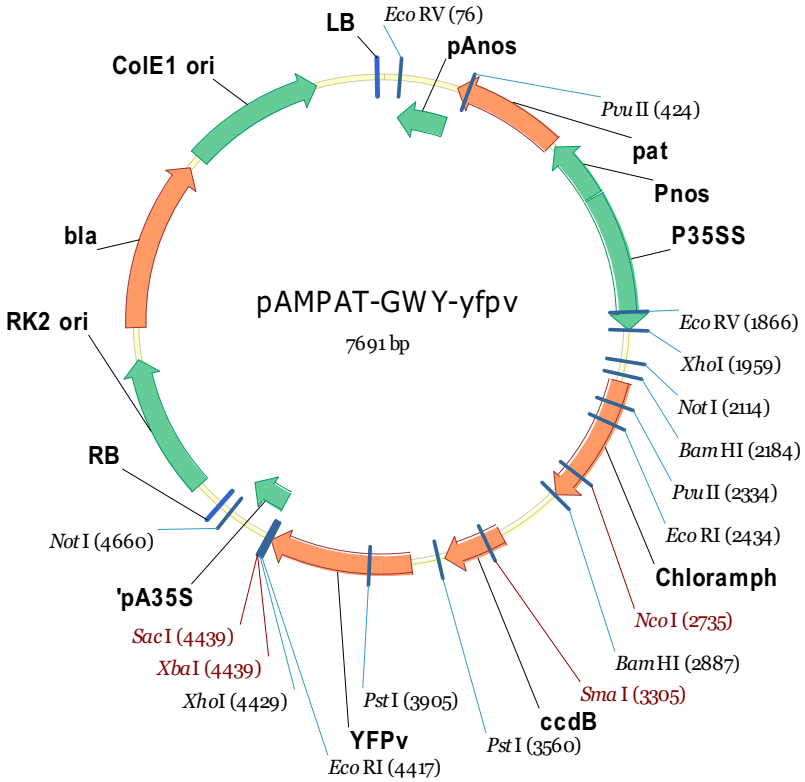
Ampicillin
AmpR promoter
2micron2 origin
XbaI (429)
FRT
pGEX 3 primer
URA3 promoter
URA3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LB
Eco RV (76)
pAnos
ColE1 ori
Pvu II (424)
pat
Pnos
P35SS
bla
RK2 ori
Eco RV (1866)
Xho I (1959)
Not I (2114)
Bam HI (2184)
Pvu II (2334)
Eco RI (2434)
Chloramph
RB
Not I (4660)
'pA35S
Nco I (2735)
Sac I (4439)
Bam HI (2887)
Xba I (4439)
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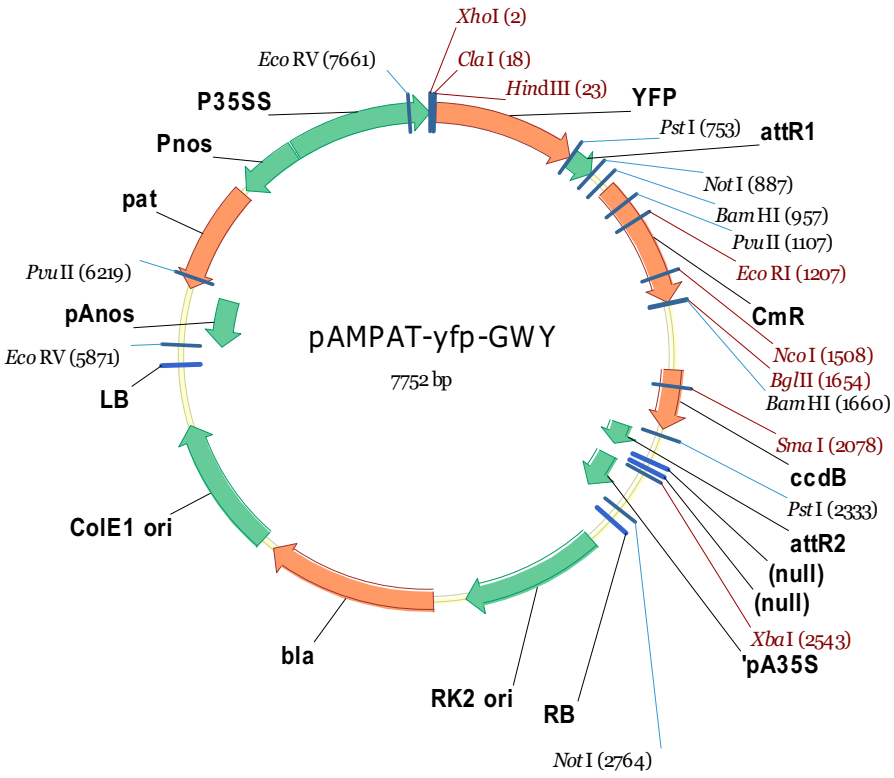
Xho I (4429)
Pst I (3905)
ccdB
YFPv
Pst I (3560)
Eco RI (4417)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Hind III (23)
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YFP
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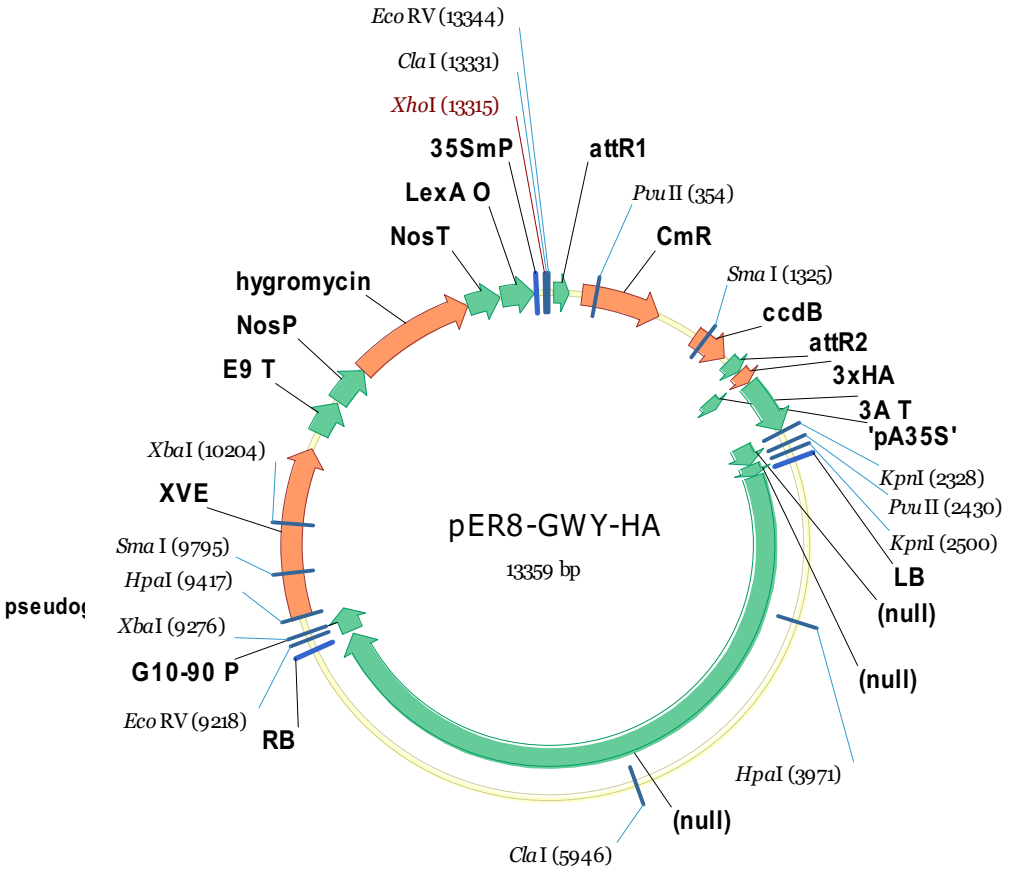
attR1
Pnos
Not I (887)
pat
Bam HI (957)
Pvu II (1107)
Pvu II (6219)
Eco RI (1207)
CmR
pAnos
Nco I (1508)
Eco RV (5871)
Bgl II (1654)
Bam HI (1660)
LB
Sma I (2078)
ccdB
ColE1 ori
Pst I (2333)
attR2
(null)
(null)
Xba I (2543)
'pA35S
bla
RK2 ori
RB
Not I (2764)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ClaI (13331)
XhoI (13315)
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LexA O
PvuII (354)
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CmR
hygromycin
SmaI (1325)
ccdB
NosP
attR2
E9 T
3xHA
3A T
'pA35S'
XbaI (10204)
KpnI (2328)
XVE
pER8-GWY-HA
PvuII (2430)
13359 bp
SmaI (9795)
KpnI (2500)
HpaI (9417)
LB
pseudo
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G10-90 P
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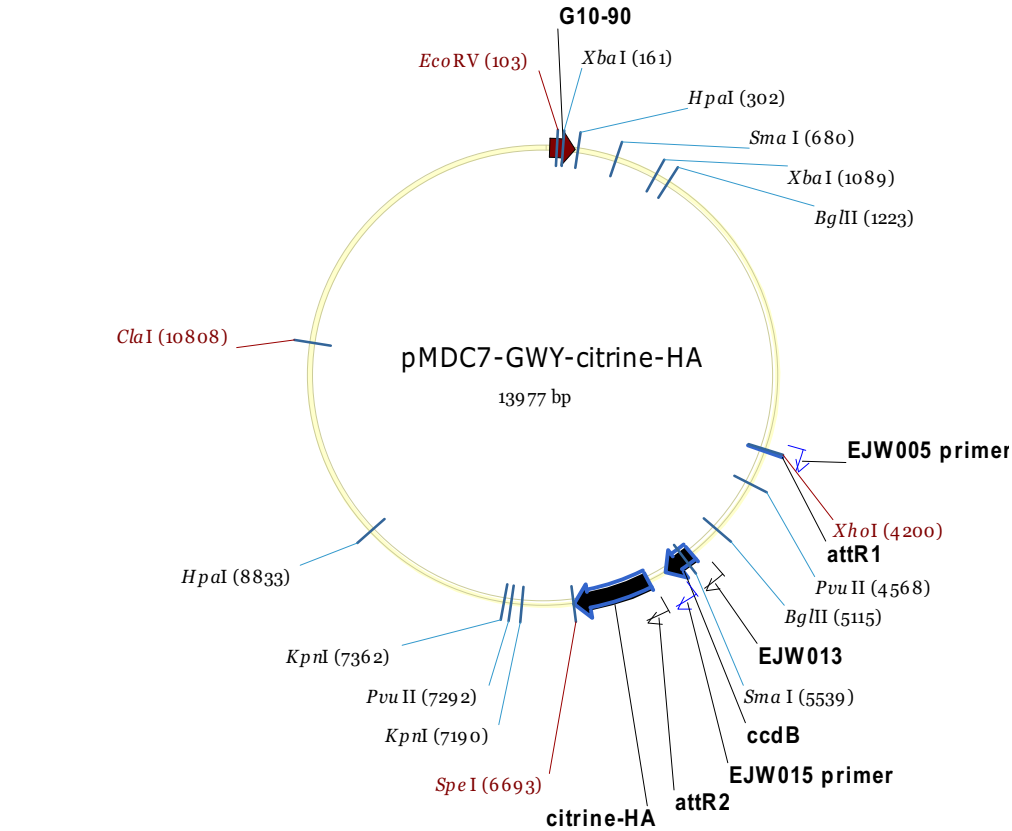
RB
HpaI (3971)
(null)
ClaI (5946)


G10-90
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XbaI (161)
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BglII (1223)
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13977 bp
EJW005 primer
XhoI (4200)
attR1
HpaI (8833)
PvuII (4568)
BglII (5115)
EJW013
KpnI (7362)
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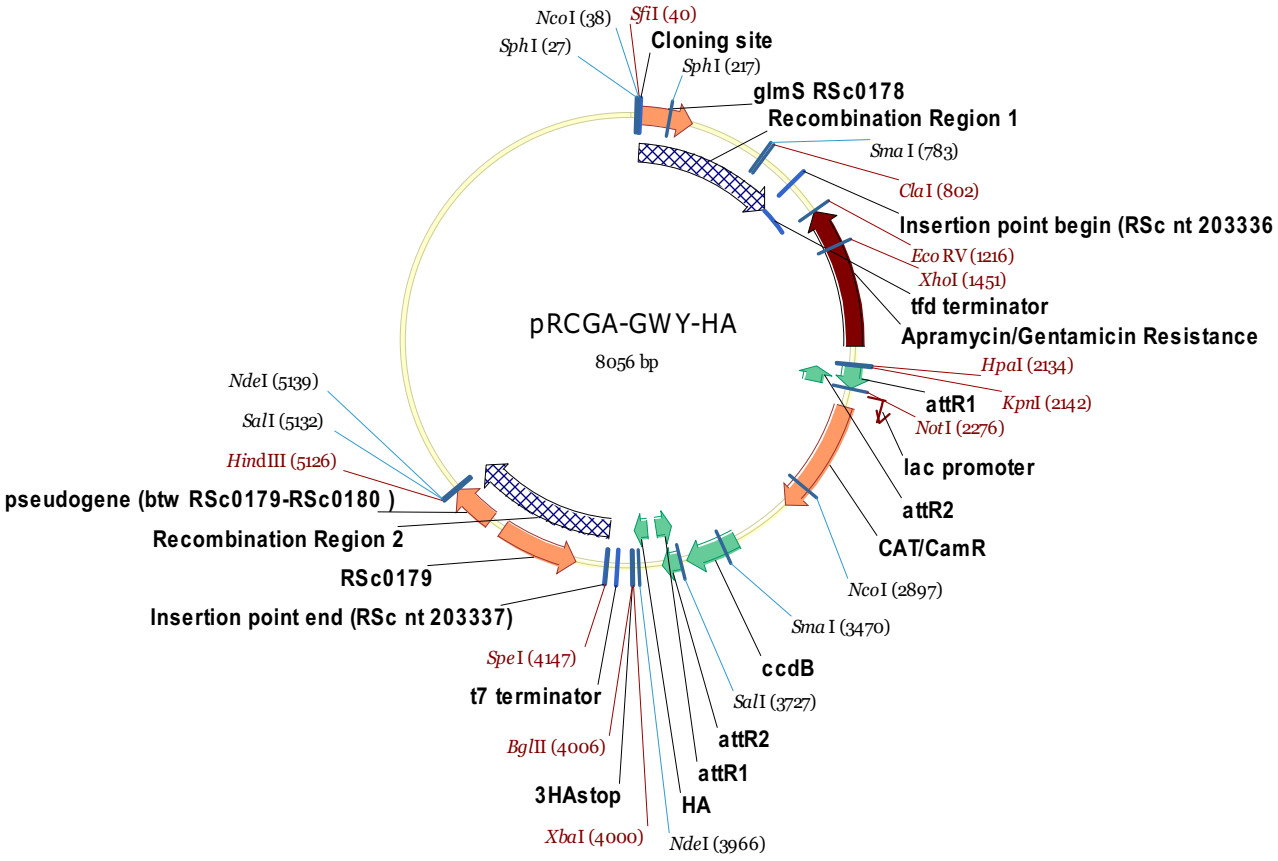
ccdB
KpnI (7190)
EJW015 primer
SpeI (6693)
attR2
citrine-HA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SfiI (40)
SphI (27)
Cloning site
SphI (217)
glmS RSc0178
Recombination Region 1
SmaI (783)
ClaI (802)
Insertion point begin (RSc nt 203336
EcoRV (1216)
XhoI (1451)
tfd terminator
Apramycin/Gentamicin Resistance
HpaI (2134)
attR1
KpnI (2142)
NotI (2276)
lac promoter
attR2
CAT/CamR
NcoI (2897)
SmaI (3470)
ccdB
SalI (3727)
attR2
attR1
HA
NdeI (3966)
XbaI (4000)
3HAstop
BglII (4006)
t7 terminator
SpeI (4147)
Insertion point end (RSc nt 203337)
RSc0179
Recombination Region 2
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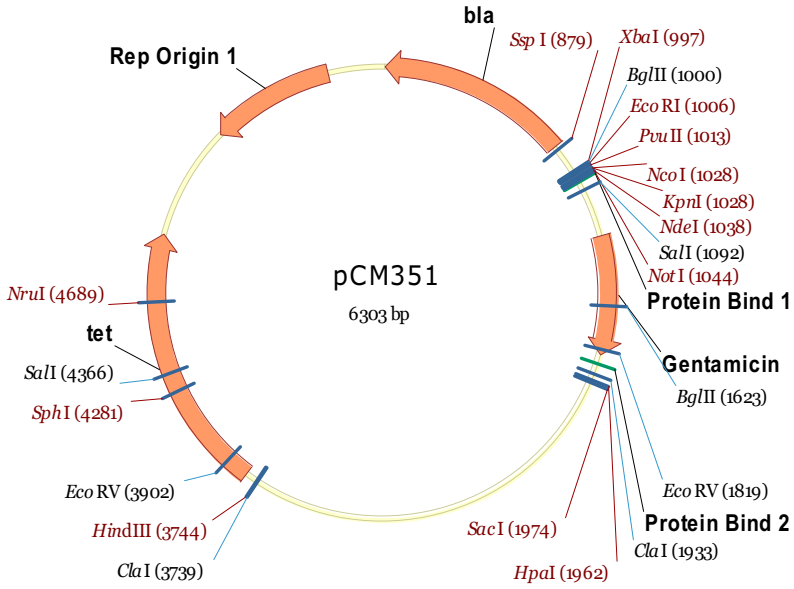
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KpnI (1028)
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NotI (1044)
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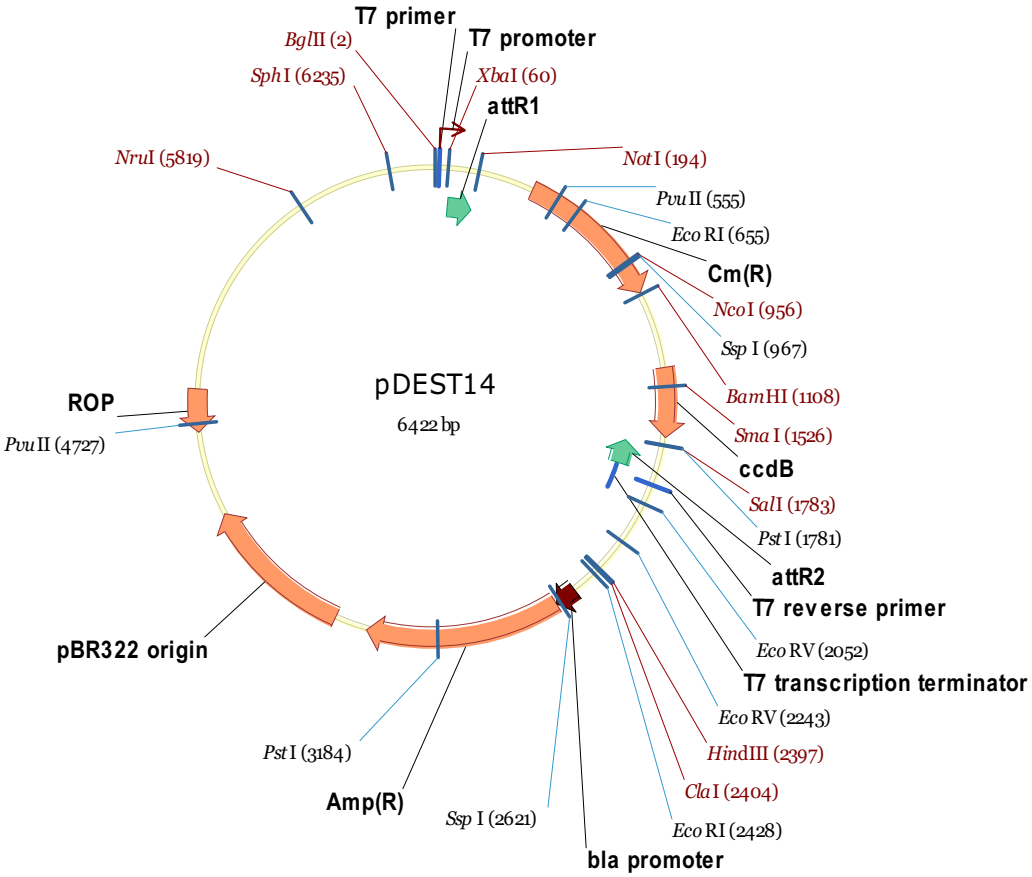
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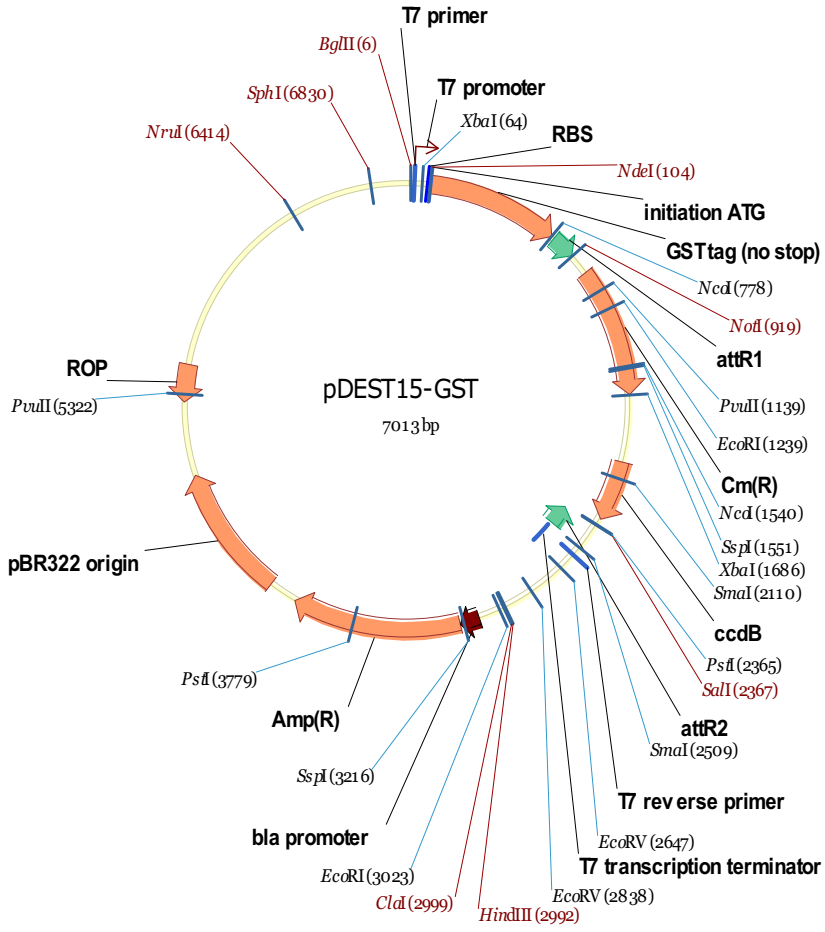
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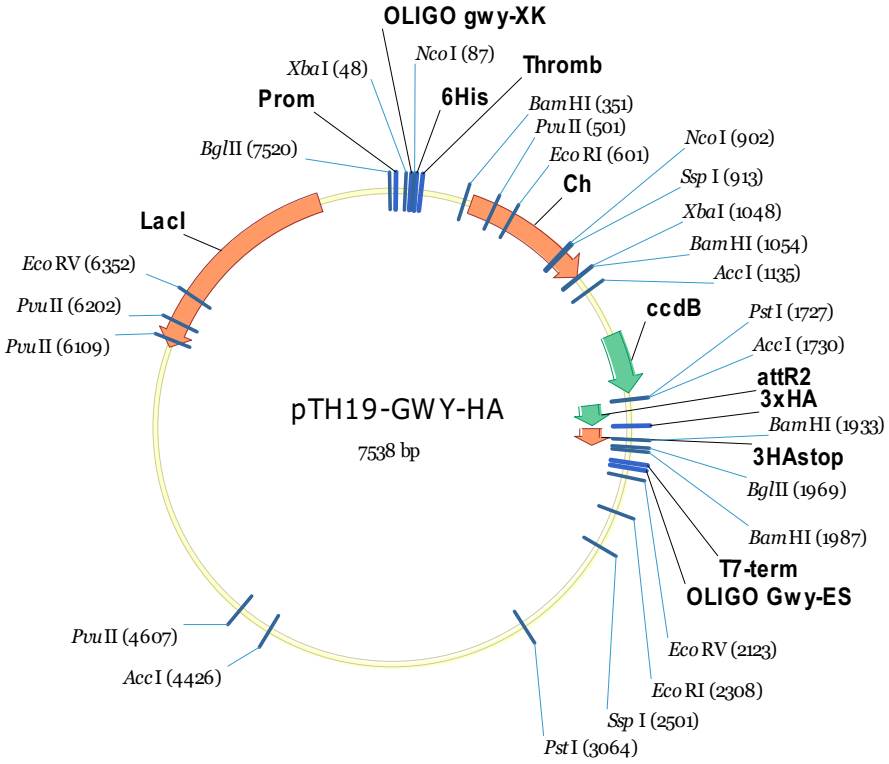
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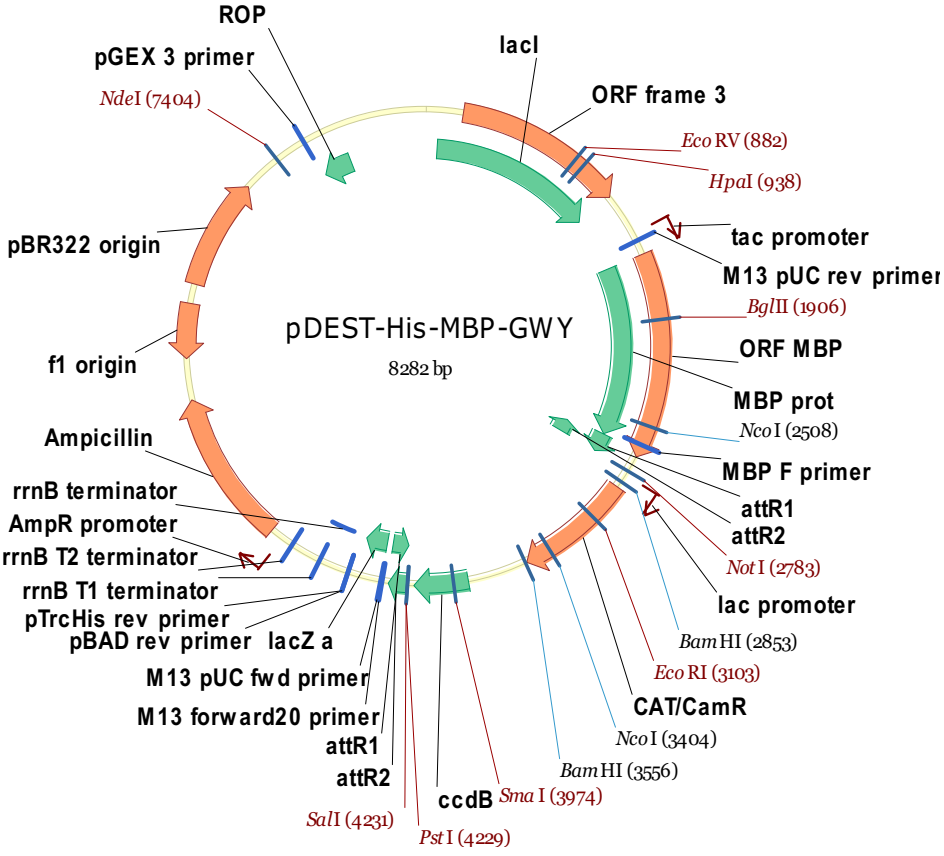
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PAPER IN COLLABORATION

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MAPK phosphatase MKP2 mediates disease responses in Arabidopsis and functionally interacts with MPK3 and MPK6

Victoria Lumbreras[1,*], Belmiro Vilela[1], Sami Irar[1], Montserrat Solé[2], Montserrat Capellades[1], Marc Valls[2], Maria Coca[1] and Montserrat Pagès[1]

[1]*Departamento de Genética Molecular, CRAG (CSIC-IRTA-UAB), 18–26 Jordi Girona, 08034 Barcelona, Spain, and*

[2]*Departament de Genètica Molecular, Universitat de Barcelona, Av. Diagonal 645 annex 2n pis, 08028 Barcelona, Spain*

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*For correspondence (fax +34 932 04 5904; e-mail vlrgmm@cid.csic.es).

SUMMARY

Mitogen-activated protein kinase (MAPK) cascades have important functions in plant stress responses and development and are key players in reactive oxygen species (ROS) signalling and in innate immunity. In Arabidopsis, the transmission of ROS and pathogen signalling by MAPKs involves the coordinated activation of MPK6 and MPK3; however, the specificity of their negative regulation by phosphatases is not fully known. Here, we present genetic analyses showing that MAPK phosphatase 2 (MKP2) regulates oxidative stress and pathogen defence responses and functionally interacts with MPK3 and MPK6. We show that plants lacking a functional *MKP2* gene exhibit delayed wilting symptoms in response to *Ralstonia solanacearum* and, by contrast, acceleration of disease progression during *Botrytis cinerea* infection, suggesting that this phosphatase plays differential functions in biotrophic versus necrotrophic pathogen-induced responses. MKP2 function appears to be linked to MPK3 and MPK6 regulation, as indicated by BiFC experiments showing that MKP2 associates with MPK3 and MPK6 *in vivo* and that in response to fungal elicitors MKP2 exerts differential affinity versus both kinases. We also found that MKP2 interacts with MPK6 in HR-like responses triggered by fungal elicitors, suggesting that MPK3 and MPK6 are subject to differential regulation by MKP2 in this process. We propose that MKP2 is a key regulator of MPK3 and MPK6 networks controlling both abiotic and specific pathogen responses in plants.

Keywords: *Botrytis cinerea,* mitogen-activated protein kinase (MAPK) phosphatase, mitogen-activated protein kinase 3 (MPK3), mitogen-activated protein kinase 6 (MPK6), oxidative stress, *Ralstonia solanacearum*.

INTRODUCTION

Reversible phosphorylation of proteins is one important mechanism that has evolved to transduce regulatory signals in eukaryotes. This mechanism often depends on relatively few types of protein kinases and signalling pathways, such as the evolutionarily conserved mitogen-activated protein kinase (MAPKs) cascade, which are reutilized in different biological contexts (Colcombet and Hirt, 2008; Pitzschke and Hirt, 2009). In plants, MAPK cascades regulate many developmental and physiological processes including the response to different types of abiotic and biotic stresses, such as the regulation of stomatal functions (Gudesblat *et al.*, 2007; MacRobbie and Kurup, 2007) stomatal density (Wang *et al.*, 2007; Lampard *et al.*, 2008) and innate immunity responses (Asai *et al.*, 2002; Menke *et al.*, 2004; Pedley and Martin, 2005; Asai and Yoshioka, 2008).

MAPKs are activated when their conserved TEY motif at the activation loop is phosphorylated by a dual specificity MAPK kinase (MAPKK) on both Thr and Tyr residues (Kiegerl *et al.*, 2000). This step can be reversed by dephosphorylation through Ser/Thr phosphatases (PP2C family), Tyr phosphatases (PTPs), or dual specificity phosphatases (DSPs) such as the MPK-specific phosphatases (MKPs; Keyse, 2000; Meskiene *et al.*, 2003; Schweighofer *et al.*, 2007). In particular, MKPs that act on both phosphothreonine and phosphotyrosine residues exert important roles in the inhibition of MAPK activity (Tonks and Neel, 2001). Indeed, MKPs are pivotal in the regulation of many physiological responses associated to MAPK cascades and their activity is tightly controlled (Luan, 2003; Dickinson and Keyse, 2006; Owens and Keyse, 2007).

Only five potential MKPs have been identified in Arabidopsis (Monroe-Augustus *et al.*, 2003; Bartels *et al.*, 2010), in contrast with the high number of MAPKs (20 different family members) identified in this species (Hamel *et al.*, 2006).

Consequently, the molecular and genetic characterization of MKPs may be particularly useful to unravel the function of MAPK cascades in plants. Recently, two MKPs, IBR5 and PHS1 have been shown to regulate MPK12 and MPK18 during auxin signalling and cortical microtubule stabilization (Lee *et al.*, 2009; Walia *et al.*, 2009). A third enzyme, MKP1, plays a role in genotoxic responses and resistance to high concentration of salt(s) through MPK6 interaction (Ulm *et al.*, 2001, 2002), and works as a repressor of salicylic acid synthesis through MPK3/MPK6 (Bartels *et al.*, 2009). The last two family members, DsPTP1 and MKP2, are structurally related enzymes that display *in vitro* dephosphorylation activity against MPK4 and MPK3/MPK6, respectively (Gupta *et al.*, 1998; Lee and Ellis, 2007). *In vivo*, MKP2 positively regulates oxidative stress functions; however, its role in disease responses remains largely unknown.

Previous studies have implicated MAPK cascades, in particular those dependent on MPK3/MPK6, as important regulators in the perception and response to pathogen infections in plants (Asai and Yoshioka, 2008; Pitzschke *et al.*, 2009). Pathogen defence responses involve a variety of mechanisms that include the rapid production of reactive oxygen species (ROS; Torres *et al.*, 2006) and induction of the so-called hypersensitive response (HR; Heath, 2000). Exactly how MAPK signalling controls HR-mediated responses is not well understood, but there is evidence suggesting that mutual cross-talk between MAPK signals, ethylene signalling and ROS levels regulates HR-like cell death (Liu *et al.*, 2008; Pitzschke and Hirt, 2009). Different results indicate that inhibition of MAPKs could prevent the progression of HR cell death (Pedley and Martin, 2005). The HR response induces a localized cell-death at the site of infection that prevents further spreading of biotrophic pathogens (e.g. different types of bacteria), which proliferate within living tissues (Heath, 2000; Delledonne *et al.*, 2001; Ren *et al.*, 2002; Greenberg and Yao, 2004). However, the role of HR in the resistance against fungal necrotrophic pathogens remains less clear, given that such pathogens normally kill the infected cells to spread throughout the host (Govrin and Levine, 2000).

Here, we identify an Arabidopsis *mkp2* mutant allele and characterize its effects in oxidative stress and pathogen responses. Our results indicate that MKP2 positively controls abiotic oxidative stress responses, and exerts differential functions in specific pathogen interactions. These differential effects appear to correlate with differences in the sensitivity of biotrophic and necrotrophic pathogens to HR and the mechanisms used by these pathogens to spread in the plant. We also provide evidence that MKP2 protein interacts with both MPK3 and MPK6 *in vivo*, and that such interaction is critical for the role of MPK6 in HR-like responses triggered by fungal elicitor. We propose that MKP2 is a key regulator of MAPK networks controlling both abiotic and specific pathogen responses in plants.

RESULTS

Role of MKP2 during biotrophic and necrotrophic infections

Our entry point for the analysis of MKP2 function was the identification of a mutant *mkp2* allele in a collection of T-DNA insertions screened for altered glucose responses (see Experimental procedures). Sequence and PCR analyses mapped the *mkp2* T-DNA insertion to the third intron of the *mkp2*/*At3g06110* gene (Figure 1a). As shown in Figure 1(b), *MKP2* mRNA levels appear severely reduced in 7-day-old *mkp2* homozygous seedlings based on quantitative PCR analyses, indicating that *mkp2* represents a loss of function mutation.

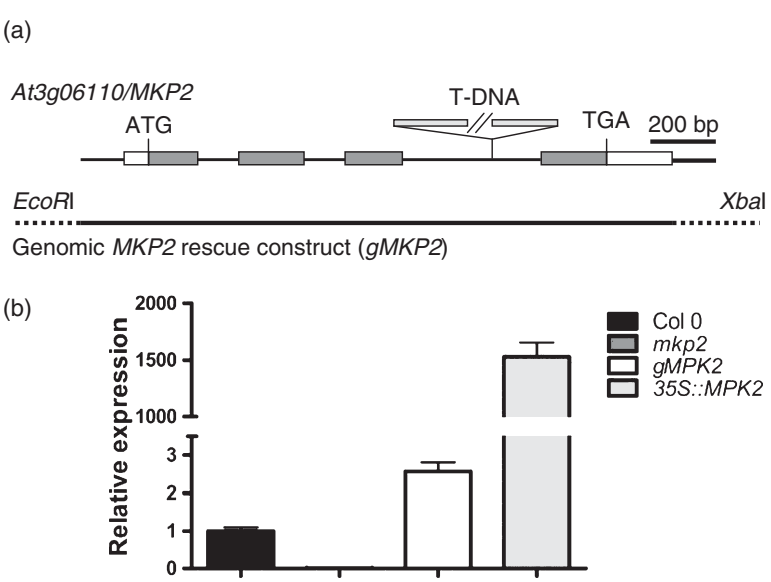

Figure 1. Structure of the *mkp2* T-DNA mutant allele.
(a) Genomic organization of the *MKP2* locus; the genomic construct used in the rescue experiment is shown below.
(b) Quantification of *MKP2* expression by RT-PCR analyses in 7-day-old seedlings grown on MS medium. *MKP2* relative expression in *mkp2* mutant is not significantly different from the non-template ($P \leq 0.05$). The experiment was conducted more than twice with similar results and representative data from one such quantification are shown.

We used *mkp2* homozygous plants to examine their response against the bacterium *R. solanacearum* GMI1000. *R. solanacearum* is a biotrophic pathogen that produces a wilt phenotype on susceptible hosts, including Col-0 (Hayward, 1991; Deslandes *et al.*, 1998). When *mkp2* plants were inoculated with strain GMI1000, we observed a delay in symptom development, as compared with controls (Figure 2a). Specifically, mutant plants show in average a delay of 1 day in the appearance of bacterial wilt symptoms. This delay was reproducible and was suppressed in plants complemented with a genomic *MKP2* transgene (*gMKP2*). In addition, a complementation experiment using an MKP2 overexpression construct (*35S::MKP2*) also restored the onset of wilting caused by *R. solanacearum* infection to wild-type response (Figure 2a). These results suggest that MKP2 plays a direct or indirect role in the response to *R. solanacearum* infection, possibly facilitating the development of plant symptoms. Furthermore, *mkp2* mutants did not show altered responses upon infection with the bacterial hemitrophic pathogen *P. syringae.* As shown in Figure S1 this pathogen showed similar rates of growth in mutant versus wild-type plants, indicating a selective interaction of *mkp2* with *S. solanacearum* but not *P. syringae* defense pathways. This different role of MKP2 in resistance to *P. syringae* versus *R. solanacearum* wilting phenotype may be due to the specific signalling cascades responding to both pathogens. For instance, jasmonic acid signalling is an important component of the susceptible disease response to *P. syringae*, but is probably not involved in the wilt symptom production by *R. solanacearum* (Hirsch *et al.*, 2002; Laurie-Berry *et al.*, 2006).

To determine whether the observed phenotype of *mkp2* mutant plants is specific to *R. solanacearum* or extends to necrotrophic pathogens, we also tested these plants with another Arabidopsis pathogen, *B. cinerea.* We have observed a differential time course in the progression of the fungus infection. Five days after infection, inoculated *mkp2* leaves showed on average smaller lesions than those of wild-type plants (Figure S2). However, 15 days after inoculation, most *mkp2* plants exhibited systemic spread of the pathogen throughout the plant, whereas the majority of wild-type plants displayed local lesions restricted to the inoculated leaves (Figure 2b). Around this time, 80% of mutant plants were dead, compared with only 42% of wild-type plants (Figure 2b). We observed that *B. cinerea* infection was restricted to the inoculated leaves in most wild-type plants while in the majority of mutant plants the infection was extended to the whole plant causing its death. These results suggest that there are different steps in the progression of *B. cinerea* infection and that the initial restriction in the symptoms observed in *mkp2* plants were over passed in a second phase in which *B. cinerea* progression through the plant is accelerated driving the death of the *mkp2* plant. Again, this effect results from *MKP2* mutation because *mkp2* plants complemented with *gMKP2* and *35S::MKP2* constructs showed a degree of susceptibility comparable to wild-type plants. Thus, loss of MKP2 function results in an increased susceptibility to fungal spreading, indicating that MKP2 acts as a positive regulator against *B. cinerea* disease. The opposite effect exerted by MKP2 during the response to the biotrophic *R. solanacearum* bacterium and the necrotrophic *B. cinerea* pathogen is consistent with current models on the host/pathogen interactions that occur during both types of plant infection (see Discussion, Rudd *et al.*, 2008).

Activation of MPK3 and MPK6 has been shown to play a role in local *Botrytis* infection (Ren *et al.*, 2008). To investigate whether MKP2 affects the activity of both kinases upon *Botrytis* infection, we performed *in vitro* MBP kinase assays using cell extracts from infected plants. In these experiments, we monitored kinase activities in wild-type, *mkp2* and *35S::MKP2/mkp2* plants during the immediate response to *Botrytis* infection. The samples corresponding to different time points were quantified and equal amounts of total protein were loaded in the gel. The MPK3 and MPK6 activity bands were identified by immunoprecipitation using anti-MPK3 and anti-MPK6 antibodies in wild-type plants collected 24 h after inoculation (Figure 2c; upper panel). Our analyses reveal three bands of activity that correspond to MPK6, MPK3 and probably MPK4, consistently with previous studies by Ren *et al.* (2008). As shown in Figure 2(c), whereas MPK6 activity was not affected during infection of the different backgrounds tested, MPK3 activity appeared significantly enhanced in both control and *mkp2* mutant plants at 30 min after infection. Furthermore, we observed a second peak of MPK3 upregulation that was specific for *mkp2* plants at 6 h after inoculation. Both the early and late (mutant-specific) peaks of MPK3 activity were absent in *35S::MKP2* plants. In addition, this background showed upregulation of a distinct MAPK activity, possibly corresponding to MPK4 (Ren *et al.*, 2008) (Figure 2c; bottom panel). Thus, our results reveal a dynamic pattern of MPK3 activation-inactivation during *Botrytis* infection that is sensitive to changes in *mkp2* function, particularly at 6 h after infection. Specifically, we suggest that MKP2 is required to maintain low MPK3 activity at this time point of infection, perhaps reflecting a role of MKP2 in desensitizing MPK3 signalling activity after the initial phase of response.

MKP2 modulates oxidative stress responses

Previous data suggest that MKP2 positively regulates the physiological responses to the oxidative stress generated during ozone treatment (Lee and Ellis, 2007). Therefore, we investigated the responses of *mkp2* plants to various abiotic stress conditions. As shown in Figure 3, the cotyledon expansion and greening in *mkp2* plants was severely inhibited in medium containing 0.6 μM of the oxidative agent methyl-viologen (MV) compared with wild-type plants, suggesting that the *mkp2* mutation disrupts

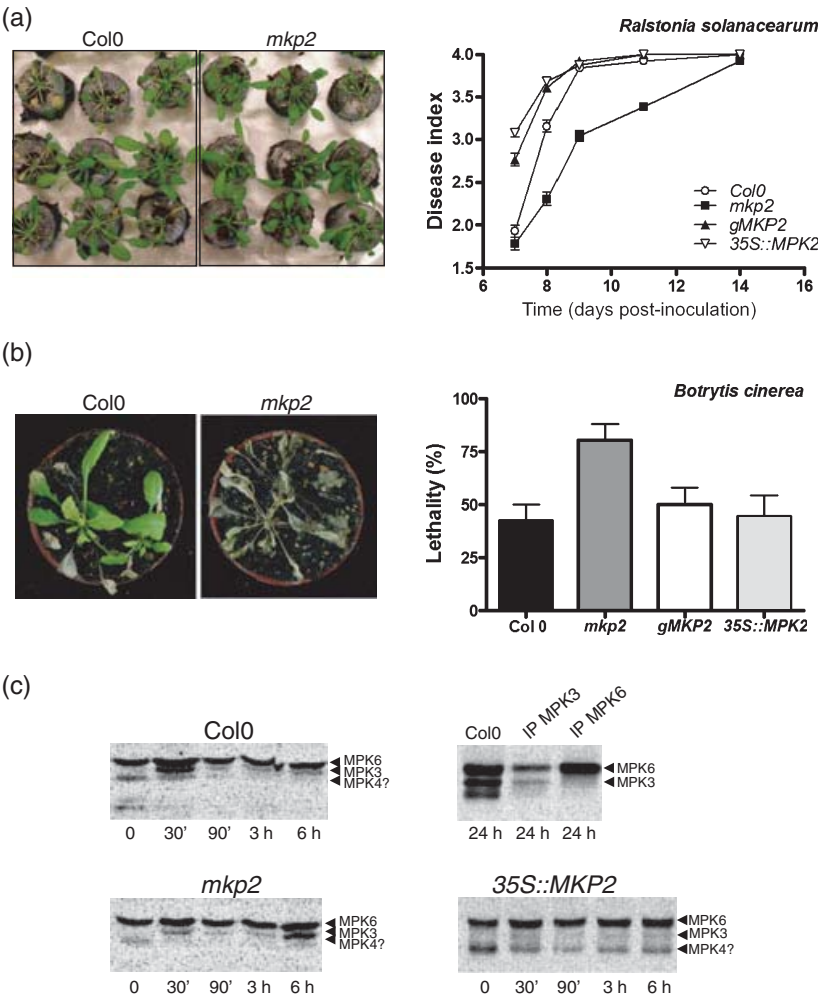

Figure 2. Progress of disease development in *mkp2* plants inoculated with *R. solanacearum* bacterium and *B. cinerea* fungus.
(a) Phenotype of Col-0 and *mkp2* plants at 8 days after root inoculation with strain GMI1000, rows of representative plants are shown (left panel). Progression of disease symptoms was recorded for each plant (1–4 scale), according to the percentage of wilted leaves and the mean for 20 inoculated plants is shown (right panel). Errors bars represent standard errors. These pathogenicity tests were repeated three times with consistent results. Col-0: wt Columbia variety, *mkp2:* MKP2 deficient line, *gMKP2* and *35S::MKP2:* complementation lines bearing the endogenous or the *35S* constitutive promoter, respectively.
(b) Phenotype of Col-0 and *mkp2* plants at 15 days after drop-inoculation (dai) with a spore suspension of *B. cinerea* (10^6 spores per millilitre). Appearance of representative plants (left panel) and percentage of lethality of indicated genotypes (right panel) at 15 dai are shown. Data are the mean (±SE) of three independent experiments.
(c) MAPK activation in *B. cinerea* inoculated plants. 2-week-old Col-0, *mkp2* and *35S::MKP2/mkp2* plants were inoculated by spraying with *B. cinerea* spore suspension (10^6 spores ml^{-1}). At the indicated times, seedlings were collected and the activity of the MAPKs was analyzed by in-gel kinase assay with MBP as a substrate. The MPK3 and MPK6 activity was determined by immunoprecipitation using anti-MPK3 and anti-MPK6 antibodies in wild-type plants collected 24 h after inoculation and then subjected to in-gel kinase assay (IP MPK3: Immunoprecipitation experiment with MPK3 antibody (Sigma), the antibody recognizes both activities, MPK3 and MPK6; IP MPK6: Immunoprecipitation experiment with MPK6, the antibody only recognizes MPK6 activity).

oxidative stress responses. Complementation with the *gMKP2* transgene efficiently rescued the MV hypersensitivity of *mkp2* mutant plants, indicating that loss of MKP2 function is indeed responsible for the *mkp2* phenotype. The *35S::MKP2* construct also complemented the MV hypersensitive phenotype, although in this case the rescue was incomplete and approximately one-third of seedlings still arrested around day 7 (Figure 3). We also assayed the responses of *mkp2* plants to osmotic stress conditions. As shown in Figure 3, *mkp2* seedlings grown on media containing 0.6 μM of ABA or 100 mM of NaCl developed similarly to wild-type plants, suggesting a specific role of MKP2 in response to oxidative stress. However, *35S::MKP2/mkp2* plants were hypersensitivity to ABA and salt treatments raising the possibility that MKP2 might directly or indirectly affect other osmotic stress responses.

Because MKP2 is involved in ROS tolerance (Lee and Ellis, 2007), it is possible that MV hypersensitivity observed in *mkp2* plants may be associated with an alteration in ROS levels. Therefore, we determined the accumulation of

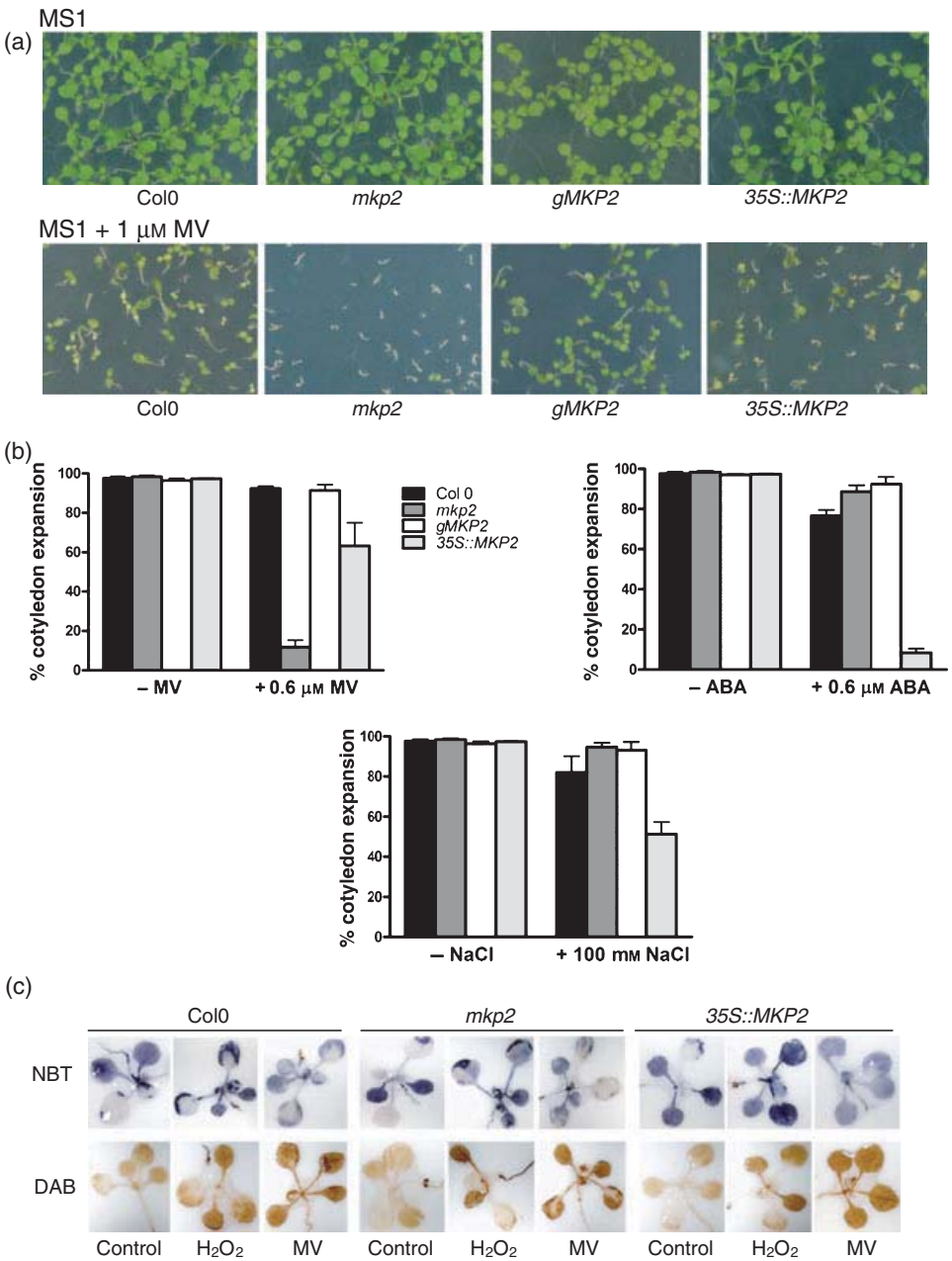

Figure 3. Response of *mkp2*, *gMKP2/mkp2* and *35S::MKP2/mkp2* plants to various stresses and abscisic acid.
(a) *mkp2* seedlings show a hypersensitive response to methyl-viologen (MV) treatment. Representative photographs were shown.
(b) Quantification of cotyledon expansion in the presence of MV 0.6 μM, ABA 0.6 μM and NaCl 100 mM. The growth rate was scored at 7 days after stratification. These data represent the average of three independent experiments evaluating about 150–200 plants. Bars indicate standard errors (±SE).
(c) *In situ* detection of O_2^- and H_2O_2 by DAB (bottom) and NBT (upper) staining in 2-week-old wild-type, *mkp2* and *35S::MKP27mkp2* plants grown on soil. Plants were treated with 20 mM H_2O_2 or 100 μM methyl-viologen (MV) for 2 h in mild light.

superoxide radical anions (O_2^-) and hydrogen peroxide (H_2O_2) in 2-week-old *mkp2*, wild type and *35S::MKP2/mkp2* seedlings using both nitroblue tetrazolium (NBT) reduction and diaminobenzidine (DAB) polymerization. As seen by the accumulation of blue (NBT) and brown (DAB) precipitates *mkp2* plants accumulate similar superoxide radical anions and H_2O_2 than control plants (Figure 3c). We also observe an overall increased NBT and DAB staining after 2 h MV and 20 mM H_2O_2 treatments (Figure 3c); this increase, clear for DAB staining, shows more variability among different leaves in the same plant for NBT staining, in particular after MV treatment. These data suggest that altered *mkp2* function does not correlate with changes in ROS levels and homeostasis; rather, we suggest that hypersensitivity of *mkp2*

mutant plants to MV treatment reflects an enhanced susceptibility of *mkp2* plants to ROS accumulation.

MKP2 expression and accumulation

To gain further insight into *MKP2* expression, we generated transgenic lines carrying the *MKP2* promoter fused to the GUS reporter gene (see Experimental procedures). Analysis of several independent lines harbouring this construct reveals a complex pattern of expression. During floral development GUS activity staining was observed in stigmatic papillae, anthers, pollen grains and floral abscission zones (data not shown). At later floral stages, GUS staining was no longer visible in mature flowers and dry-embryos (Figure 4a,b) but persisted in the abscission zones and in the septum of the siliques (Figure 4a). In 2 day-old germinating plants, GUS staining was detectable in the cotyledons, but was completely absent in young roots (Figure 4c,d). Expression in roots appeared by days 5–7 and was detected in vascular organs and at branching points in lateral roots (Figure 4h). In adult leaves, *MKP2-GUS* expression appeared particularly localized in vascular tissues and hydathodes (Figure 4f). The higher expression in both young (Figure 4e) and senescent leaves (Figure 4g) compared with adult leaves suggests a role of *MKP2* in tissues undergoing developmental transitions.

At the protein level, we tested MKP2 accumulation during development using polyclonal antibodies raised against GST-MKP2 (Figure S3). Although, our anti-MKP2 antibodies readily detect recombinant MKP2 protein *in vitro* and MKP2 overexpressed in transgenic plants (lane 2), no endogenous MKP2 was detected in the plant tissues in wild-type Col-0 plants (data not shown and lane 1; Figure S3). In addition,

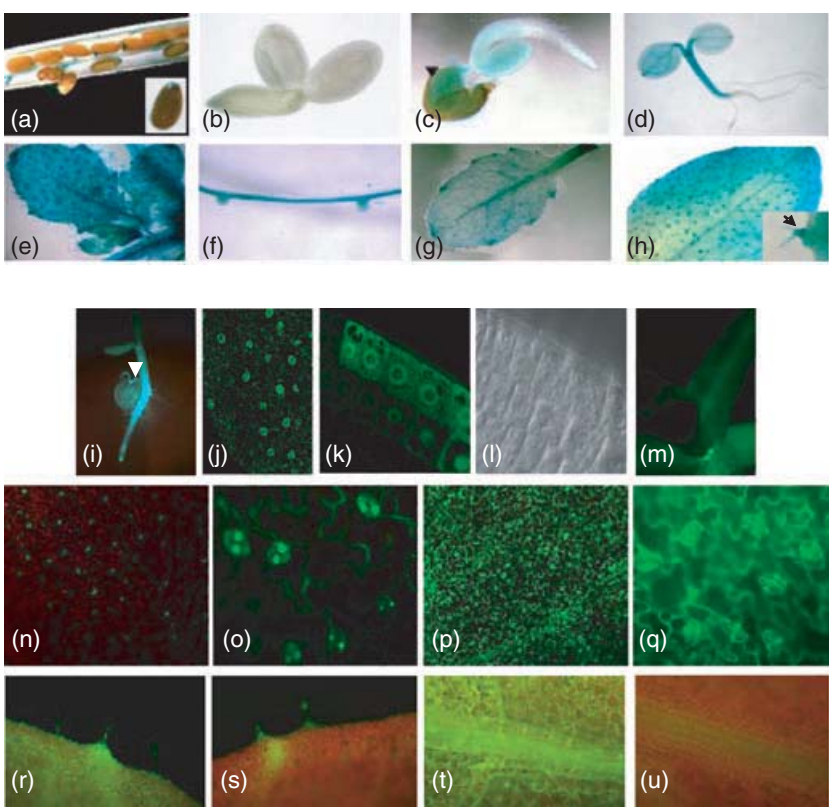

Figure 4. *MKP2* expression and protein accumulation during development.
(a–h) Expression of the *MKP2::GUS* reporter in transgenic plants. (a) Staining of mature siliques showing GUS expression in the hilum and the valve margin. Intense GUS expression is also detected in the seed coat, being absent from dry embryos. (c) Two-day-old seedlings show GUS expression in the aerial part and in the endosperm (black arrow). (d) Three-day-old seedlings display strong GUS expression in the hypocotyl. In cotyledons the higher staining is observed in the hydathodes and the apical meristem. (e) In young plants, a broad pattern of GUS staining is observed in leaves while in roots GUS detection begins at day 6–7, in which the higher staining is observed in vascular tissues and at the sites of emerging secondary roots (f). As the leaf develops the staining declines and becomes more restricted to hydathodes at the adult stage (g). (h) In senescent leaves, strong GUS expression is detected with localized GUS staining at the base of trichomes (black arrow).
(i–u) Expression of the MKP2–GFP fusion in *35S::MKP2–GFP* transgenic plants. (i) Three-day-old seedlings show GFP fluorescence in the upper hydathode, the epidermal and vascular tissues, the apical meristem and the basal region of the hypocotyl. Fluorescence is also detected in the endosperm layer during heterotrophic growth (arrowhead). (j) Detail of MKP2–GFP localization in epidermis and stomata. (k) Confocal micrograph of 3-day-old young roots showing nuclear and cytoplasmic GFP accumulation. (l) The same root detail under bright field. (m) Detail of GFP fluorescence in meristematic tissues of young leaves. (n–u) Localization of MKP2–GFP fusion in adult leaves. (n) MKP2–GFP localizes mainly in leaf specific tissues, including guard cells (n), trichomes, leaf margin hydathodes (s), and vasculature (u). (p, q, r, t) Leaf constitutive GFP accumulation in *35S::MKP2–GFP* plants.

MKP2 protein accumulation was also surveyed by the generation of transgenic lines expressing a *MKP2–GFP* fusion protein under the control of the constitutive *35S* promoter (Figure 4). As a control, we analysed lines carrying a *35S::GFP* transgene, which showed constitutive expression at all developmental stages (Figure 4q,r,s,u). However, we found that *MKP2–GFP* plants showed a more restricted pattern of MKP2–GFP accumulation in both germinating seedlings and adult plants. During seed development MKP2–GFP is mainly detected in the endosperm layer, where it persists until the fourth day after germination (Figure 4i). In young seedlings and adult leaves, MKP2–GFP accumulates in specific cell types, such as, the epidermis, trichomes, stomata, hydathodes and vascular tissues (Figure 4j,k,o,p,t,v). At the subcellular level MKP2–GFP protein was readily detected in both nucleus and cytoplasm of all cell types examined in young roots, but was absent from nucleoli (Figure 4l), suggesting that MKP2 could be function in both sub-cellular compartments.

Finally, during the characterization of *35S::MKP2–GFP* plants, we noticed that such plants display altered osmotic stress responses (Figure S4). *MKP2–GFP*-expressing plants are hypersensitive to MV, ABA and salt treatments, exhibiting reduced cotyledon expansion and greening compared with wild-type plants. Although overexpression constructs need to be interpreted with caution, our results at the level of protein localization and phenotypic effects raise the possibility that MKP2 could also be involved in ABA and salt responses.

Dynamic interactions of MKP2 with MPK3 and MPK6

It has been previously shown that MKP2 is able to dephosphorylate MPK3 and MPK6 *in vitro* (Lee and Ellis, 2007). To test this interaction *in planta* and to examine the subcellular localization of MKP2/MAPK complexes in the cell, we performed BiFC assays. We generated fusion proteins for MKP2, MPK3 and MPK6 proteins that were co-infiltrated in tobacco leaves. Reconstituted YFP signals were observed in leaves co-expressing YFP N-terminal and C-terminal chimeras, but not in control leaves infiltrated with single constructs. As shown in Figure 5, MKP2 and MPK3/MPK6 tagged proteins interact to reconstitute YFP fluorescence, indicating the *in vivo* relevance of MKP2 and MPK3/MPK6 associations. We also found that YFP fluorescence localizes

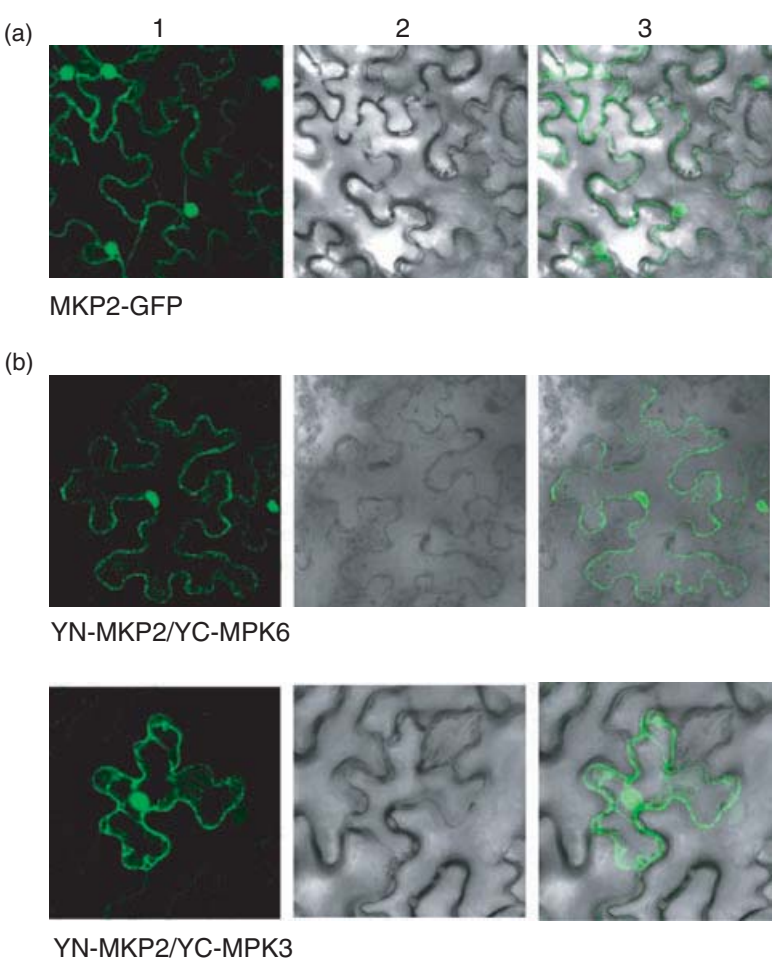

Figure 5. Subcellular localization of MKP2 and interaction with MPK3/MPK6.
(a) Subcellular localization of MKP2–GFP fusion in *Agrobacterium*-infiltrated tobacco leaves. Epifluorescence (1), bright-field (2) and bright-field merged with epifluorescence images (3) of representative epidermal leaf cells were shown.
(b) Bimolecular fluorescence complementation (BiFC) reveals MKP2 interaction with MPK3/MPK6 in the nucleus and the cytosol. *35S::YN* and *35S::YC* vectors expressing split YFP domains alone were used as controls.

in both the nucleus and the cytoplasm (Figure 6b), indicating that the complex occurs in these two subcellular compartments.

Next, we investigated whether MKP2 and MKP2/MAPK interactions are regulated in response to pathogen infection. For these experiments, we tested MKP2, MKP2/MPK3, and MKP2/MPK6 subcellular localization upon treatment with fungal elicitors. A careful examination of MKP2 upon this treatment revealed that GFP relocalizes surrounding spherical structures that could correspond to epiplasts (Figure 6a). Although the BiFC results do not provide quantitative information, treatment with fungal elicitors caused a differential effect on the MKP2/MPK3 and MKP2/MPK6 interactions, reducing the MKP2/MPK3 interaction and enhancing the MKP2/MPK6 association (Figure 6b) when the same cells were monitored before and after elicitor treatment using the same laser intensity. Evaluation of fluorescence changes in MKP2 interaction with MPK3/MPK6 suggests a degree of specificity in the interaction and indicates that MKP2 could act differentially on both kinases. In order to verify the expression of the tagged fusion proteins, western blot analyses were performed before and after 1 h of treatment with elicitors (Figure 6c).

We also analyzed the biological responses associated with MKP2 and MPK3/MPK6 during pathogen infection. Previous studies have shown that activation by MEK2 of MPK6/SIPK cascade leads to HR-like cell death in tobacco leaves (Ren *et al.*, 2002; Jin *et al.*, 2003). We used this biological response to determine whether the activation of MPK3 and/or MPK6 by elicitor treatment was affected by the MKP2 interaction in tobacco agro-infiltrated plants. As shown in Figure 7, we found a dramatic HR-response in MPK6 agro-infiltrated plants upon elicitor treatment. After 24 h of treatment, MPK6-expressing leaves lost turgor and collapsed, followed by dehydration in a response that is similar to pathogen-induced HR cell death (Figure 7a). In contrast to MPK6, plants agro-infiltrated with MPK3 plays a minor role in the HR response (Figure 7a). Similarly, agro-infiltration of MKP2 did not cause significant effects in these assays. However, co-infiltration with MKP2 and MPK6 constructs resulted in a clearly reduced HR response compared with the effects caused by the expression of MPK6 alone (Figure 7a). The area of cell death produced 24 h after infiltration was reduced by co-expression of MKP2 and MPK6 in about 75% (Figure 7b). Taken together, these experiments indicate that MKP2 and MPK6 functionally

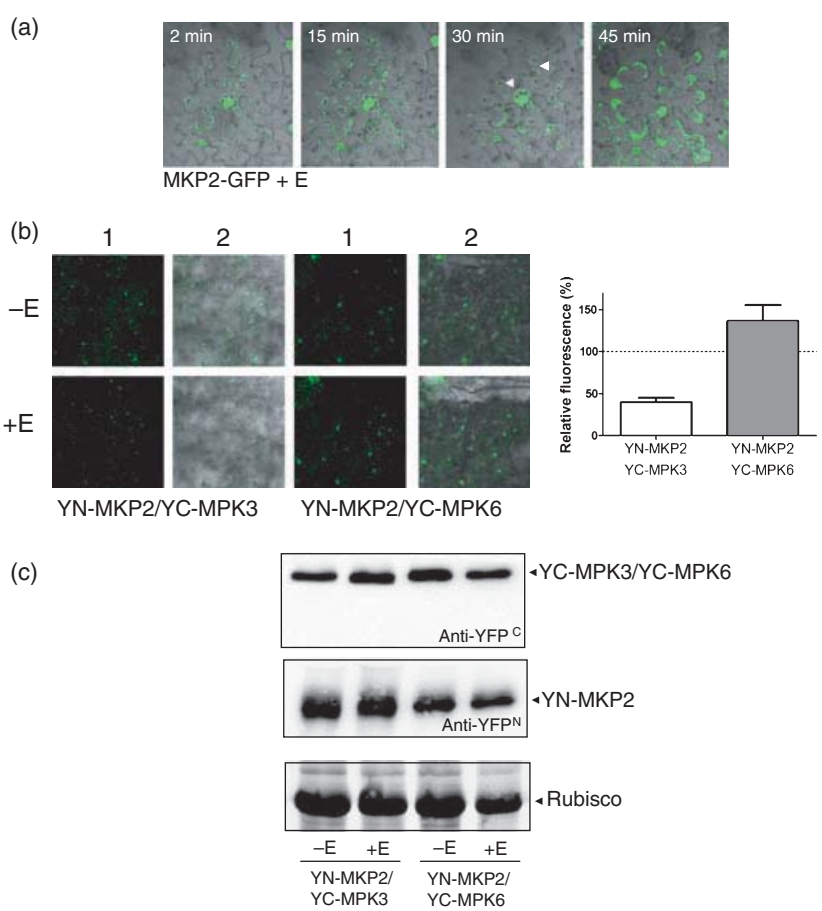

Figure 6. MKP2 interaction with MPK3/MPK6 in response to fungal elicitors.
(a) Detail of MKP2–GFP subcellular localization at the indicated times after elicitor treatment (+E). Arrows indicate epiplasts surrounded by MKP2–GFP signal. Epifluorescence (1), bright-field (2) and bright-field merged with epifluorescence images (3) of representative epidermal leaf cells were shown.
(b) MKP2 interaction with MPK3 and MPK6 before (–E) and after (+E) 1h elicitor treatment (left). Epifluorescence (1) and bright-field merged with epifluorescence images (2) of representative epidermal leaf cells were shown. Data points represent average BiFC interaction intensity of the same microscopic field before and after 1 h of elicitor treatment (right; n = 10 areas). Bars indicate standard errors (±SE).
(c) Protein extracts obtained from tobacco leaves infiltrated with *Agrobacterium* suspensions harbouring constructs YN-MKP2/YC-MPK3 and YN-MKP2/YC-MPK6 has been monitored before and after 1 h of elicitor treatment by western-blot analysis.

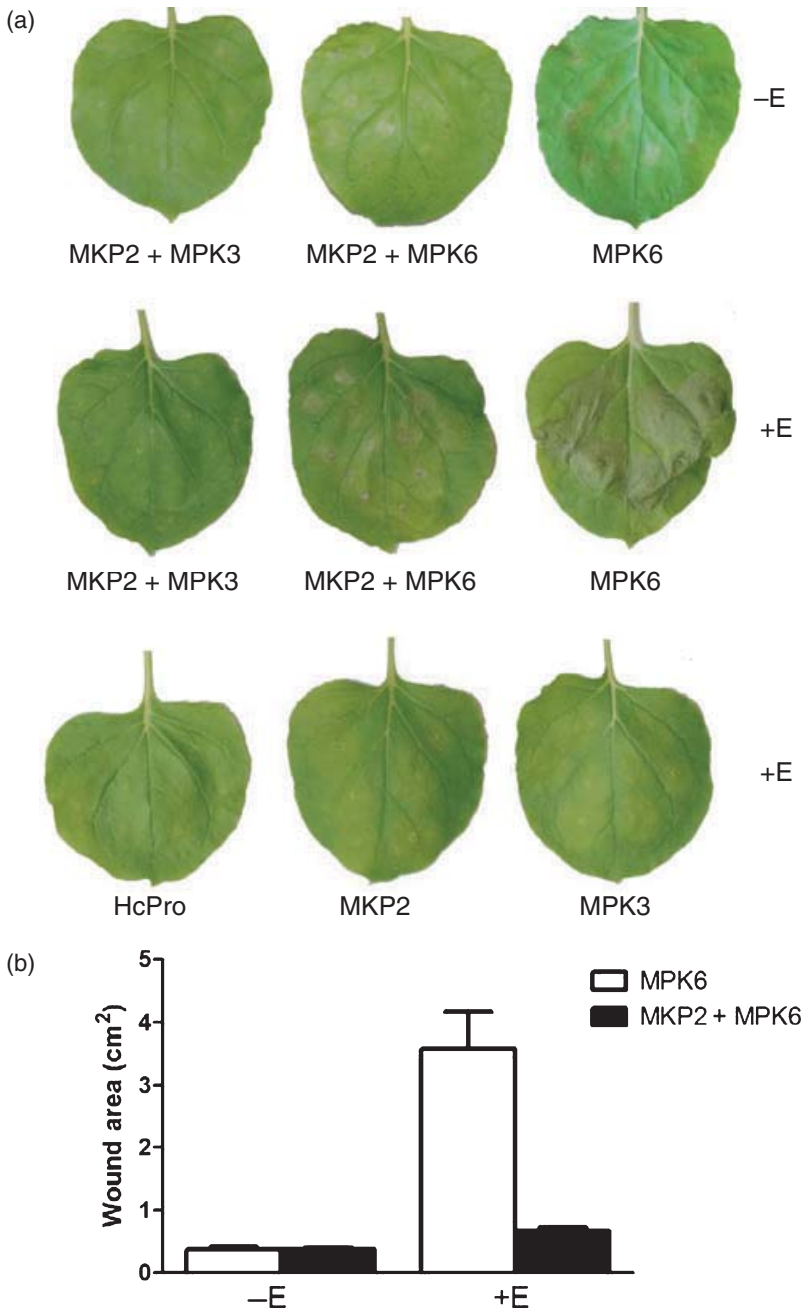

Figure 7. HR-like cell death in YC-MPK6 agroinfiltrated *N. benthamiana* leaves is negatively regulated by MKP2.
(a) HR response in YN-MKP2; YC-MPK3; YC-MPK6; YN-MKP2/YC-MPK6 and YN-MPK2/YC-MPK3 agro-infiltrated leaves before (–E) and after 24 h treatment with the elicitor (+E).
(b) Quantitative analyses of HR-like cell death response. The area of lesion was scored at time 0 and 24 h after infiltration. These data represent the average of three independent experiments. Bars indicate standard errors (±SE).

interact in the context of HR-like responses triggered by fungal infection.

DISCUSSION

The complete sequencing of the Arabidopsis genome has revealed a bewildering complexity of putative MAPK cascades and signalling components, which poses a challenge for assigning individual MAPK functions to specific biological processes (Andreasson and Ellis, 2010). In this context, the analysis of a small family of MAPK phosphatases present in Arabidopsis is proving particularly fruitful for our understanding of MAPK pathways and their interactions with other regulatory signals *in vivo* (Luan, 2003; Bartels *et al.*, 2010). In this report, we use *in vivo* genetic analyses to explore the functional roles of MKP2 in biotic and abiotic stress responses in relation to specific MAPK proteins.

Resistance to pathogen infection involves a complex set of defence mechanisms, which are at least partly controlled by specific MAPK proteins such as MPK3 and MPK6 (reviewed in Pitzschke *et al.*, 2009). Previous studies indicate that these MAPKs are positively involved in disease resistance toward *R. solanacearum;* for example, infection by

this pathogen leads to development of wilt symptoms that appear increased in *MPK3* virus-induced gene silencing (Chen *et al.*, 2009). Recently, physiological and biochemical analyses have shown that MPK3 and MPK6 activities are inhibited via dephosphorylation by MKP2, raising the possibility that this phosphatase was involved in regulatory pathways controlling pathogen responses (Lee and Ellis, 2007). Consistent with this hypothesis, we now show that mutation of MKP2 shows delayed symptom development in response to *R. solanacearum* infection. The severity of disease symptoms was reduced in mutant plants compared with wild-type and *gMKP2* or *35S::MKP2* complemented plants, suggesting that in Arabidopsis MKP2 is a negative modulator of wilt disease symptom development caused by *R. solanacearum.* Thus, it is conceivable that MKP2 controls the balance of MPK3 activities during the response to *R. solanacearum* infection, although such function is likely to depend on additional phosphatase inputs (Leung *et al.*, 2006; Schweighofer *et al.*, 2007), as indicated by the fact that *35S::MKP2* lines expressing high levels of MKP2 do not exhibit a clear hypersensitive response to *R. solanacearum* that would phenocopy the effects of susceptibility produced by MPK3 down regulation.

In contrast to the *R. solanacearum* effects, we have observed enhanced susceptibility of *mkp2* mutant plants against the necrotrophic fungus *B. cinerea*, which causes gray mold disease (van Kan, 2006). This systemic susceptibility is observed by the decay of plants 15 days after infection, but not for local response. Previous studies indicate that early (local) and late (systemic) responses to *B. cirenea* infections are subject to differential regulation (Asai and Yoshioka, 2009), involving distinct signalling pathways, such as the salicylic acid- and ethylene-mediated pathways (Ferrari *et al.*, 2003). Therefore, it is conceivable that our results reflect the differential impact of *mkp2* disruption on those pathways. As in the case of *R. solanacearum* infections, resistance to *B. cinerea* in Arabidopsis appears to be regulated by MPK3 and MPK6 activities (Asai *et al.*, 2002; Alonso *et al.*, 2003; Schweighofer *et al.*, 2007; Ren *et al.*, 2008). However, there are major differences in the host–pathogen mechanisms of interaction that occur during biotrophic and necrotrophic pathogen infection (Glazebrook, 2005; Yoshioka *et al.*, 2009); whereas defence responses involving HR-induced cell death are effective against biotrophic infections, they conversely facilitate the spread of necrotrophic pathogens upon death of host cells (Govrin *et al.*, 2006). Thus, the opposite phenotypes displayed by *mkp2* mutant plants in response to *R. solanacearum* and *B. cinerea* infections suggest that MKP2 normally functions as a negative regulator of HR-dependent responses. Indeed, recent studies have shown that the Arabidopsis LOV1 protein, a NB-LRR disease-resistance protein which mediates HR responses upon pathogen infection, behaves as a positive determinant of susceptibility against the necrotrophic fungus *Cochliobolus victoriae* (Lorang *et al.*, 2007). Also, successful infection by the necrotrophic fungus *Mycosphaerella graminicola* has been shown to involve a mechanism of cell death in the host response with features related to the HR-like response, more commonly seen during resistance to avirulent plant pathogens (Rudd *et al.*, 2008). Taken together, these observations support the view that HR responses represent a powerful mechanism of defence that requires precise regulation in order to prevent excessive killing of host cells (Heath, 2000). We suggest that MKP2 participates in this negative regulation of cell death responses possibly acting as an inhibitor of MPK6 activity, and that this function also could represent a mechanism of resistance against the systemic expansion of necrotrophic pathogens such as *B. cinerea.*

That MKP2 controls MPK3 and MPK6 activities is supported by changes in the activation of MPK3 after *B. cinerea* infection and by our finding that MKP2 interacts with MPK3 and MPK6 in BiFC experiments. Presumably, MKP2 binds both kinases and dephosphorylates regulatory residues in their pTEpY motif (Lee and Ellis, 2007). This negative regulation is consistent with the genetic interactions observed between MKP2 and MPK6 during HR-like responses triggered by fungal elicitors (Figure 7). However, our results argue against a simple model in which MKP2 controls both kinases through exactly the same mechanism. We find that treatment with a fungal elicitor reduces the MKP2/MPK3 interaction but enhances MKP2/MPK6 binding (Figure 6), suggesting that MKP2 regulates both kinases differentially. This result is unexpected given the extensive functional overlap of MPK3 and MPK6 in several cellular and physiological contexts (Asai *et al.*, 2002; for review, see Nakagami *et al.*, 2005). Nevertheless, there is evidence that both kinases exert individual mechanisms in specific processes, such as the ABA dependent opening and closure of stomata mediated by MPK3 and MPK6, respectively (Pitzschke and Hirt, 2009). The changes in MKP2 interaction observed during elicitor treatment could reflect differences in MPK3 and MPK6 activities during specific plant defence responses, for instance the HR-cell death response. Also, the binding of MKPs to their MAPK substrates sometimes enhances the catalytic activity of MKPs (Camps *et al.*, 1998). In plants, for specific MAPK-MKP modules, catalytic activity of NtMKP1 was strongly increased by the binding of SIPK (Katou *et al.*, 2005) and the activity of AtMKP2 was found to be stimulated by incubation with either MPK3 or MPK6 *in vitro* (Lee and Ellis, 2007), suggesting also that differences in MKP2 and MPK3/MPK6 interaction during elicitor treatment could also reflect changes in the activity of the phosphatase. Furthermore, the nucleo-cytoplasmic localization of the phosphatase-MAPK complexes is consistent with the proposed shuffling of MAPKs to the nucleus upon stress (Ahlfors *et al.*, 2004) and with the involvement of MAPKs in the

phosphorylation of distinct targets, namely ACS6 in the cytosol and EIN3 in the nucleus reported for MPK6 function (Yoo *et al.*, 2008).

Finally, the interactions between MPK3/MPK6 and MKP2 are likely to modulate other processes such as the response to abiotic stress. Previous studies have shown that overexpression of both kinases in tobacco causes hypersensitivity to ozone exposure (Samuel and Ellis, 2002), suggesting that oxidative-stress signalling depends on a fine-tuned balance of these MAPK activities. Furthermore, Lee and Ellis (2007) have shown that RNAi-mediated suppression of MKP2 function leads to extended activation of MPK3 and MPK6 in response to ozone treatment, arguing that MKP2 inhibits MPK3/MPK6 signalling in this context. In support of this idea, we now show that of *mkp2* mutant plants exhibit hypersensitivity to oxidative stress induced by methyl-viologen in germination. In contrast, other abiotic stress responses remain largely unaffected in the mutant. As in the case of pathogen responses, this MKP2 function may reflect a common feedback mechanism that maintains adequate levels of MPK3/MPK6 activation. Similarly, these shared responses point to a central role of MKP2 at the intersection of biotic and abiotic signalling pathways in Arabidopsis (Lee and Ellis, 2007). Future studies will be aimed at deciphering the precise molecular mechanism(s) of this regulatory function and the possible differential effects of MKP2 on MPK3 and MPK6 signalling pathways.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions and treatments

Arabidopsis Col-0 plants were maintained in controlled growth chambers (24 ± 2°C, 16 h:8 h light:dark photoperiod). Seeds were germinated in medium containing 1 × Murashige and Skoog (MS) basal salt mixture supplemented with 0.05% MES. Seeds were incubated at 4°C for 3 days to break dormancy prior to germination. Salt treatments were carried out by growing plants in 0–150 mM of NaCl. ABA was added to the growth medium at concentrations between 0.1 and 10 μM. Methyl-viologen was used at 0.1–1 μM. Plant treatments were monitored in MS medium supplemented with 1% sucrose medium.

Analysis of the *MKP2* mutant line

The *mkp2* insertion allele was from a Spanish project T-DNA line collection selected in a screening for mutants with a glucose insensitive phenotype. In a first step, the *mkp2* T-DNA mutation was found in an *aba1* background, responsible of a glucose insensitive phenotype. After cleaning the T-DNA from the *aba1* background, and tested that the single *mkp2* allele does not show glucose insensibility. The insertion in the gene was identified by using inverse PCR with two T-DNA left border primers. The T-DNA insertion in the *mkp2* mutant was confirmed by PCR and its exact position was determined by sequencing. The homozygous *mkp2* mutant was further analyzed by RT-PCR using genomic *MKP2* primers (gMKP2.1–5′- GTGGCAGGGTACTATTCTACC-3′; gMKP2.2–5′-CCTCCTAGACATTCCCATGAAGC-3′ and gMKP2.3–5′-CACGTTCGATCAAGATATCGG-3′) to confirm the insertion of the T-DNA in the gene. Plants homozygous for *mkp2* were used for further analysis.

Quantitative PCR analysis

RNA isolation was conducted using RNeasy kits (Qiagen, http://www.qiagen.com/) following the manufacturer's instructions. Total RNA (5 μg) was DNaseI-treated (Roche, http//www.roche.com/) and used for cDNA synthesis with SuperScript III (Invitrogen, http://www.invitrogen.com/). The reaction was diluted to a final volume of 20 μl and 2 μl were used as PCR template by using master mix SYBR Green Premix Ex-Taq Takara qPCR kit and *MKP2* primers (R1–5′-GCCGACGACATCAGGCATAT-3′ and R2–5′-GCTGGAGCTGCGAAATGAA-3′) at 0.3 μM. The raw threshold cycle values were normalized to Ubiquitin and products obtained from the PCR were run on 1%-agarose gels to ensure that there was only one band of the expected size.

Constructs and Arabidopsis transformation

To generate the *MKP2*::*GUS* construct, Arabidopsis Col-0 genomic DNA was used as a template to amplify a 620-bp *MKP2* promoter fragment carrying *Bam*HI and *Nco*I ends. This fragment *Bam*HI–*Nco*I was subcloned into the *pCambia 1300* vector (Clontech, http://www.clontech.com/). The *35S::MKP2–GFP* construct was made by amplifying the *MKP2* coding sequence (with *Nco*I ends) and subcloning this fragment into the *pCK–GFP–S65C* vector. The *35S::MKP2–GFP* cassette was released with *Hind*III and transferred to the *pCambia 1300* vector (Clontech). The *35S::MKP2* construct was made similarly by subcloning the *MKP2* coding region into the vector *pJIT60* (using *Eco*RI), and transferring the *35S::MKP2* sequences as a *Kpn*I–*Sph*I fragment to *pCambia 1302* (Clontech). The genomic *MKP2* rescue construct was made by amplifying a 1784-bp fragment carrying *Eco*RI (5′) *Xba*I (3′) ends and subcloning this fragment in *pCambia 1300* (Clontech). These constructs were transformed in Arabidopsis by the floral dip method (Clough and Bent, 1998). Transgenic seedlings were selected in MS medium containing kanamycin 50 μg ml^{-1} or hygromycin 40 μg ml^{-1}. For each construct, 10 independent lines were selected and a minimum of five to six homozygous lines were analyzed. For the *35S::MKP2* plants, only lines with over-accumulation of MKP2 protein were selected for phenotypic analyses (Figure S3).

Pathogenicity tests on *A. thaliana*

For plant infection with *R. solanacearum*, *A. thaliana* Col-0 seeds were sterilized during 15 min in 25% sodium hypochlorite solution, washed with sterile water and sown on MS-agar plates. The plates were incubated at 4°C during 2 days (wrapped with aluminum foil) and then transferred to a growth chamber (24°C, 16 h/8 h light/dark). After 8–10 days, seedlings were planted in Jiffy-7 peat pellets and grown for approximately 3 weeks until inoculated (24°C, 8 h/16 h light/dark, 50% humidity). Pathogenicity tests were performed by *R. solanacearum* root inoculations on *A. thaliana* plants, as described in Deslandes *et al.*, 1998;. Symptom appearance was recorded independently for each plant according to a wilting scale 0–4 (0: no wilting; 1: 25% leaves wilted; 2: 50%; 3: 75%; 4: death). Results are given as the medium of wilting for the 20–30 plants assayed in the experiment. For plant infection with *B. cinerea* inoculation was performed on 4-week-old soil-grown plants as described by Thomma *et al.*, 1998. One needle-prick wound was applied to two leaves per plant, and the fresh wounds were covered with 5 μl drops of 10^6 conidial spores per ml in 0.2% glucose water solution. Progression of infection was followed macroscopically by viewing the disease symptoms, and the percentage of decayed plants 15 days after inoculation was determined. At least 12 plants per genotype were inoculated in each experiment, and three independent experiments were done. Activation of MPK3 and MPK6 after *B. cinerea* inoculation was assessed in 2-week-old seedlings.

Seedlings collected at times 0, 30, 90 min, 3 and 6 h were frozen and analyzed by in-gel kinase assay. Determination of which band corresponded to MPK3 and MPK6 activities was performed by immunoprecipitation of wild-type seedling after 24 h infection (Ren *et al.*, 2008).

In gel kinase assay

Proteins were extracted in 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 25 mM NaF, 1 mM Na_3VO_4, 50 mM, 50 mM β-glycerophosphate, 20% glycerol, 1 mM PMSF, 10 μM leupeptin, 1μg ml^{-1} aprotinin and 10 μg ml^{-1} pepstatin and 50 mM HEPES-KOH, pH 7.5. In gel kinase assay was performed according to Fujii *et al.* (2007). Proteins (50 μg/lane) were separated on a 12.5% SDS-PAGE gel containing 0.25 mg ml^{-1} of myelin basic protein (Sigma). The gels were washed 3 × 30 min with 0.5 mM DTT, 5 mM NaF, 0.1 mM Na_3VO_4, 0.5 mg ml^{-1} BSA, 0.1% Triton X-100, and 25 mM Tris–HCl, pH 7.5 and proteins were renatured with 1 mM DTT, 5 mM NaF, 0.1 mM Na_3VO_4, and 25 mM Tris–HCl, pH 7.5, for 2 × 30 min and 16 h at 4°C prior to the reaction. The gel was incubated for 90 min at room temperature with 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4, and 25 mM Tris–HCl, pH 7.5, supplemented with 50 mCi of [γ-^{33}P]ATP and 250 nM cold ATP. Finally, the gel was washed with 5% trichloroacetic acid (TCA) and 1% sodium pyrophosphate at least five times for 30 min and dried. Radioactivity was quantified using a Storm 820 imager (GE Healthcare).

Protein immunoprecipitation

Immunoprecipitation of MPK3 and MPK6 was done with Anti-AtMPK3 and Anti-AtMPK6 antibody (Sigma) respectively. Proteins (1.5 mg) were incubated at 4°C overnight with the desired antibody, loaded on a Protein-A Sepharose CL-4B resin (GE Healthcare) and incubated for 3 h with IP buffer (20 mM Tris–HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 10 μM leupeptin, 1 μg ml^{-1} aprotinin and 10 μg ml^{-1} pepstatin). The slurry was washed 3 × 15 min with IP buffer and the supernatant was removed prior to the in gel kinase assay.

Histochemical ROS staining

H_2O_2 accumulation was detected by the DAB and O_2^- using NBT staining methods (Scarpeci *et al.*, 2008). Two-week-old (Col-0, *mkp2*, *gMKP2/mkp2* and *35S::MKP2/mkp2*) Arabidopsis seedlings grown on soil were treated for 2 h either with MS solution (control) or MS supplemented with 20 mM H_2O_2 or 100 μM methyl-viologen in the light (120 μmol photons m^{-2} s^{-1}) and then incubated in 5 mM 3,3′- diaminobenzidine (DAB) at pH 3.8 for 6 h or 6 mM nitroblue tetrazolium (NBT) for 6 h to detect H_2O_2 and O_2^-, respectively. After clearing the chlorophyll with 80% ethanol at 60°C, images of representative plants were taken with a Leica stereomicroscope.

Histochemical GUS staining

In situ assay of GUS activity was performed as described by Jefferson (1989). Whole plants were immersed in 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid solution in 100 mM sodium phosphate, pH 7, 0.1 mM EDTA, 0.5 mM ferricyanide, and 0.5 mM ferrocyanide, and 0.1% Triton X-100, and after applying vacuum for 5 min, they were incubated at 37°C over-night. Chlorophyll was cleared from the plant tissues by immersion in 70% ethanol.

GFP localization and BiFC by confocal microscopy

GFP observation of transgenic Arabidopsis seedlings expressing *MKP2–GFP* was examined by using a Leica fluorescence stereomicroscope, a Zeiss Axiophot epifluorescence microscope and a Leica TCS SP confocal laser-scanning microscope. For *Agrobacterium tumefaciens*-mediated transient expression in *N. benthamiana* the full-length *MKP2*, *MPK3* and *MPK6* cDNA sequences were cloned in the GATEWAY-compatible vector *pENTRY3C* (Invitrogen). The three *pENTRY3C* plasmids were transferred to a BiFC GATEWAY-modified vector developed by A. Ferrando (unpublished results; López-Paz *et al.*, 2009) to produce *35S::YN-MKP2; 35S::YN-MPK6; 35S::YN-MPK3; 35S::YC-MKP2; 35S::YC-MPK6; 35S::YC-MPK3. N. benthamiana* plants were transiently transfected for YFP detection. For the co-infiltration, equal volumes of the three *Agrobacterium* cultures (the two truncated YFP constructs; and the strain expressing the HcPro protein) were mixed (Dunoyer *et al.*, 2004). Observations were performed 3 days after infiltration.

Elicitors were derived from liquid cultures of the tobacco pathogen *Fusarium tabacinum* (Mycotheque de l'Universite Catholique de Louvain-La-Newe, MUCL 9701 strain), the anamorph of *Plectosphaerella cucumerina.* Elicitors were crude preparation of culture filtrates sterilized by autoclave and lyophilized as previously described (Casacuberta *et al.*, 1992). Treatment with fungal elicitors was performed by infiltration of previously transfected *N. benthamiana* leaves with an elicitor solution (0.5 μg ml^{-1}) 1h prior to confocal microscopy or by direct application of the elicitor solution on leaves. BiFC interaction intensity was analysed by measuring the average fluorescence of the same microscopic field before and after 1 h of elicitor treatment. To monitor the same field, the plant tissue was fixed on the cover slip using a Medical Adhesive (Hollister, http://www.hollister.com/) that keeps the epidermal cells viable while making them immobile. After the initial scanning of fluorescence (time 0), we added the fungal elicitor to the system and we scanned the same field one hour after the treatment, keeping the initial settings of the laser and of the image acquisition unchanged. Expression levels of MPK3, MPK6 and MKP2 before and after elicitor treatment was performed by western blot analysis of infiltrated leaves. Approximately 40 μg of total protein from several independent transfected leaves was loaded per lane and transferred to a nitrocellulose membrane. Homogenous protein transfer was confirmed by Pounceau red staining. Anti-GFP, N-Terminal antibody (Sigma) and monoclonal antibody, JL-8 (Living Colors) were used to detect YFN- and YFC-conjugated proteins, respectively. Rubisco was used as input control. For the HR assay, *N. benthamiana* leaves were transiently transfected for 3 days with the different constructs and then infiltrated with the same fungal elicitor solution mentioned above. Leaves were photographed before the elicitor treatment and 24 h after infiltration.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Bacterial growth in *Ps. syringae*-inoculated leaves after infection.

Figure S2. Necrotic lesions in *B. cinerea* -inoculated leaves at 5 days after drop inoculation (dpi).

Figure S3. Analysis by western blot of MKP2 protein accumulation in *35S::MKP2/mkp2* plants.

Figure S4. Phenotype of *35S::MKP2–GFP* transgenic plants in stress responses.

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