UNIVERSIDAD POLITÉCNICA DE MADRID

**ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRARIA, ALIMENTARIA
Y DE BIOSISTEMAS**

Departamento de Biotecnología-Biología Vegetal

Centro de Biotecnología y Genómica de Plantas (UPM-INIA)

**Characterization of the ERECTA-YODA MAPK functional cascade and
other phosphorylation events in innate immunity**

Ph.D. Thesis:

SANJAY SWAMI

Bachelor in Biotechnology, M.Sc. in Life Sciences.

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SUMMARY

Phosphorylation cascades are important signal transduction mechanisms that mediate the perception of signals and the activation of multiple responses in plants. YODA (YDA) is a MAPK Kinase Kinase that functions downstream the Receptor Like Kinase (RLK) ERECTA (ER). Besides controlling stomatal patterning and other plant developmental-associated processes, YDA-ER pathway regulates Arabidopsis immune responses. Constitutive activation of YDA in Arabidopsis (CA-YDA plants) confers broad-spectrum disease resistance. A transcriptomic analysis showed that CA-YDA plants constitutively overexpress several cell wall-related and defence-associated genes. In addition, some putative *SMALL SECRETED PEPTIDES* (SSP1-8) and *PUTATIVE RECEPTOR PROTEINS* (PRP1-9) coding for RLKs, were upregulated in CA-YDA plants. Disease resistance analysis on single mutants in these genes revealed that *SSP1*, *SSP2*, *SSP6*, *SSP7*, *SSP8*, *PRP1*, *PRP4* and *PRP7* are required to trigger Arabidopsis immune responses against the fungus *Plectosphaerella cucumerina* (PcBMM), the bacteria *Pseudomonas syringae* pv. *tomato* DC3000 or the oomycete *Hyaloperonospora arabidopsidis* Noco2. Moreover, mutants in *SSP1* and *PRP1* displayed enhanced susceptibility towards *Cauliflower Mosaic Virus*, highlighting their putative role in immunity against a broad range of pathogens. Synthetic peptides corresponding to the C-terminal regions of SSP1 (PEP-A and PEP-E), SSP2 (PEP-O) and SSP7 (PEP-I) were generated to determine their ability to trigger PAMP-triggered immune (PTI) responses. Preliminary data indicate that SSP7 and, to a lesser extent, SSP1 could act as activators of defence responses, as treatments with PEP-I and PEP-E are able to trigger some PTI events in WT plants (Col-0). Interestingly, neither these small signalling peptides nor PRP1 seem to be involved in regulating developmental parameters such as plant height and pedicel and silique length, and only SSP3 may have a role in stomata patterning, as *ssp3* mutants present higher stomatal index. Thus, the contribution of most of the selected SSPs and PRP1 remains unique to Arabidopsis defence responses. We therefore hypothesize that changes in the cell wall of CA-YDA plants may trigger novel immune responses in parallel to canonical disease resistance pathways regulated by phytohormones. This new

defence surveillance system would rely on immunogenic peptides (SSPs)-RLK pairs that upon perception of pathogens or elicitors would act in a positive feedback loop maximizing the level of immunity.

Arabidopsis heterotrimeric G protein complex modulates PTI and regulates disease resistance to pathogens. To identify new immune response elements, a mutational screening was performed to identify suppressors of the enhanced susceptibility to *PcBMM* displayed by a mutation in the gene encoding the heterotrimeric G protein β subunit (*AGB1*). Four *sgb* (*suppressor of G* protein *beta1*) mutants (*sgb10*, *sgb11*, *sgb12* and *sgb13*) were identified based on their ability to fully rescue *agb1-2* susceptibility. This study was mainly focused on the characterization of *sgb10*. This mutation mapped on chromosome 3 and corresponded to a G to A transition in the coding sequence of *MAP KINASE PHOSPHATASE 1* (*MKP1*; *At3g55270*). Interestingly, *mkp1/sgb10* mutant displayed elevated reactive oxygen species (ROS) levels that were compromised in the NADPH oxidase mutant *rbohD*, suggesting that the enhanced ROS production is generated by this oxidase. Moreover, an epistatic, genetic interaction was observed between *mkp1/sgb10* and *rbohD* in *Arabidopsis* resistance response to *PcBMM*, suggesting that MKP1 and RBOHD are components of the same immune pathway and that this NADPH oxidase could be a downstream target of MKP1.

RESUMEN

Las cascadas de fosforilación son importantes mecanismos de transducción de señales que median entre la percepción de las señales y la activación de múltiples respuestas en las plantas. YODA (YDA) es una MAPK quinasa quinasa que funciona aguas abajo del receptor tipo quinasa (RLK) ERECTA (ER). Además de controlar el patrón estomático y otros procesos del desarrollo de la planta, la vía mediada por YDA-ER regula respuestas inmunitarias de Arabidopsis. La activación constitutiva de YDA en Arabidopsis (plantas *CA-YDA*) confiere resistencia a la enfermedad de amplio espectro. Un análisis transcriptómico mostró que las plantas *CA-YDA* sobreexpresan constitutivamente varios genes relacionados con la pared celular y asociados con la respuesta de defensa. Además, algunos supuestos *SMALL SECRETED PEPTIDES* (*SSP1-8*) y *PUTATIVE RECEPTOR PROTEINS* (*PRP1-9*), que codifican RLKs, se inducen transcripcionalmente en plantas *CA-YDA*. El análisis de resistencia a enfermedades realizado en mutantes individuales en estos genes reveló que *SSP1*, *SSP2*, *SSP6*, *SSP7*, *SSP8*, *PRP1*, *PRP4* y *PRP7* son necesarios para activar las respuestas inmunes de Arabidopsis frente al hongo *Plectosphaerella cucumerina* (*PcBMM*), la bacteria *Pseudomonas syringae* pv. *tomato* DC3000 o el oomiceto *Hyaloperonospora arabidopsidis* Noco2. Por otra parte, los mutantes en *SSP1* y *PRP1* mostraron una mayor susceptibilidad al virus del mosaico de la coliflor, destacando así su papel en la inmunidad frente a una amplia gama de patógenos. Además, se generaron péptidos sintéticos correspondientes a las regiones C-terminales de *SSP1* (PEP-A y PEP-E), *SSP2* (PEP-O) y *SSP7* (PEP-I) para determinar su capacidad de desencadenar respuestas inmunes activadas por PAMPs (*PAMP-triggered immunity*, PTI). Datos preliminares indican que *SSP7* y, en menor medida, *SSP1* podrían actuar como activadores de respuestas de defensa, ya que los tratamientos con PEP-I y PEP-E son capaces de desencadenar algunos eventos de PTI en plantas silvestres (Col-0). Curiosamente, ni estos pequeños péptidos de señalización ni *PRP1* parecen estar implicados en la regulación de parámetros del desarrollo vegetal tales como la altura de la planta o la longitud del pedicelo y la silicua. Además, sólo *SSP3* parece tener un papel regulador en el patrón de desarrollo de estomas, ya que los mutantes *ssp3* presentan un

índice estomático mayor. Por lo tanto, la mayoría de los *SSPs* seleccionados y *PRP1* parecen estar implicados exclusivamente en las respuestas de defensa de *Arabidopsis*. Estos resultados apoyan la hipótesis de que cambios en la pared celular de las plantas *CA-YDA* podrían desencadenar respuestas inmunes novedosas que actuarían en paralelo a las vías canónicas de resistencia a enfermedades reguladas por fitohormonas. Este nuevo sistema de vigilancia de la defensa se basaría en pares de péptidos inmunogénicos (*SSP*) y sus receptores (*LRKs*) que, tras la percepción de patógenos o elicitores, actuarían en un ciclo de retroalimentación positiva que maximizaría el nivel de inmunidad.

El complejo de proteína G heterotrimérica de *Arabidopsis* modula la PTI y regula la resistencia a la enfermedad causada por patógenos. Para identificar nuevos elementos de la respuesta inmune de las plantas, se realizó un cribado de mutantes para identificar supresores de la susceptibilidad a *PcBMM* mostrada por una mutación en el gen que codifica la subunidad β de la proteína G heterotrimérica (*AGB1*). Cuatro mutantes *sgb* (supresores de la proteína G beta 1; *sgb10*, *sgb11*, *sgb12* y *sgb13*) fueron identificados por su capacidad para rescatar completamente la susceptibilidad de *agb1-2*. Este estudio se centró principalmente en la caracterización de *sgb10*. Esta mutación se localizó en el cromosoma 3, correspondiendo a una transición de G a A en la secuencia codificante de *MAP KINASE PHOSPHATASE 1* (*MKP1*; At3g55270). Curiosamente, el mutante *mkp1/sgb10* presentó niveles elevados de especies reactivas de oxígeno (ROS) que se mostraron ausentes en el doble mutante con la NADPH oxidasa *rbohD*, lo que sugiere que esta producción aumentada de ROS es generada por esta oxidasa. Además, se observó una interacción genética epistática entre *mkp1/sgb10* y *rbohD* en la respuesta de resistencia de *Arabidopsis* a *PcBMM*, lo que sugiere que MKP1 y RBOHD son componentes de la misma vía de señalización de la inmunidad y que esta NADPH oxidasa podría ser una diana aguas abajo de MKP1.

ABBREVIATIONS

ABA	Absciscic acid
AEQ	Aequorin
AGB1	Arabidopsis β subunit of heterotrimeric G protein
AGG1	Arabidopsis γ subunit of heterotrimeric G protein 1
AGG2	Arabidopsis γ subunit of heterotrimeric G protein 2
ANOVA	Analysis of Variance
AP2/ERF	Apetala2/ethylene-response element binding factor
ATP	Adenosine triphosphate
AtPEP, PEP	Arabidopsis endogenous danger peptide
AtRBOH	Arabidopsis thaliana respiratory burst oxidase homologue
Avr	Avirulence factor
BAK1	BRI1-associated kinase 1
BIK1	<i>Botrytis</i> induced kinase 1
BIR1	BAK1-Interacting Receptor-like kinase 1
BRI1	Brassinosteroid insensitive 1
BRs	Brassinosteroids
bZIP	Basic domain leucine zipper
Ca ²⁺	Calcium ion
CaM	Calmodulin
CA-YDA	Constitutive active form of YDA
CBEL	Cellulose-binding elicitor lectin
CBL	Calcineurin B-like
cDNA	Complementary deoxyribonucleic acid
CPK	Calcium-dependent protein kinase
CEBiP	Chitin elicitor-binding protein
CERK1	Chitin elicitor receptor kinase 1
CESA9	Cellulose synthase A9
cfu	Colony forming units
CKs	Cytokinins
Cl ⁻	Chloride ion
CLV	CLAVATA
CMV	<i>Cucumber mosaic virus</i>

Col-0	<i>Arabidopsis thaliana</i> accession Columbia-0
COS	Chitin oligosaccharide
CWI	Cell wall integrity
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
DR	Disease rating
ESK1	Eskimo1
EFR	Elongation Factor-thermo unstable (EF-Tu) Receptor
EF-Tu	Elongation factor-thermo unstable
EGF	Epidermal growth factor
EPFs	Epidermal Patterning Factor Peptides
EXT3	Extensin 3
elf18	Elongation factor-Tu epitope 18
<i>elk</i>	ERECTA-like mutant
<i>er</i>	ERECTA mutant
ER	ERECTA receptor like kinase
ERf	ERECTA family
ERL	ERECTA-like receptor
ET	Ethylene
ETI	Effector-triggered immunity
flg22	Flagellin epitope 22
FLS2	Flagellin sensing 2
FUT6	Fucosyltransferase 6
FUT8	Fucosyltransferase 8
FW	Fresh weight
G protein	Guanine nucleotide binding protein
Gβγ	β and γ subunit heterodimer of heterotrimeric G protein
GDP	Guanine dinucleotide phosphate
gDNA	Genomic deoxyribonucleic acid
GO	Gene ontology
GPA1	<i>Arabidopsis</i> α subunit of heterotrimeric G protein
GPCR	G protein coupled receptor
GSH	Glutathione (reduced form)

GTP	Guanine trinucleotide phosphate
H ⁺	Hydrogen ion
H ₂ O ₂	Hydrogen peroxide
<i>Hpa</i>	<i>Hyaloperonospora arabidopsidis</i>
hpi	Hours post-inoculation
HR	Hypersensitive response
HRP	Horseradish peroxidase
IG	Indol glucosinolate
JA	Jasmonic acid
JAZ	JASMONATE-ZIM-DOMAIN PROTEIN
K ⁺	Potassium ion
La-0	<i>Arabidopsis thaliana</i> accession Landsberg
Lec	Lectin
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LysM	Lysin motif
MAMP	Microbe-associated molecular pattern
MAP3K, MEKK	Mitogen-activated protein kinase kinase kinase
MAPK, MPK	Mitogen-activated protein kinase
MAPKK, MKK	Mitogen-activated protein kinase kinase
MKP	Map kinase phosphatase
MS	Murashige and Skoog medium
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NO ³⁻	Nitrate ion
NOX	NADPH oxidase
OD ₆₀₀	Optical Density (600 nm)
OG	Oligogalacturonide
PAMP	Pathogen-associated molecular pattern
<i>Pc, PcBMM</i>	<i>Plectosphaerella cucumerina</i> isolate BMM
PCR	Polymerase chain reaction
PEPR	AtPEP Receptor
PGN	Peptidoglycan
PP2A	Protein phosphatase 2A

PP2C	Protein phosphatase 2C
PR	Pathogenesis related
PRP	Putative receptor protein
PRR	PAMP recognition receptor
PTI	PAMP-triggered immunity
<i>Pto</i>	<i>Pseudomonas syringae</i> pathovar <i>tomato</i>
PUB	Plant U-box protein
qPCR	Quantitative real-time polymerase chain reaction
qRT-PCR	Quantitative real-time reverse transcription polymerase chain reaction
QTL	Quantitative trait locus
R protein	Resistance protein
RBOHD	Respiratory burst oxidase homologue D
RBOHF	Respiratory burst oxidase homologue F
RGS1	Regulator of G protein signalling-1
RLCK	Receptor-like cytoplasmic kinase
RLK	Receptor-like kinase
RLP	Receptor-like protein
RLU	Relative light units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM1	Resistance to <i>Pseudomonas syringae</i> pv. <i>Maculicola</i> 1
RPS2	Resistance to <i>Pseudomonas syringae</i> pv. <i>Maculicola</i> 2
RT	Room temperature
SA	Salicylic acid
SAR	Systemic acquired resistance
SD	Standard deviation
SE	Standard error
SERK	Somatic embryogenesis receptor kinase
<i>sgb10</i>	<i>Suppressor of G protein beta1-10</i>
<i>sgb11</i>	<i>Suppressor of G protein beta1-11</i>
SOBIR1	Suppressor of BIR1-1
SSP	Small secreted peptide
TB	Trypan blue staining
TF	Transcription factor

TIR-NBS-LRR	Toll/Interleukin 1 receptor-like-NBS-LRR protein
TMM	Too many mouths
Trp	Tryptophan
WAK	Wall-associated kinase
WRKY	Transcription factor with a W-box binding domain
WT	Wild-type
YDA	YODA
[Ca ²⁺]cyt	Cytoplasmic calcium concentration

INTRODUCTION

INTRODUCTION

1.1. Plant innate immunity defence response against pathogens.

Plants have a constant exposure to their environment throughout their life cycle. Interaction of a plant with its immediate environment, including the attack of microbial communities, requires the plant to sense and respond accordingly. Thus, the ultimate outcome of plant-pathogen interaction depends on the communication established at molecular level between plant and pathogen (Nimchuk *et al.*, 2003). Accordingly, plants have a complex immunity system to detect pathogen attack and to prevent causing disease (Dodds and Rathjen, 2010; Zipfel, 2014). Unlike mammals, plants lack specialized mobile defenders, immune cells and molecules that mediate the more sophisticated adaptive immunity. Therefore, immunity in plants is dependent upon innate immunity, being mostly based on a system with each individual cell performing the detection of the pathogen and mounting the immune response (Jones and Dangl, 2006). However, once the pathogen is perceived and the cell defence triggered there are signalling pathways emerging from the infection site, often mediated by different plant hormones, which allow the establishment of a broad disease resistance affecting the whole plant.

Plant pathogens broadly can be divided into two groups based on their lifestyle. On the one hand necrotrophs, which actively kill their host tissues during the infection, so they can feed on them. These pathogens typically, deploy diverse phytotoxic compounds and cell-wall degrading enzymes to induce cell damage and nutrient leakage. On the other hand, biotrophs, which rely partially or entirely on their intact host to complete their life cycle. Thus, biotrophs, although they may partially damage the host cell, and particularly its cell wall, they maintain host viability to fulfil their nutrient requirements (Dangl and Jones, 2001). These two lifestyles have important implications on the detection by the plant. Moreover, immune responses against necrotrophs and biotrophs may be activated through independent pathways (typically mediated by different plant hormones), although signal transduction cross-talk may exist (Andolfo and Ercolano, 2015).

Regardless of the pathogens mode of infection, any invasion attempt will activate plant immune responses, which involve complex cellular, histological and molecular events that prevent the pathogen proliferation or disease progression (Kemmerling *et al.*, 2007). Plant defence mechanism is complex and is composed of multilayers that are effective against a wide array of pathogens. Thus, for any organism to be pathogenic, it requires to override several of these defensive layers. Often, first, they must access the plant interior either by penetrating the leaf or root surface or by natural openings like stomata or hydathodes. Plant cell wall acts as the next barrier for the microbe to breach. Then plasma membrane is laced with extracellular surface receptors that recognize common elements present in the pathogens (Chisholm *et al.*, 2006). Finally, in the case of pathogens that release effectors that penetrate inside the cell, an additional intracellular layer of receptors and some intracellular defences need to be evaded for the pathogen to succeed.

The structure of the plant cell wall is a singular characteristic that distinguishes plant cells from all other eukaryotic cells. The cell wall is like an exoskeleton surrounding the plant cell that is composed of a complex matrix of polysaccharides, proteins and different polymers secreted by the cell. Cell wall can be classified as primary, present in all plant cells and with its major constituents being cellulose, hemicellulose and pectin, and secondary cell wall, present in certain cell types and that additionally contains suberin and lignin (Miedes *et al.*, 2014). Cell wall provides both structural support and protection against biotic and abiotic stresses and is under the control of a dedicated cell wall integrity maintenance mechanism. Thus, plant cell wall is able to sense perturbations and mediate a response with regards to growth and development as well as different stresses (Sanabria *et al.*, 2010). Hence, the cell wall usually represents the first barrier to an invading pathogen. If the invasion is halted, cellular damage is minimized, and no other defensive actions are required. However, a recurrent pathogen attack can lead to the release of cell wall elements such as oligomers of α -1,4-linked galacturonosyl residues, called oligogalacturonides (OGs), that can be released after partial degradation of plant pectins, either mechanically or more commonly by the action of hydrolytic enzymes secreted by pathogens. These signals released are detected by the cell wall integrity

maintenance mechanism that possibly includes Wall-Associated Kinases (WAKs), potential cell wall receptors for sensing and signalling from the cell wall (Kohorn and Kohorn, 2012). Thus, exogenous OGs treatments can induce innate immune responses, including activation of Mitogen-Activated Protein Kinase (MAPK) cascades, callose deposition, production of reactive oxygen species (ROS), elevated cytosolic Ca^{2+} and altered expression of defence genes (Ferrari *et al.*, 2013). Plant cell wall also acts as a reservoir for antimicrobial compounds such as thionins, defensins, calmalexin, indole glucosinolates (IG) and lipid transfer proteins that are released upon an infection and can destabilize pathogen membranes (Miedes *et al.*, 2014; Sanchez-Vallet *et al.*, 2009).

1.1.1. Pathogen-associated molecular pattern-triggered immunity and effector-triggered immunity.

Plants have evolved two cell autonomous strategies to detect pathogen. The first one relies on protein receptors, called Pattern Recognition Receptors (PRRs), present at the cell surface and that typically detect conserved parts of the invading organisms called Pathogen (or Microbial)-Associated Molecular Patterns (PAMPs or MAMPs). PAMPs are essential and evolutionary conserved parts of a whole class of pathogens. Typical examples of PAMPs are chitin from the fungal cell wall and flagellin from gram (-) bacteria. Apart from this direct recognition of a group of pathogens, plants also respond to endogenous molecules produced by the pathogen invasion called Danger Associated Molecular Patterns (DAMPs). These include cuticle or cell wall fragments (such as OGs mentioned above) and released small extracellular peptides that are also recognized through PRRs. Stimulation of PRRs by these PAMPs or DAMPs leads to the activation of a first layer of plant defence system called PAMP-triggered immunity (PTI; Figure 1.1.; Jones and Dangl, 2006).

A second tier of pathogen perception involves detection of secreted pathogen virulence molecules (termed effectors) by a set of intracellular immune receptors, called R-proteins (for Resistance-proteins), which are mostly Nucleotide-Binding site Leucine-Rich Repeats (NB-LRR) proteins to perceive these effectors. This recognition, that can be direct or indirect, induces a second defence

layer called Effector-Triggered Immunity (ETI; Figure 1.1; Dangl and McDowell, 2006; Dodds and Rathjen, 2010).

Figure 1.1. General overview of the plant immune system. Plant cell surface receptors (Pattern Recognition Receptors, PRRs) recognize the conserved Pathogen-Associated Molecular Patterns (PAMPs) from different pathogens upon colonization or the Damage-Associated Molecular Patterns (DAMPs) that are released by plant cell wall upon cellular damage. Perception of PAMPs and DAMPs leads to the activation of PAMP-Triggered Immunity (PTI). To facilitate virulence and suppress PTI, pathogens can deliver effectors directly into the cytoplasm. Effectors are detected by specific intracellular immune receptors that activate Effector-Triggered Immunity (ETI). Adapted from Dodds and Rathjen, 2010.

Host-pathogen interaction is an important force shaping organismal diversity. The usual large populations of invading pathogens allow the continuing generation of new effectors that modify the defences. As a counterpart, in the plant genomes *R*-genes exist in large multigene families that undergo rapid adaptive diversification, apparently, in response to intense selection for new resistance specificities. The resistance effectiveness is limited because the pathogens are, in turn, polymorphic for virulence genes that can help evading plant resistance. All these data support the hypothesis of an

arm race of selection affecting *R* genes and effector genes in the coevolution of hosts and pathogens (Bishop *et al.*, 2000; Frank, 1992; Flor, 1971).

In contrast to PAMPs, effectors are variable and dispensable. Adapted pathogens have initially evolved the effectors to reprogram the host's physiology towards infectious compatibility. Generally, PTI is more effective against general non-adapted pathogens in a phenomenon called non-host resistance, whereas ETI is more effective against specific adapted pathogens. Responses in the host are similar for both PTI and ETI, although ETI is qualitatively more rapid, stronger and often involves a localized form of cell death called hypersensitive response (HR; Macho and Zipfel, 2014; Dodds and Rathjen, 2010).

1.2. PAMP-triggered immunity, first line of defence.

PTI is based upon activation of host cell surface PRRs following recognition of conserved PAMPs/MAMPs from the pathogen or endogenous DAMPs released from the host upon pathogen attack (Monaghan and Zipfel, 2012; Jones and Dangl, 2006). PAMPs represent structural components that are essential for microbial survival and that are typically harboured by groups and families of invading pathogens. These PAMPs generally include constituents of the microbial surface but can also be molecules located in the cytoplasm. A large number of PAMPs have been described, so far, from fungi, oomycetes and bacteria, and include a wide variety of peptides, oligosaccharides and glycopeptides (Choi and Klessig., 2016).

Two classes of proteins work at the host-cell surface as PRRs to perceive these PAMPs/MAMPs and DAMPs: Receptor-Like Kinases (RLKs); and Receptor-Like Proteins (RLPs). Structurally, RLKs possess an extracellular ligand-binding domain, a single transmembrane domain and an intracellular serine/threonine kinase-signalling domain. RLPs share this basic conformation except they lack an intracellular signalling domain. For this reason, RLPs are thought to be dependent upon other regulatory receptor kinases to transduce the intracellular signalling after perception of ligand. Plant genomes contain a large number of these two classes of PRRs and many of them are transcriptionally

induced upon PAMP treatment, illustrating the large diversity of such perception systems and suggesting their potential role in defence (Couto and Zipfel, 2016; Zipfel *et al.*, 2004).

1.2.1. PAMPs in PAMP-triggered immunity.

Molecular patterns that trigger PTI are conserved among pathogens and benign microbes (Boller and Felix, 2009). Several examples on the perception of bacterial PAMPs have been characterized in detail. For example, flagellin which is a major component of flagella and is a potent inducer of PTI. Perception of bacterial flagellin by its corresponding PRR, the LRR-RLK FLS2 (FLAGELIN SENSING 2), is very well understood in *Arabidopsis* (Zipfel *et al.*, 2004). FLS2 recognizes and binds a conserved N-terminal 22 amino acid immunogenic epitope in flagellin (flg22; Felix *et al.*, 1999). Functional orthologs of FLS2 has been identified in several plant species, including a wild relative of tobacco (*Nicotiana benthamiana*), rice (*Oryza sativa*), tomato (*Solanum lycopersicum*) and grapevine (*Vitis vinifera*), indicating that the same flg22 peptide is recognized by most higher plants (Zipfel, 2014). However, flg22 is not the exclusive epitope from flagella recognized by plants. Some field isolates of *Pseudomonas syringae* carry a 28-amino acid epitope, flgII-28, which induces immune responses in several *Solanaceae* species. But this PAMP is not perceived by the *Arabidopsis* flg22 receptor, FLS2 (Clarke *et al.*, 2013).

Other bacterial PAMP/PRR pairs have been well characterized. Elf-18, an N-acetylated peptide comprising the first 18 amino acids of the bacterial Elongation Factor Tu (EF-Tu) also acts as a PAMP in *Arabidopsis* (Kunze *et al.*, 2004). Perception of elf-18 is mediated by the *Arabidopsis* LRR-RLK EFR (ELONGATION FACTOR-Tu RECEPTOR; Figure 1.1). However, in rice, a fragment from the central region of bacterial EF-Tu, termed EFa50, acts also as a PAMP and is perceived by a yet unknown PRR (Furukawa *et al.*, 2014). Also, peptidoglycans (PGNs), major constituents of bacterial cell walls, represent classical PAMPs perceived by plants. In *Arabidopsis*, this perception involves two RLPs with lysine motif (LysM) containing ectodomains (LysM Domain-Containing GPI-Anchored Protein 1 -LYM1- and LYM3), AtLYM1 and AtLYM3, which specifically bind to PGNs (Willmann *et al.*, 2011). Also, the lipopolysaccharide (LPS; also, called 'endotoxin'), abundant in the outer cell envelope of Gram (-)

bacteria, can act as a PAMP and induce early PTI responses including ROS production and expression of defence genes (Ranf *et al.*, 2015). Conserved lipid A of LPS serves as the main PAMP, although the oligosaccharide core and the O-antigen structure are also recognized as PAMPs (Ranf *et al.*, 2015; Erbs and Newman, 2012).

Chitin, the main component of fungal cell wall, has been studied for decades as a classic PAMP in plants. Chitin is composed of long chain polymers of β -1,4-N-acetyl-D-glucosamine, that can be degraded by plant chitinases generating shorter chitin oligosaccharides (4 to 8-mer), which are able to induce defence responses in several plant systems (Shibuya and Minami, 2001). Interestingly, different plants have evolved distinct mechanisms that employ common components for chitin perception. In Arabidopsis, Chitin Elicitor Receptor Kinase 1 (AtCERK1) binds chitin which in turn induces its homodimerization and consequent immune signalling (Liu *et al.*, 2011). However, affinity of AtCERK1 to chitin is very low, which suggests that other major receptors with higher chitin binding affinity might be present. Lysin Motif Receptor Kinase 5 (AtLYK5) was found to be the primary receptor for chitin perception in Arabidopsis and, after binding of chitin, subsequently induces a complex formation with AtCERK1 to induce PTI (Cao *et al.*, 2014). Another well characterized fungal PAMP is the Cellulose-Binding Elicitor Lectin (CBEL) from *Phytophthora parasitica* var. *nicotianae*, which was shown to trigger defence responses in Arabidopsis and tobacco (Gaulin *et al.*, 2006). The two cellulose-binding domains of CBEL are essential and sufficient to stimulate defence responses.

Plant viruses extensively use the intracellular host cell environment for replication of their genome, viral gene expression and infection establishment. Although no viral PAMP that could be detected by classical PRR receptors has been documented, plants are able to detect the attack of viruses and devote multiple counter mechanisms to overcome viral infection. RNA silencing represents the major mechanism of plant immune response against viruses, that also include hormone mediated defence, protein degradation and metabolism regulation (Incarbone and Dunoyer, 2013). Plants use R-proteins with NB-LRR domains to recognize viral effectors and activate ETI against virus (Mandadi and Scholthof, 2013). However, recent studies indicate that Arabidopsis mutants in the LRR-RLK BAK1

(BRI1-Associated Receptor-Kinase 1) are more susceptible to viral infection, suggesting that PRRs, and thus unidentified PAMPs or DAMPs, may be involved in antiviral defence. Furthermore, NIK1 (Nuclear Shuttle Protein (NSP)-Interacting Kinase 1), a member of the RLK family, was found to have an important role in defence response against geminiviruses (Fontes *et al.*, 2004). NIK1 is structurally similar to BAK1, but the mechanism of defence action is completely different from BAK1 mediated immune response (Machado *et al.*, 2015).

1.2.2. DAMPs in PAMP-triggered immunity.

The ability to distinguish self from non-self is the most fundamental aspect of an immune system. In addition to the PAMPs and MAMPs, pathogen infection causes release of endogenous DAMPs from the cell wall or the cytoplasm into the extracellular space, where, following perception by PRRs, can induce immune responses resembling PTI (Hou *et al.*, 2014; Huffaker *et al.*, 2006). Examples of DAMPs generated upon pathogen attack are OGs, cutin monomers and short peptides.

A well characterized DAMP is PEP1, identified as an extracellular 23-aa peptide derived from the C-terminus of the 92-aa Arabidopsis Precursor of Peptide 1 (PROPEP1, Huffaker *et al.*, 2006). The full length amino acid sequence of the precursor protein lacks an N-terminal signal peptide, therefore, the release of PEP1 into the apoplast could result from cellular damage caused by pathogen attack or wounding (Hou *et al.*, 2014). PROPEP1 belongs to a seven-member gene family in Arabidopsis that display a low overall amino acid sequence identity but with 35% to 65% amino acid identity at the C-terminal. Two LRR-RLKs, PEP1 Receptor 1 (PEPR1) and PEP1 Receptor 2 (PEPR2), were shown to act as PEP1 receptors (Figure 1.1) to activate PTI and enhance host resistance against the pathogens *P. syringae* and *Pythium irregulare* (Yamaguchi *et al.*, 2006). Other peptides acting as DAMPs include a new gene family of PAMP-Induced secreted Peptides (prePIPs), which has been identified recently in Arabidopsis (Hou *et al.*, 2014). Expression of some members of the family, including prePIP1 and prePIP2, is induced by different pathogens and elicitors and they all share the hallmarks of post-translationally modified secreted peptides: i) a signal peptide at the N-terminus; ii) a highly-conserved

cysteine-poor region at the C-terminus (SGPS motif); iii) a variable region between the signal peptide and the SGPS motif (Matsubayashi, 2011). prePIP1 and prePIP2 induce immune responses and enhance resistance against *P. syringae* and *Fusarium oxysporum*. It was found that LRR-RLK RLK7 is required for PIP1- and PIP2-triggered responses and RLK7 binds PIP1 thus acting as the PIP1 receptor (Hou *et al.*, 2014).

As stated above, OGs are oligomers of alpha-1,4-galacturonosyl residues released from the pectin of plant cell walls. OGs are thought to be released upon partial degradation of homogalacturonan by microbial Polygalacturonases (PGs) during infection or by the action of endogenous PGs induced by mechanical damage (Ferrari *et al.*, 2013). OGs can elicit defence responses, including accumulation of ROS and Pathogenesis-Related (PR) proteins, and protect plants against pathogen infections. WAKs are RLKs with an extracellular domain containing epidermal growth factor (EGF) motifs that act as receptors for the OGs (Figure 1.1; Kohorn and Kohorn, 2012).

Interestingly, some ligands that have been recognized as important mediators of developmental processes could also play an important role in plant immunity. This is the case of several secreted peptides such as CLAVATA3 peptide (CLV3p). This peptide was previously characterized in relation to stem cell homeostasis in the shoot apical meristem (Fletcher *et al.*, 1999). Recent data suggest that CLV3p can also be recognized by FLS2 and activate FLS2-dependent immune responses in the shoot meristem (Lee *et al.*, 2011). Moreover, the sulfated peptide Phytosulfokine (PSK) and plant Peptide containing Sulfated Tyrosine 1 (PSY1) were initially identified as promoters of cell proliferation and tissue growth, and have been recently shown to enhance susceptibility to a biotrophic pathogen and resistance to a necrotrophic pathogen (Mosher *et al.*, 2013).

1.2.3. Perception of PAMPs and DAMPs by pattern recognition receptors in plants.

Perception of environmental signals and generation of a response accordingly is essential for organisms to defend themselves against potential pathogens. Thus, plants rely on membrane-

localized receptors to detect apoplastic non-self and damaged-self patterns to activate effective immune responses.

Plant genome encodes a plethora of RLKs and RLPs. For instance, *Arabidopsis* genome encodes ~410 different RLKs and ~170 RLPs that could work as PRRs. Plant PRRs typically contain highly variable ligand binding ectodomains that can recognize a wide range of ligands including peptides, oligosaccharides, lipopolysaccharides and steroids. Thus, PRRs can be sub divided based on the nature of their ectodomains : LRR , LysM, Lectin or Epidermal Growth Factor (EGF) like domains (Zhou *et al.*, 2017; Couto and Zipfel, 2016; Macho and Zipfel, 2014). All characterized LRR-type PRRs are able to bind peptides and protein ligands in nature. Some examples of these ligands are bacterial flagellin (flg22) that is perceived by FLS2, elongation factor (elf-18) that is bound by EFR (Figure 1.2) and the proteinaceous DAMP PEP that is bound by AtPEPR1 and AtPEPR2 (Zipfel *et al.*, 2006; Yamaguchi *et al.*, 2006; Gómez-Gómez and Boller, 2000). The sub-group belonging to LysM-PRRs are known to act as receptors of sugar containing PAMPs, such as fungal chitin, bacterial PGNs and other N-acetylglucosamine containing glycans (Figure 1.2). For example, as stated above, in *Arabidopsis* chitin is perceived by three LysM-RLKs, CERK1, LYK4 and LYK5, with the last one showing the highest affinity for chitin (Cao *et al.*, 2014). However, rice appears to contain homologous yet distinct chitin receptors. In this species, chitin induced defence pathway requires the rice counterpart of CERK1, the LysM-RLP Chitin Oligosaccharide licitor-Binding Protein (CEBiP; Shimizu *et al.*, 2010; Kaku *et al.*, 2006). PGNs are also perceived by CERK1 in collaboration with two *Arabidopsis* LysM-RLPs (Cao *et al.*, 2014; Willmann *et al.*, 2011; Miya *et al.*, 2007). On the other hand, an *Arabidopsis* Lectin domain RLK called LORE (Lipooligosaccharide-specific Reduced Elicitation), is known to recognize the lipid A moiety of bacterial LPS (Ranf *et al.*, 2015). Finally, receptor WAK1 with EGF-like ectodomains recognizes plant cell-wall derived OGs (Brutus *et al.*, 2010; Decreux and Messiaen, 2005).

Figure 1.2. Cell surface pattern recognition receptors (PRRs) for detection of PAMPs. Different regulatory receptor kinases are recruited according to the ectodomains of PRRs in response to pathogens. Leucine-rich repeat (LRR)-containing PRRs preferentially bind pathogen derived proteins or peptides, such as bacterial flagellin or elongation factor Tu (EF-Tu), or endogenous peptides such as AtPEP. PRRs containing lysine motifs (LysM) bind carbohydrate-based ligands, such as fungal chitin or bacterial peptidoglycan. BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1; also known as SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3, SERK3), related SERKs and CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) are recruited upon ligand perception by LRR-receptor kinases and LysM-receptor kinases or receptor-like proteins, respectively. Adapted from Couto and Zipfel, 2016.

PAMP/DAMP recognition elicits classical defence signal transduction events that lead to the establishment of PTI. Early cellular events include a rapid influx of Ca^{2+} , ROS production, callose deposition, stimulation of MAP kinase cascades and phosphorylation by Calcium-Dependent Protein Kinases (CPKs), and transcriptional activation of defence response genes (Wu and Zhou, 2013). In PTI/DTI activation, since perception occurs extracellularly, receptors typically associate with other proteins acting as coreceptors that facilitate the transduction of the signal to the interior of the cell. Thus, upon ligand binding, activation of the signalling requires homo- or hetero-dimerization between immune receptors and coreceptors. Both, RLKs and RLPs, are able to interact with many Receptor-Like

Cytoplasmic Kinases (RLCKs) for rapid activation of downstream signalling pathways. One of the best characterized coreceptors is BAK1. BAK1 is an LRR-RLK with only five LRRs, member of the family of Somatic Embryogenesis Receptor Kinases (SERKs) and initially identified as an interactor and positive regulator of the Brassinosteroid (BR) Receptor BRI1 (Couto *et al.*, 2016; Nam and Li, 2002). This coreceptor is recruited by many PRRs of the LRR group upon binding with the ligand to rapidly form ligand-induced complexes (Figure 1.2). E.g., direct binding of flg22 to the FLS2 ectodomain containing 28 LRRs leads to recruitment of BAK1 to form a heterodimer complex resulting in rapid phosphorylation of BAK1 and FLS2 and activation of downstream signalling (Chinchilla *et al.*, 2007; Chinchilla *et al.*, 2006). Other LRR-PRRs such as EFR, which recognizes EF-Tu (epitopes elf18 or elf26), and PEPR1 and PEPR2, which recognize the endogenous AtPEP1 (and related peptides), all are associated with the regulatory co-receptor BAK1 (Couto and Zipfel, 2016; Roux *et al.*, 2011). In the case of LRR-RLPs, that lack a cytoplasmic kinase domain, the association with RLKs is particularly pertinent for signal transduction to the cytoplasm. One of such LRR-RLKs is Suppressor of BIR1 (SOBIR1), originally identified as a component required for autoimmunity caused by loss-of-function of BAK1- Interestingly, Receptor-Like Kinase 1 (BIR1) functions as a common adaptor for multiple LRR-RLK-type PRRs (Gust and Felix, 2014). Also, other types of PRR require dimerization. E.g., in Arabidopsis, chitin signalling is mediated by the LysM-RLK CERK1. CERK1 contains three extracellular LysM domains that bind to chitin oligomers longer than six sugars and requires homodimerization to generate an active receptor complex to trigger chitin-induced immune signalling (Liu *et al.*, 2011).

1.3. Effector-triggered immunity, second layer of protection.

ETI is the second layer of plant defence, which acts largely inside the cell. Plants have developed intracellular immune receptors named R proteins that can detect the effector molecules directly or indirectly to induce ETI. Effectors that are recognized by the corresponding R proteins are called Avirulence (Avr) factors, and pathogens carrying Avr factors are known as avirulent pathogens. ETI is equivalent to the classical gene-for-gene resistance (Flor, 1971).

Receptors of ETI are typically polymorphic NB-LRR proteins with characteristic Nucleotide Binding (NB) site and LRR domains. Based on their N-terminal sequences, plant NB-LRRs can be mainly divided into two subclasses. One class shares homology to *Drosophila* Toll and human Interleukin-1 Receptor (TIR) intracellular signalling domains, therefore being called TIR-NB-LRR receptors. The second class possesses a predicted Coiled-Coil (CC) or an uncharacterized domain (Bonardi and Dangl, 2012). NB-LRR mediated response is more effective against biotrophic or hemibiotrophic pathogens than against necrotrophs (Jones and Dangl, 2006; Glazebrook, 2005; Dangl and Jones, 2001).

Effectors are proteinaceous molecules produced by the pathogen that modify the metabolism of the host cell. Effectors target central hubs important for cell functioning or for activation of immunity, thus acting as virulent factors. Often, the targets of the effectors are proteins involved in PAMP recognition. AvrPto and AvrPtoB from *P. syringae* are good examples of effector molecules whose targets have been well characterized. AvrPto interacts with the kinase domain of EFR and FLS2 and inhibits PTI signalling (Xiang *et al.*, 2008). AvrPtoB mediates degradation of various PRRs including FLS2, BAK1 and CERK1. The N-terminal of AvrPtoB binds these PRRs, whereas its C-terminal domain has structural homology to U-box E3 ubiquitin ligase proteins and target these PRRs to proteasomal degradation (Gimenez-Ibanez *et al.*, 2009; Göhre *et al.*, 2008). Defense signalling intermediates can also be targets of these effectors. Enhanced Disease Susceptibility (EDS1), which is considered a central regulator of basal resistance and of ETI mediated by several TIR-NB-LRR proteins is the target for the bacterial effectors AvrRps4 and HopA1 (Heidrich *et al.*, 2012; Bhattacharjee *et al.*, 2011).

R proteins can detect the presence of the pathogen by directly interacting with these effectors (Innes *et al.*, 2007). However, most frequently R proteins guard the integrity of the target cellular proteins from proteinases degradation or modification that the virulence proteins inflict, and they will initiate the immunity cascade. This scenario has conceptually been described in the guard hypothesis. The *P. syringae* AvrPto and AvrPtoB effectors target tomato Pto, a serine/threonine kinase that trans-phosphorylates and activates the NB-LRR PRF (for *Pseudomonas* Resistance and Fenthion sensitivity) to activate ETI (Ntoukakis *et al.*, 2013; Salmeron and Vernooij, 1998). Arabidopsis RPM1-Interacting

Protein 4 (RIN4) is also a very well characterized 'guardee' that is targeted in different ways by multiple bacterial effectors under surveillance mechanism of different R proteins. For instance, the *P. syringae* AvrB or AvrRpm1 effectors induce RIN4 phosphorylation by a plasma membrane-localized RIN4-Interacting Receptor-Like Kinase RIPK. Phosphorylation of RIN4 is detected by the CC-NB-LRR R protein RPM1 (Resistance to *P. syringae* Expressing *AvrRpm1*) thus activating ETI (Liu *et al.*, 2011; Bernoux *et al.*, 2011). An additional effector, *avrRpt2* also targets RIN4, causing in this case its degradation. Disappearance of RIN4 is detected by a second CC-NB-LRR R protein, RPS2 (RESISTANCE TO *P. syringae* EXPRESSING *AvrRpt2*; Belkadir *et al.*, 2004) leading to ETI.

Among the indirect mechanisms of effector recognition, a quite sophisticated scenario has been observed with some transcription factors (TF) of the WRKY family (characterized by the presence of a DNA-binding domain called WRKY). In some cases the R protein, instead of detecting the modification of a functional guardee, recognizes the modification of a decoy with no cellular function but that presents structural similarities to the effector target (Sarris and Jones, 2015). This is the case of the dual system conformed by the TIR-NB-LRR proteins RRS1 (Resistance to *Ralstonia solanacearum* 1) and RPS4 (Resistance to *P. syringae* Expressing *avrRps4*). The corresponding bacterial effectors PopP2 and AvrRps4 target WRKY proteins, causing acetylation of their lysines and interfering with DNA binding and their regulatory function. RRS1 contains a WRKY domain (decoy) that is also targeted by these effectors. Modification of this WRKY domain in RRS1 promotes dimerization of the two TIR-NB-LRR and activation of RPS4 downstream signalling leading to ETI (Roux *et al.*, 2015).

1.4. Signalling in immunity.

Even the mechanisms of PTI and ETI activation are different, both, PAMP/DAMP or effector recognition lead to similar profound and traceable signalling events to activate the defences (Cui *et al.*, 2015; Katagiri and Tsuda, 2010). These chain reaction responses include very early responses occurring few minutes after PAMP/DAMP detection such as changes of ion fluxes (e.g. K⁺) and increased Ca²⁺ concentration in the cytoplasm, apoplastic ROS production, activation of signalling

cascades mediated by MAPK and CPKs (Figure 1.3). Early responses, which can be detected in next 5-30 mins, are activation of defence gene expression, endocytosis of receptors and hormone signalling events such as ethylene synthesis (Figure 1.3). Late response processes, which can be detected after hours or days, include callose deposition, biosynthesis of antimicrobial compounds and cell wall remodelling (Boller and Felix, 2009). The following subsections will describe the most important defence signalling events that have been characterized as markers of PTI activation, although equally occur during ETI establishment.

1.4.1. Signalling cascade mediated by Ca^{2+} .

An ion flux across the plasma membrane is among the earliest physiological response to PAMPs and DAMPs is (Boller, 1995). A characteristic increase of cytosolic Ca^{2+} concentration can be detected within 0.5-2 mins of elicitation and it usually reaches the maximum peak around 4-6 mins (Boller and Felix, 2009). Ca^{2+} ions are delivered into the cytosol from extracellular and vacuolar pools. Pre-treatment with the Ca^{2+} channel blocker LiCl_3 or the membrane-impermeable Ca^{2+} chelator BAPTA (1,2-bis(o-aminophenoxy) ethane tetraacetic acid) completely abolished the MAMP/DAMP-induced cytosolic Ca^{2+} elevations, showing that an initial influx of Ca^{2+} is required for activation of cytosolic Ca^{2+} responses (Ranf *et al.*, 2011). The process is positively regulated by BIK1 (*Botrytis* Induced Kinase 1) and members of the BIK1 family, though the ion channels that facilitates this process are not well-characterized (Li *et al.*, 2014b). Ca^{2+} influx through plasma membrane channels will generate the opening of other membrane transporters leading to influx of H^+ and efflux of K^+ , Cl^- , and NO_3^- , which results in depolarization of the plasma membrane (Jeworutzki *et al.*, 2010; Nürnberger *et al.*, 2004; Boller, 1995). Change in cytosolic concentration of Ca^{2+} are detected by specific Ca^{2+} sensors. Plants possess three classes of sensors: CPKs, calmodulins (CaMs) and calcineurin B-like proteins (CBLs). The best characterized sensors are CPKs that have the unique feature of containing both, sensing Ca^{2+} and responding activities, within a single protein to directly translate Ca^{2+} signals into phosphorylation events (Romeis and Herde, 2014; Harper and Harmon, 2005).

Figure 1.3. Snapshot of key players involved in the establishment of the immune response. Plants recognize pathogen derived elicitors/effectors and mount response signalling cascades. The elicitors (PAMPs, MAMPs, DAMPs) are recognized by host membrane-localized PRRs (RLKs, RLPs, WAKs), while the effectors are recognized by NB-LRRs produced by *R* genes. Upon perception of pathogen, plant cell triggers activation of receptor complexes, phytohormone signaling, MAPKs, transcription factors, eventually to produce resistance-related (RR) metabolites and proteins. Elicitors produced mainly by hemibiotrophs and necrotrophs are perceived by RLKs and RLPs to activate downstream defence response through MAPK kinase kinase (MAPKKK) pathway. Necrotrophs produce elicitors such as enzymes and toxins, which damage the plant cell walls accumulating cell wall fragments and contents (DAMPs), which activate plant defence responses through wall-associated receptor kinases (WAKs). This recognition initiates DAMP-triggered immunity (DTI). On the other hand, biotroph pathogens deliver virulence effectors to the apoplast to block PAMP perception that are often recognized by NB-LRR proteins, which activate effector-triggered immunity (ETI). Concurrently, several secondary messengers such as Ca^{2+} , ROS, and plant hormone-mediated defence pathways (ethylene, salicylic acid, and jasmonic acid)

are activated following biotic stress, which also trigger downstream genes resulting in hypersensitive response or reduced susceptibility. Overall, the signal perceived by receptor kinases are transmitted efficiently through cytosolic protein kinases such as MAPKKK pathway, to activate an array of plant transcription factors, which regulate several *R* genes to produce resistance-related metabolites and proteins to sustain the pathogen to initial infection area. Adapted from Kushalappa *et al.*, 2016.

Structurally, CPKs are characterized by a CPK-activation domain (CAD), a protein kinase domain, and an N-terminal variable domain. The CAD domain contains a calmodulin-like Ca^{2+} binding domain with up to four canonical EF-hand Ca^{2+} binding motifs and an autoinhibitory junction region. At basal cytoplasmic Ca^{2+} levels, the kinase site is blocked by the autoinhibitory region. A rise in Ca^{2+} concentration allows binding of Ca^{2+} to the CAD domain that induces conformational changes and results in the release of the autoinhibitory region (pseudo substrate segment). Thus, CPK bound to Ca^{2+} adopts the active state and can catalyse auto-phosphorylation and phosphorylation of the targets (Boudsocq *et al.*, 2012). Through this mechanism, CPKs mediate ROS production and transcriptional reprogramming of defence response genes. CPKs also play a key role in jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) hormonal signalling (Boudsocq and Sheen, 2014).

1.4.2. Production of reactive oxygen species.

Stepwise reduction of molecular oxygen (O_2) by high-energy exposure or electron-transfer reactions leads to production of highly reactive ROS. ROS include free radicals such as superoxide anion ($\text{O}_2^{\bullet-}$), hydroxyl radical ($\bullet\text{OH}$), as well as nonradical molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1\text{O}_2$). Hydrogen peroxide and superoxide anion are the most common and broadly detected species in many studies (Tripathy and Oelmüller, 2012; Torres, 2010). ROS are constantly being produced in the plant cell as a by-product of numerous metabolic processes via the electron transport chain in photosynthesis and respiration occurring in chloroplasts, mitochondria and peroxisomes. The extreme reactivity of these molecules could lead to detrimental effects by causing lipid peroxidation in cellular membranes, protein denaturation, carbohydrate oxidation, pigment breakdown and DNA damage. However, plants have evolved scavenging and detoxifying systems to prevent these

deleterious effects (Mittler *et al.*, 2004). This allows the use of ROS as signalling components, probably through redox modification of proteins. Thus, controlled production of ROS is an important factor in many developmental processes including transition from dormant to metabolic active tissues during seed germination and bud burst, cell division and morphogenesis patterning, cell expansion and orientation of growth (Considine and Foyer, 2014; Swanson and Gilroy, 2010; Clore *et al.*, 2008). Additional enzymatic mechanisms allow the tight production of ROS with signalling role in response to abiotic and abiotic stresses (Liu and He, 2016; Bose *et al.*, 2014). Thus, increased ROS production has been reported in response to a large variety of abiotic stresses including salinity, heat, drought or induced by ozone, heavy metals and UV radiation (Jalmi and Sinha, 2015; Suzuki *et al.*, 2011). And an apoplastic ROS production, the so called oxidative burst, is among the fastest defence responses observed after pathogen perception.

Although some cell-wall peroxidases could account for some of this localized ROS production, the enzymes that have been generally associated to this ROS production are plasma membrane NADPH oxidases. These enzymes, called in plants Respiratory Burst Oxidase Homologues (RBOHs), were first identified based on the homology to gp91^{phox}, the mammalian phagocyte NADPH oxidase, also termed NOX2 or Respiratory Burst Oxidase (Torres and Dangl, 2005). Structurally, NADPH oxidases contain six transmembrane domains with two asymmetrical hemes present in the third and fifth ones. Each heme group harbours two histidine coordinated with a ferrous ion and both are required to transfer electrons to O₂ to generate the superoxide anion. Electrons would come from NADPH through the NADPH and FAD binding sites present at the C-terminal domain (Bedard and Krause, 2007).

Arabidopsis has 10 RBOHs that produce ROS in many different developmental processes as well as in response to the environment (Suzuki *et al.*, 2011; Torres and Dangl, 2005). For example, two *Arabidopsis* NADPH oxidases, *RBOHD* and *RBOHF* are pleiotropic and play different roles in response to abiotic and biotic stress responses, often showing synergistic effects (Miller *et al.*, 2010). Although *AtRBOHF* has an important role in defence response and cell death, *AtRBOHD* is responsible for most of the ROS production observed after pathogen detection. The N-terminal of the RBOH proteins, a

domain that is absent in the mammalian gp91^{phox}, has been postulated as the main regulatory domain of the activity of these plant oxidases. This 300-aa extension is the target of GTPase binding localized in intimate association with EF-hand Ca²⁺ binding motifs (Kurusu *et al.*, 2015) and some lipid-signals derived from phospholipase Dα1 (Zhang *et al.*, 2009). However, phosphorylation of specific N-terminal residues mainly regulates the activation of these proteins. Thus, upon PAMP perception RBOHD is known to associate with FLS2 and EFR for the full activation of RBOHD by BIK1 and CPK mediated phosphorylation (Kadota *et al.*, 2015; Kadota *et al.*, 2014; Li *et al.*, 2014).

A mutual relationship between ROS and Ca²⁺ plays a key role in signalling amplification and long distance propagation from cell to cell to establish calcium and ROS signalling waves in response to biotic and abiotic stresses (Mittler *et al.*, 2011). Full activation of RBOHD requires pattern-triggered elevated level of intracellular Ca²⁺, binding of Ca²⁺ ion into the EF-motifs and activation of several CPKs (CPK5, Qi *et al.*, 2017; Dubiella *et al.*, 2013; Boudsocq *et al.*, 2012). Conversely, H₂O₂ produced by RBOHD may trigger further activation of Ca²⁺ channel(s), leading to the full activation of Ca²⁺ signalling and Ca²⁺-based regulation of RBOHD. However, recent studies have indicated the existence of an additional Ca²⁺ independent, direct regulation of RBOHD upon PAMP perception by a BIK1, a protein present in the PRR complex (Figure 1.4; Li *et al.*, 2014; Kadota *et al.*, 2014). This direct BIK1 mediated phosphorylation that is required for ROS production also occurred in absence of Ca²⁺ or in *cpk5 cpk6* and *cpk11* mutants (Li *et al.*, 2014; Kadota *et al.*, 2014). This direct phosphorylation by BIK1 may trigger several conformational changes in RBOHD, leading to an increased Ca²⁺ binding affinity for the EF hand motifs in the N-terminal of RBOHD and/or an increased ability of CPKs to phosphorylate and fully - activate RBOHD (Kadota *et al.*, 2014; Kimura *et al.*, 2012; Ogasawara *et al.*, 2008).

Figure 1.4. A two-step activation model for RBOHD during immunity. Upon PAMP (elf18/flg22) perception, PRRs, such as EFR and FLS2, and their co-receptor BAK1 directly phosphorylate and activate the cytoplasmic kinase BIK1. Phosphorylated BIK1 has a higher binding affinity and phosphorylates some specific sites present at the N-terminal of RBOHD. BIK1-mediated phosphorylation may ‘prime’ the Ca^{2+} based regulation of RBOHD by inducing conformational changes that could lead to increased Ca^{2+} binding affinity for EF-hand motifs and/or increased accessibility for CPK-mediated phosphorylation. At the same time, PRRs together with BIK1 also activate Ca^{2+} channel(s) and induce Ca^{2+} influx directly or indirectly. This leads to Ca^{2+} binding to EF-hand motifs in RBOHD and activation of CPKs, which in turn phosphorylates RBOHD (Kadota *et al.*, 2015).

1.4.3. Activation of mitogen-activated protein kinase cascades.

MAPK signalling cascades are important signal transduction nodes in plants and other eukaryotes (Asai *et al.*, 2002). MAPK signalling links the environmental and developmental cues to downstream targets via sequential phosphorylation cascades involving MAPK Kinase Kinases (MAP3Ks or MEKKs), MAPK Kinases (MAPKKs or MEKs) and MAPKs (MAPKs or MPKs; Rasmussen *et al.*, 2012). The phosphorylated (activated) MAPK in turn interacts with and alters the phosphorylation status of target proteins, including enzymes, TFs and other proteins, ultimately influencing gene expression, metabolism, cell division and growth (Pitzschke *et al.*, 2009). MAPK cascades play essential roles in plant innate immunity by controlling expression of defence genes and the biosynthesis of antimicrobial compounds. Based on sequence homology, Arabidopsis genome encodes around 20 MAPKs, 10

MAPKKs and approximately 60 MAP3Ks (Meng and Zhang, 2013b). Of the three groups of kinases in the cascade, MAP3Ks are the most divergent group. MAP3Ks are serine/threonine kinases that phosphorylate two serine and/or threonine residues (S/T-X-S/T) within the activation loop of the MAPKKs. MAPKKs are dual-specificity kinases that, in turn, trigger MAPK activation through phosphorylation of conserved threonine and tyrosine residues in their T-X-Y activation motifs, where X is typically a glutamic or an aspartic acid. MAPKs are serine/threonine kinases that subsequently phosphorylate a wide range of substrates, including other kinases and TFs, to alter patterns of gene expression or modulate the activity of other proteins (Meng and Zhang, 2013b; Zhang and Klessig, 2001).

Several PRRs, including the very well characterized FLS2, EFR and CERK1, have been shown to trigger MAPK signalling upon PAMP perception (Figure 1.3). In Arabidopsis, flg22, elf-18, chitin and PGNs can induce a strong and transient activation of MPK3, MPK6, MPK4 and MPK11 (Bethke *et al.*, 2012; Ranf *et al.*, 2011; Miya *et al.*, 2007), all of them key regulators of defence responses to bacterial and fungal pathogens (Colcombet and Hirt, 2008). Recognition of flg22 leads to the activation of two MAPK cascades: i) a positive regulatory cascade including the activation of MEKK1 (a MAP3K), MKK4/MKK5 (two redundant MAPKKs) and MPK3/MPK6 (two partially redundant MAPKs); ii) a negative regulatory pathway inducing MEKK1, MKK1/MKK2 (two redundant MAPKKs) and MPK4 (Kong *et al.*, 2013; Petersen *et al.*, 2000). However, the components linking the receptor complexes with MAPKs are still unknown. One potential downstream regulator of the FLS2-BAK1 complex is the cytoplasmic protein kinase BIK1, which interacts with and is phosphorylated by the FLS2-BAK1 complex and is required for PTI responses (including RBOHD-dependent ROS production) upon flg22 perception (Lu *et al.*, 2010). Despite being recognized by different receptors, elf-18 and flg22 do not show an additive effect on MPK3/MPK6 activation and also regulate the expression of similar sets of genes, suggesting that these MAPKs are converging points of PAMP signalling (Zhang and Klessig, 2001).

MAPK cascades are also important signalling hubs mediating ETI. Several effector proteins target MAPK cascades for downregulation of PTI. The *P. syringae* effector HopAI1 suppresses immune

responses by targeting MPK3, MPK4 and MPK6 (Zhang *et al.*, 2007). In turn, some R proteins guard these pathways. For example, the MEKK1-MKK1/MKK2-MPK4 negative regulatory cascade is guarded by the R protein SUMM2 (Suppressor of *mkk1 mkk2*) and disruption of the cascade induces ETI (Zhang *et al.*, 2012). In addition, the same MAPK cascades involved in immunity also mediate diverse developmental processes including leaf abscission and stomatal development as well as responses to abiotic stresses (Colcombet and Hirt, 2008). For example, MAP3K YODA (MAPKKK4), which functions upstream of MKK4/MKK5-MPK3/MPK6 and whose role in immunity we are characterizing, is also involved in developmental pathways including inflorescence architecture, zygote elongation and stomatal patterning (Bergmann, 2004; Lukowitz *et al.*, 2004).

1.4.4. Transcriptional regulation.

Transcriptional reprogramming can be considered as the main link between pathogen recognition and implementation of induced defence mechanisms, from the activation of the signal transduction pathways to the production of antimicrobial compounds. In plants, PAMPs and DAMPs have a massive role in reprogramming genome wide expression. For instance, flg22 perception in Arabidopsis can modulate the expression of thousands of genes (Bigeard *et al.*, 2015; Denoux *et al.*, 2008). Transcriptional changes take place as early as 15 minutes after perception as a part of early gene modulation (within one hour). These early transcripts are functionally enriched on genes encoding enzymes for the synthesis of antimicrobial compounds and for proteins involved in signal perception and transduction, including RLKs, regulatory TFs, kinases and phosphatases (Zipfel *et al.*, 2006; Zipfel *et al.*, 2004). In Arabidopsis, TFs belonging to certain families are customarily dedicated to immune responses (Tsuda and Somssich, 2015).

WRKY TFs are considered as the major players regulating responses to the environment and particularly to biotic stresses. WRKYs are a large family of TFs that bind to specific W-box and W-box like sequences present in the promoter of the genes that regulate (Bakshi and Oelmüller, 2014). Members of this family play roles in both the activation and repression of immunity processes.

AtWRKY33 provides resistance to *B. cinerea* and, upon infection with this necrotrophic fungi, WRKY33 binds to its target loci and directly regulates the expression of more than 300 genes (Birkenbihl *et al.*, 2017). Interestingly, WRKY33 can bind its own promoter, suggesting that a positive feedback regulatory loop occurs in the regulation of *WRKY33* expression (Mao *et al.*, 2011). WRKY TFs orchestrate these responses by interacting with other signalling components. E.g., a regulated connection between WRKY33 with the redox regulator Glutathione (GSH), and the plant hormone ET was recently uncovered. Elevated levels of GSH induced the expression of the ET biosynthetic genes *ACS2* and *ACS6* in a *WRKY33*-dependent manner (Datta *et al.*, 2015). In contrast, other WRKYs act as negative regulators of immunity. Thus, *WRKY22* is a negative regulator of the Arabidopsis susceptibility to aphids (Kloth *et al.*, 2016). Also, *WRKY57*, negatively affects resistance to *B. cinerea* by activating the expression of repressors of the JA signalling pathway (*JASMONATE-ZIM-DOMAIN PROTEIN (JAZ)* genes; Lai *et al.*, 2011). Interestingly, WRKY33 also binds to the promoter motifs of these *JAZ* genes. Thus, competition between WRKY33 and WRKY57 to bind to the same target sequences coordinate the regulation of the Arabidopsis response to *B. cinerea* (Jiang and Yu, 2016).

In addition, some TFs families typically involved in developmental phenomena have also members that function in immunity. TFs of the TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP) family play vital roles in development and in modulating hormone activities. However, recent studies have identified TCP13, TCP14, TCP15, TCP19 and TCP21 as central regulators of signalling pathways targeted by diverse pathogen-derived effectors. Moreover, TCP8, TCP14 and TCP15 have been shown to have a positive effect on ETI mediated by several *R* genes (Nicolas and Cubas, 2016; Webling *et al.*, 2014). Also, over expression studies with members of the large family of APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) TFs such as ERF1, ORA59, and ERF6 point towards a vital role for some of these TFs in defence regulation (Catinot *et al.*, 2015; Zhang *et al.*, 2015; Berrocal-Lobo *et al.*, 2002).

Along with TFs, specific TF-associated co-regulators also participate in modulating the expression of immune-related genes. For example, one of these transcriptional co-regulators is Nonexpressor of Pathogenesis-related Proteins 1 (NPR1), which is required for basal resistance towards biotrophic and

hemibiotrophic pathogens and orchestrates SA- and JA-mediated systemic defences. NPR1 is a focal point of posttranslational modifications that regulate its activity thus modulating transcriptional defence outputs (Saleh *et al.*, 2016).

1.4.5. Downstream responses.

The onset of PTI and ETI often triggers and induces resistance in distal tissues from the site of infection and involves long-distance signals that propagate and enhance defensive capacity in still unchallenged parts of the plant (Shah and Zeier, 2013). Plants produce a range of hormones whose role is widely distributed among plant growth, development, reproduction and survival to a wide variety of biotic and abiotic stresses (Bari and Jones, 2009). The relevant role of SA, JA and ET mediated signalling pathways is well established in biotic and abiotic stresses (Seilanianitz *et al.*, 2011). These three hormones regulate a large transcriptional reprogramming that mediates the establishment of immunity (Figure 1.3). Although there are exceptions, SA usually functions as a positive regulator of plant defence against biotrophic, whereas ET/JA are required for resistance to necrotrophic pathogens and herbivorous pests (Bari and Jones, 2009; Glazebrook *et al.*, 2005). A regulated cross-talk between these phyto-hormone pathways allows the plant to adjust the defences to the type of pathogen as well as to balance growth and resistance. Thus, a negative crosstalk between SA and JA/ET pathways helps to fine tune the responses to the different pathogens (Gimenez-Ibanez and Solano, 2013; Berrocal-Lobo and Molina, 2004). However, there are exceptions for this rule. For example, elevation of ET and SA levels have been shown to mediate an amplification cascade in response to bacterial PAMPs leading to PTI (Spoel and Dong, 2012; Bari and Jones, 2009). Moreover, ET, JA and SA signalling cooperate to arrest the progression of the colonization of *Arabidopsis* by the necrotrophic fungus *Plectosphaerella cucumerina* (Sánchez-Vallet *et al.*, 2012).

Although, SA/ET/JA are considered the most relevant regulators for immunity, new findings reveal the role of additional hormones related to developmental functions such as ABA, auxins, gibberellic acid and brassinosteroids, in the modulation of the response to plant-pathogen interactions (Denancé *et*

al., 2013; Xu *et al.*, 2013; Yasuda *et al.*, 2008). Thus a complex network of different phytohormones is emerging in balancing plant innate immunity and development (Pieterse *et al.*, 2012).

1.5. Heterotrimeric G protein role in plant innate immunity.

One of the most important signal transduction systems in Eukaryotes is mediated by heterotrimeric G protein. This G protein classically consists of three subunits $G\alpha$, $G\beta$ and $G\gamma$ that are associated to a 7-transmembrane spanning G protein-coupled receptor (GPCR). When the GPCR binds the ligand, the heterotrimer gets activated by the energy exchange of GDP for GTP on the $G\alpha$ subunit. $G\alpha$ -GTP binding results in dissociation of $G\alpha$ and the heterodimer $G\beta\gamma$. These two functional signalling elements of the activated complex independently interact with multiple downstream effectors mediating specific signal transduction pathways (Figure 1.5; Aranda-Sicilia *et al.*, 2015; Gautam *et al.*, 1998). Plant heterotrimeric G proteins have structural similarities to the corresponding molecules in animals, although they display important signalling peculiarities. In animals, multiple genes encode the three canonical subunits and diverse gene combinations regulate different responses. However, plants possess a limited set of genes for each subunit, which implies that the different regulatory functions are not determined the specific combination of them. The Arabidopsis genome encodes a single $G\alpha$ subunit (*GPA1*), a single $G\beta$ (*AGB1*) and three $G\gamma$ subunits (*AGG1*, *AGG2* and *AGG3*). Although some 7-transmembrane proteins have been documented in plants, no functional GPCR has been identified. This, together with the fact that plant heterotrimeric G protein is self-activating, led to propose that regulation of heterotrimeric G protein activation in plants occurs at the deactivation step (Urano and Jones, 2014). In fact, Regulator of G protein Signalling, RGS1 protein, has been described to interact with the G-protein complex to maintain the inactive state and RLKs, not GPCRs, phosphorylate RGS1 in a ligand-dependent manner to induce its endocytosis allowing G protein signalling (Tunc-Ozdemir *et al.*, 2017).

In plants, heterotrimeric G-proteins mediate multiple developmental processes, such as cell proliferation as well as responses to abiotic stresses including light perception, ion channel regulation

and stomata control (Wang and Assmann, 2007; Jones and Assmann, 2004; Ullah, 2001). Moreover, they play an important role in the immune response to diverse pathogens. The G $\beta\gamma$ heterodimer has a prominent role in immunity. In Arabidopsis, the G β subunit AGB1 and the G γ AGG1/AGG2 subunits are required for full resistance against *P. cucumerina*, *Alternaria brassicicola*, *Fusarium oxysporum*, *B. cinerea*, *Pseudomonas* and viruses, since *agb1-2* and *agg1 agg2* double mutant plants exhibit enhanced susceptibility to all these pathogens (Brenya *et al.*, 2016; Klopffleisch *et al.*, 2011; Torres *et al.*, 2013; Trusov *et al.*, 2006; Llorente *et al.*, 2005). On the other hand, the role of G α was found to be circumstantial during these defence responses (Trusov *et al.*, 2009; Llorente *et al.*, 2005). Moreover, the analysis of the Arabidopsis heterotrimeric G-protein interactome revealed connections to cell wall carbohydrates metabolism. Interestingly, a detailed glycome profiling performed in WT and heterotrimeric G protein mutants found significant differences in the sugar content of the cell wall (Klopffleisch *et al.*, 2011). And alterations in cell wall structure were observed in mutants in the G β and G γ subunits that display enhanced susceptibility to *P. cucumerina* (Delgado-Cerezo *et al.*, 2012). All these data suggest that heterotrimeric G proteins could play an important role in cell wall remodelling and that this feature could account for the signalling role displayed by these proteins in immunity. Heterotrimeric G proteins also play an important part in bacterial infection. Analyses of mutants indicate that the G β and G γ subunits are required for full resistance against the hemibiotrophic bacterial pathogen *P. syringae* (Torres *et al.*, 2013). Interestingly, recent studies have shown that G $\beta\gamma$ actually partners with Extra-Large G-Proteins (XLG2 and XLG3) to facilitate resistance against fungal and bacterial pathogens (Maruta *et al.*, 2015).

Although G β and G γ are the subunits important for immunity in most of the plant-pathogen interactions evaluated, there are cases where the other signalling subunit may have a preeminent role. For example, analysis of a rice *dwarf1* mutant in the G α subunit indicates that this subunit is required for resistance to avirulent races of the rice blast fungus *Magnaporthe grisea* (Suharsono *et al.*, 2002).

Figure 1.5. Model for the G-protein cycle in plants. (A) This model assumes that $G\alpha$ is bound to GTP by default based on the reported kinetic properties for GPA1. The GTP-bound heterotrimer has the proper conformation to allow interaction with downstream effectors but is not functionally active. (B) Upon binding of an agonist, RLKs (or other associated kinases) phosphorylate the G-protein subunits activating and initiating the signalling cycle. (C) Signalling proceeds by the two functional subunits ($G\alpha$ and the $G\beta\gamma$ dimer) until the phosphate groups are removed by phosphatases (D), rendering the heterotrimer inactive and associating again with a RLK to complete the cycle (Trusov and Botella, 2016).

Also, a recent study shows that both $G\alpha$ and $G\beta\gamma$ subunits mediate a surveillance system that recognizes and responds to pathogen-secreted proteases that could release host polypeptides to function as potential DAMPs (Benedetti *et al.*, 2015). Additionally, both subunits play an important function in response to bacterial infection through the control of stomata opening. For instance, flg22 was unable to inhibit inward K^+ channels of guard cells and stomata closure in *gpa1* single mutant plants, suggesting that GPA1 functions downstream of FLS2 to regulate flg22-induced stomata closure (Zeng and He, 2010). And both $G\alpha$ and $G\beta$ subunits were shown to be required for stomata closure and cell death after exposure to different elicitors in *N. benthamiana* (Zhang *et al.*, 2012).

OBJECTIVES

OBJECTIVES

The aim of the current PhD Thesis is the **“Characterization of the ERECTA-YODA MAPK functional cascade and other phosphorylation events in innate immunity”**. To this end, the following specific objectives have been pursued:

- Identification of molecular components of YODA MAP Kinase Kinase Kinase mediated immunity pathway.
- Identification and characterization of new signalling elements of the heterotrimeric G protein defence signalling.

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1. Plant materials and growth conditions.

Three ecotypes of *Arabidopsis thaliana*, Columbia-0 (Col-0, TAIR_CS76778), Landsberg (TAIR_CS76538) and Landsberg *erecta* (Ler, TAIR_CS77020) were used in these studies. Mutants and transgenic lines used, and their respective background are detailed in table 3.1.

Table 3.1. Arabidopsis mutants and transgenic lines with respective background.

Mutant	Accession	Reference
<i>agb1-2</i>	Col-0	Ullah <i>et al.</i> , 2003
<i>CA:YDA</i>	Ler	Lukowitz <i>et al.</i> , 2004
<i>CA:YDA</i>	Col-0	Sopeña-Torres <i>et al.</i> , 2018
<i>cerk1-2</i>	Col-0	Miya <i>et al.</i> , 2007
<i>cpr5</i>	Col-0	Bowling <i>et al.</i> , 1997
<i>eds1-2</i>	Col-0	Bartsch <i>et al.</i> , 2006
<i>er105</i>	Col-0	Torii <i>et al.</i> , 1996
<i>fls2</i>	Col-0	Zipfel <i>et al.</i> , 2004
<i>irx1-6</i>	Col-0	Hernandez-Blanco <i>et al.</i> , 2007
<i>mkp1-1</i>	Col-0	Bartels <i>et al.</i> , 2009; Ulm <i>et al.</i> , 2001
<i>NahG</i>	Col-0	Delaney <i>et al.</i> , 1994
<i>rbodD</i>	Col-0	Torres <i>et al.</i> , 2002
<i>sid2</i>	Col-0	Nawrath and Métraux, 1999
<i>yda11/elk2</i>	Col-0	Lease <i>et al.</i> , 2001
<i>35S::Pyo-MKP1</i>	Col-0/ <i>mkp1</i>	Bartels <i>et al.</i> , 2009
<i>35S::RBOHD</i>	Col-0	Kadota <i>et al.</i> , 2014; Torres <i>et al.</i> , 2006

Genes coding *SMALL SECRETED PEPTIDES* (SSPs) and *PUTATIVE RECEPTOR PROTEINS* (PRPs) were obtained from NASC (Alonso, 2003) and homozygous lines were selected by Polymerase Chain Reaction (PCR) genotyping with specific oligonucleotides.

For experiments with pathogens, *Arabidopsis* plants were grown in sterilized and hydrated soil-vermiculite 3:1 mixture under short-day conditions (10 h day/14 h night schedule). Temperature (21°C daytime, 20°C night time), light intensity (approximately 120 $\mu\text{E}/\text{m}^2 \text{ sec}$) and humidity (65%) were maintained constantly with standard *Arabidopsis* growth conditions. For the measurement of

developmental parameters, plants were grown under a 16 h day/8 h night (long-day condition) photoperiod under the same temperature, light intensity and humidity conditions.

For *in vitro* growth, seeds were first surface sterilized in a Laminar Air Flow hood with ethanol-bleach 1:1 mixture for 7 minutes, then washed for 7 minutes in 80% ethanol, followed by 7 minutes in absolute ethanol, and finally dried over a sterile filter paper. Seeds were transferred to sterilized 2 ml of 0.5 X Murashige and Skoog (MS) basal salt medium (Duchefa, Holland) supplemented with 0.25% sucrose and 1 mM MES (pH 5.7). Seeds were, then, stratified in the dark at 4°C for 3 days before being transferred to a 16 h day/8 h night photoperiod at 22°C constant temperature. When seedlings were growth for a long period (12-14 days), media was renewed after 10 days.

3.2. Pathogens, inoculation and assessment of pathogen growth.

This study involves four different pathogens: A) the necrotrophic fungus *Plectosphaerella cucumerina* isolate *BMM* B) the oomycete *Hyaloperonospora arabidopsidis* isolate Noco2; C) the hemibiotrophic bacteria *Pseudomonas syringae* pv *tomato* DC3000; and D) *Cucumber mosaic virus*.

3.2.1. *Plectosphaerella cucumerina*.

Spores of *Plectosphaerella cucumerina* isolate *PcBMM (PcBMM)*; were kindly provided by Dr B. Mauch-Mani (University of Fribourg, Switzerland; Ton and Mauch-Mani, 2004; Tierens *et al.*, 2001;). To obtain the fungal spores, primary fungal spores were grown on potato-dextrose agar plates (Difco, USA) at 28°C for 21 days. Spores were harvested in sterile water, quantified and stored at -80°C in 20% Glycerol solution (Berrocal-Lobo and Molina, 2004). A spore suspension of 4×10^6 spores/ml was used to inoculate 18-20 days old plants grown under short day condition. High humidity was maintained throughout the experiment. At least 20 plants were inoculated for each genotype to be evaluated. Tissues from 4 plants per genotype and time were harvested at 3 and 5 days-post-inoculation (dpi) to extract gDNA in order to quantify fungal growth by qPCR (LightCycler 480 system, Roche). To quantify fungal biomass, the amount of *PcBMM* β -*tubulin* was normalized to that of the constitutive

Arabidopsis gene *UBC21* (At5g25760), as described previously (Delgado-Cerezo *et al.*, 2012; Sanchez-Vallet *et al.*, 2010). N-fold expression relative to the wild-type (WT) plants were represented. Oligonucleotides used are detailed in table 3.2.

Additionally, disease progression was also monitored visually, and symptoms quantified using the following 0-5 disease rating (DR): 0, no symptoms; 1, plant with some necrotic spots; 2, one or two necrotic leaves; 3, three or more leaves showing necrosis; 4, more than half of the plant showing profuse necrosis; 5, decayed/dead plant.

Table 3.2. Oligonucleotide sequences used to assess *PcBMM* growth.

Gene	Gene accession	Primer combination (5'-3')
<i>Pcβ-tubulin</i>	-	CAAGTATGTTCCCCGAGCCGT
		GGTCCCTTCGGTCAGCTCTTC
<i>UBC21</i>	At5g25760	AAAGGACCTTCGGAGACTCCTTACG
		GGTCAAGAATCGAACTTGAGGAGGTT

3.2.2. *Hyaloperonospora arabidopsidis*.

The oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) was kindly provided by Dr. Jane Parker (Max Planck Institute for Plant Breeding Research, Köln, Germany). Frozen spores were propagated on a genetically susceptible host (*NahG*) under a 10 h day/14 h night photoperiod (20°C day/18°C night, 75-80% of relative humidity). For inoculation experiment, 12 days old plants were sprayed with a conidiospore suspension of 1×10^5 spores/ml. At least 30-35 plants were inoculated for each genotype to be evaluated. Plants were kept under high humidity and short-day condition for 7 days. For each genotype, aerial parts of the plants were harvested into a 50 ml Falcon tube and its fresh weight measured. Subsequently, the same amount of dH₂O was added to each Falcon and the tube was vortexed thoroughly. The conidiospore suspension was filtered through Miracloth (Calbiochem, Milipore) from plant debris and spores were counted under a light microscope using a Neubauer improved haemocytometer. As a part of the result, spore counts were normalised to the fresh weight.

To assess tissue viability and fungal growth, trypan blue (TB) staining was performed. A stock solution was prepared by mixing 10 ml dH₂O, 10 ml acetic acid (glacial), 10 ml glycerol, 10 ml phenol and 10 mg TB. 7 dpi leaf tissues were harvested from inoculated plants and boiled with 1:1 diluted TB solution (TB stock:100 % ethanol) for 2-3 minutes. Leaves were destained by repetitive washes with 2.5 g ml⁻¹ chloral hydrate. Finally, destained leaves were transferred to 50% glycerol and were mounted on slides for microscopic visualization.

3.2.3. *Pseudomonas syringae* pv. *tomato* DC3000.

***Pseudomonas syringae* pv. *tomato* (Pto) DC3000** was obtained from Novartis Corporation collection (Novartis Crop Protection Inc., Research Triangle Park, USA). *P. syringae* was grown on King's B (KB) liquid medium with the antibiotics Rifampicin (Rif) and Kanamycin (Km). Stocks were kept in 20% glycerol at -80°C. 48 h prior to plant inoculation, bacteria were revived at 28°C on KB media plates carrying double antibiotic selection (Rif, Km). Overnight propagated bacteria were resuspended in 10 mM MgCl₂ to a final O.D.₆₀₀ of 0.2 (approximately 10⁸ colony forming units (cfu)/ml) with 0.0035% silwet. 3 to 4-week-old plants were sprayed for the experiment. At least 10 plants were inoculated for each genotype. High humidity was maintained by covering the trays with plastic foil throughout the experiment.

Leaves from 4 plants each genotype were harvested at 2 dpi and 4 dpi and surface sterilized (30 s in 70% ethanol followed by 30 s in distilled water). Leaf discs were collected and ground in tubes containing 1 ml of 10 mM MgCl₂. Four replicates were taken per genotype and time, each one with 4 leaf discs. Serial dilutions were plated on KB plates supplemented with appropriate antibiotics. Plates were placed at 28°C for 2 days and, subsequently, colonies were counted.

3.2.4. *Cucumber mosaic virus*.

***Cucumber mosaic virus* (CMV)** strain LS, belonging to subgroup II of CMV strains, was derived from biologically active cDNA clones (Zhang *et al.*, 1994). The strain was kindly provided by Prof. Fernando

Garcia-Arenal, (CBGP, UPM-INIA, Madrid). Virions were purified as in Lot *et al.* (1972) and viral RNA was extracted by disruption of virions with phenol and sodium dodecyl sulphate. RNA was stored at -80°C.

LS-CMV was primarily inoculated in *Nicotiana clelandii* (3 weeks-old plants) using 100 ng/ul viral RNA suspended in 0.1 M Na₂HPO₄. After 15 dpi, characteristic mosaic symptoms were observed in inoculated and systemic leaves of the infected plant. Then, the youngest leaf of the infected *N. Clelandii* plant was taken for the inoculation in Arabidopsis. Leaves were ground in a sodium phosphate buffer (0.5 M Na₂HPO₄, 1 M NaH₂PO₄ pH 7.0) with DIECA and plant sap was prepared. An abrasive, carborundum (silicon carbide), was sprinkled on Arabidopsis leaves prior to mechanical inoculation of the plantlets at stage 1.04-1.05 (Boyes *et al.*, 2001) with 15 µl of the sap per leaf. Mock inoculated controls were inoculated with buffer to equalize inoculation damage. 15 replicates per genotype were used to perform the experiment. Plants were then fully randomised in the greenhouse module and grown on a long day 16 h day/ 8 h night regime at 23-25°C and 60% humidity. At 15 dpi, plants were collected, and fresh weight was measured for each plant. Plants were frozen in liquid N₂ and stored in -80°C.

For virus detection/quantification, RNA was extracted using Trizol® reagent (Life Technologies, Carlsbad, CA, USA; Material and Methods 3.5.4). Virus detection was performed by qRT-PCR on 0.16-4 ng of total RNA of each sample by triplicate by using Brilliant III Ultra-fast SYBR Green qRT-PCR Master Mix (Agilent Technologies) according to manufacturer's recommendations in a 10 µl reaction. Viral RNA was used in serial dilutions to obtain a standard curve as detailed in table 3.3. Oligonucleotides and qRT-PCR conditions used for quantification are detailed in table 3.4 and 3.5. Mock controls and water were used as negative controls.

Table 3.3. Range of concentrations included in the standard curve used for virus detection by qRT-PCR.

Dilution	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10
Concentration (ng/μl)	100	10	1	0.1	0.01	0.001	0.0001	0.00001	0.000001	0.0000001

Table 3.4. Oligonucleotide sequences used for quantification of viral accumulation using qRT-PCR.

Viral Gene	Amplicon Size (bp)	Primer combination (5'-3')
Coat protein	106	TAAGAAGCTTGTTTCGCGCATTC
		CGGAAAGATCGGATGATGAAGG

Table 3.5. Template protocol for detection of LS-CMV by qRT- PCR.

Step	Temperature	Time	Nº of cycles
Reverse transcription	50°C	10 minutes	1
Denaturation	95°C	5 seconds	1
Amplification	95°C	5 seconds	40
	60°C	10 seconds	
Melting	95°C	30 seconds	1
	60°C	1 minute	
	95°C	Continuous	
Cooling	37°C	1 minute	1

3.3. Nucleic acid extraction.

3.3.1. Plant genomic DNA extraction.

Plant tissues were homogeneously grinded using mortar and pestle in Extraction Buffer (0.35 M sorbitol, 100 mM TRIS -HCL, 5 mM EDTA, pH 7.5) and transferred to a 2 ml Eppendorf tube, where Lysis Buffer (200 mM TRIS-HCl, 50 mM EDTA, 2 M NaCl and 2 % CTAB) was added along with 10 % sarcosyl. Homogenized tissues were heated at 60°C for 30 min with shaking. Next, 1 volume of

chloroform was added and vortexed thoroughly. Samples were centrifuged at 8000 rpm for 10 min at RT. The supernatant was collected in a fresh tube with 0.7 volume of isopropanol and centrifuged for 15 min at 13.000 rpm at RT. Finally, the pellet was washed with 70% ethanol and air dried before re-suspension in 50 µl dH₂O. Quantity of extracted DNA was measured on a nanodrop ND-1000 spectrophotometer (NanoDrop Technologies Wilmington, USA). Plant genomic DNA extracted from this protocol was used for genotyping and performing qPCR to quantify fungal biomass.

3.3.2. Plasmid DNA extraction.

Overnight grown bacterial culture with appropriate antibiotic was pelleted down in a 1.5 ml Eppendorf tube. Cell mass was resuspended in 200 µl of Resuspension Buffer (50 mM TRIS -HCL, 10 mM EDTA, and 10 µg/ml Rnase A, pH 8) for 5 mins. Same volume of Lysis Buffer (1 % SDS, 0.2 M NaOH) was added and mixed by inversion for 5 mins. Pre-chilled neutralization buffer (3.0 M potassium acetate, pH 5.5) was added and mixed by inverting and the tube was kept for 5 mins on ice. Mixture was centrifuged at 13000 rpm for 10 mins at 4°C and supernatant was transferred into a fresh tube. Plasmid DNA was precipitated by adding 0.7 volumes of isopropanol and centrifuged at maximal speed for 5 mins at RT. Finally, pellet was washed with 70% ethanol, air dried and resuspended in 50 µl of TE buffer, before being quantified using a NanoDrop ND-1000 spectrophotometer.

3.3.3. Arabidopsis RNA extraction and cDNA synthesis.

For extraction of total RNA, tissues were previously frozen in liquid Nitrogen. Frozen samples were ground with pre-chilled mortar and pestle to a fine powder state. 400 µl of Extraction Buffer (0.2 M TRIS-HCl, 0.4 M LiCl, 25 mM EDTA and 1% SDS) was used for homogenization. Next, tissues were treated twice with 1 volume of phenol (vortexed vigorously) and pelleted down at 13000 rpm for 2 mins at RT followed by 1 volume of chloroform wash (13000 rpm for 2 min at RT). Supernatant was mixed with 1/3 volume of 8M LiCl for at least 2 h on ice. Further, samples were centrifuged at 13000 rpm for 30 min at 4°C and pellet was re-suspended in 300 µl of dH₂O with 30 µl of 3M sodium acetate

(pH 5.2), 600 μ l of 100 % ethanol and precipitated overnight at -20°C. Next day, samples were centrifuged at 13000 rpm for 20 mins at 4°C, washed with cold 80 % ethanol and subsequently air dried. Finally, RNA was dissolved in 30 μ l of dH₂O. RNA quality was assessed in a 2% agarose gel and quantification of each sample was performed using a NanoDrop ND-1000 spectrophotometer.

cDNA synthesis was carried out with a cDNA Synthesis Kit (Roche Applied Science, USA). First, equal amount of RNA (2 μ g) was treated with DNase I (TURBO DNA-free *TM* Kit, Ambion, Life Technologies) to remove any possible DNA contamination. Then, cDNA was synthesized using oligo-dT oligonucleotide following manufacturer's instructions (60 min at 50°C and 5 min at 85°C).

Table 3.6. Oligonucleotides sequences used in RT-PCR to confirm null mutants in the T-DNA lines.

Gene accession	Mutant /T-DNA insertional mutant	Primer combination (5'-3')
At1g65295	SALK_120534	TCAATCTTCATCAGTTCCAGTC
		AAGGAGAAGGCAAGCAAAGAC
At2g25510	SALK_132861	AGGTGCAGAAAAGACTTACCA
		AAAGATCCGCCTATTCCGGA
At2g37750	SALK_016668	CACACGCGCTTTTTGAAGGA
		TCACGTGACAACCTGACGA
At3g48640	GABI_807H09	CAAACAACCTTAAGCTTGATTGC
		AAGAGCATGACATGATCCCTG
At3g49550	SALK_011318C	CTGCTATATTGGGTCACGAA
		AATAGCGAAAACAAGAACGTCC
At4g31130	SALK_143902	TGCGTCTGGGACTCCAATAA
		TTGAGATTCACATCCCTGGAG
At5g20790	SALK_083842	TTGTCATCCCATACCTTACC
		AGCTTCTGGAGGATCAGAG
At5g44570	SALK_020569	ATGGCTACAAAGACATCAAAT
		AACGAAGGAGGTGGATACT
At1g51890	SALK_200112	CGCTGAAATCGAGAATCTTGA
		TGATAAATCTCAAGGCCGTTG
At1g56120	SALK_043782	ATCAGCGGCGAACTTTGCTC
		TGTTAAGGTCCAGAGCTCTGG
At1g67000	SAIL_842_D05	AACACCTGTTAATGCACCCAG
		CTTCAAGCTCAACTGTAGCGG
At1g16030	SAIL_241_G12	GGTGATGAGAACTATGGTGGA
		TTACCTCTTTGTAACTGCGG
At1g70690	SAIL_46_E06	CGTGACTACCACCAACACTG
		TGGATCTTACAGGACAGGTGG
At1g73500	SAIL_60_H06	CTTTGTAAACAATCCCGCCGT
		GGCTTTAGTACGTGAACGTCG
At2g13800	SALK_147275	CACCTGGATGTGAAAGCTGCA
		GATGAGCTCGAGAAGCATGAC
At4g23230	SALK_067729	GATTGCCAGACAATTCCTATC
		AAGCAAGGTGGCATTGTGTAC
At5g59660	SAIL_760_G02	GGATCGCCTTGCCAGTTGA
		TAGTGTCCAAAGTGTGACCCC
At5g59680	SALK_001929	AATGGCTGCAGAACCAACTG
		AACATGAAATCGCTGTTGGTC
At5g25760	UBC21	AAAGGACCTTCGGAGACTCCTTACG
		GGTCAAGAATCGAACTTGAGGAGGTT

3.3.4. RNA extraction for virus accumulation.

Frozen samples were homogenized with mortar and pestle and liquid N₂ until complete powder was obtained. Trizol Reagent was added accordingly and transferred to an 1.5 ml Eppendorf. Samples were vortexed thoroughly and then incubated at RT for 5 mins. Chloroform (1/5th of Trizol) was added and samples were vortexed thoroughly and then incubated at RT for 5 mins, followed by a centrifugation at 4°C for 15 mins at 13000 rpm. During this centrifugation, the mixture separates into lower chloroform phase containing proteins, interphase with DNA, and upper aqueous phase with RNA. Then, the aqueous phase is pipetted into a new Eppendorf tube. RNA was then precipitated by adding an equal volume (1:1) of isopropanol, samples were briefly vortexed and incubated at RT for 15 mins. Tubes were centrifuged at 4°C for 20 mins at 13000 rpm and supernatant was discarded. Samples were washed with cold 75% ethanol and centrifuged at 4°C for 5 mins at 9000 rpm. The pellet was air-dried at RT and resuspended in 200 µl of autoclaved MilliQ water.

To further purify the RNA, one volume (200 µl) of phenol:chloroform:Isoamylalcohol (25:24:1) was added, samples were then well vortexed and centrifuged at RT for 5 mins at 13000 rpm. Aqueous phase was transferred to a new tube. For secondary precipitation of RNA, 0.1 volumes of 3 M sodium acetate pH 5.2 and 2.5 volumes of 100% ethanol stored at -20°C was added. Samples were kept at -20°C for overnight precipitation. Next day, samples were centrifuged at 4°C for 20 mins at 13000 rpm. Supernatant was discarded and 75% ethanol stored at -20°C was added to wash the pellet. Samples were centrifuged at 4°C for 5 mins at 9000 rpm. Then, the supernatant was discarded and the pellet air-dried and resuspended in 50 µl of nuclease free water.

3.4. Genetic Mapping.

F2 populations of mutants *sgb10* and *sgb11* (Col-0 background) crossed with La-0 were generated to map the genes *SGB10* and *SGB11* genes. The *sgb10* mutation was mapped to chromosome 3 between markers cer451778 (BAC F28P10, one recombinant) and cer479349 (BAC T5P19, two recombinants), using the primers described in table 3.7. The *sgb11* was mapped to chromosome 3 between markers

AthCDC2BG (BAC F24B22, one recombinant) and nga707 (BAC T20N10, four recombinants), using the primers described in Table 3.7. To further position the *sgb10* and *sgb11* mutations, genomic DNA from *sgb10* and *sgb11* plants was fully sequenced using the Illumina technology (BGI, Hong Kong).

Table 3.7. Oligonucleotide sequences used in this study to map *SGB10* and *SGB11*.

Primer	Purpose	Primer combination (5'-3')
nga128-F	Mapping Chromosome 3	GGTCTGTTGATGTCGTAAGTCG
nga128-R		ATCTTGAAACCTTTAGGGAGGG
AtBIO2b-F	Mapping Chromosome 3	TGACCTCCTCTTCCATGGAG
AtBIO2b-R		TTAACAGAAACCCAAAGCTTTC
nga162-F	Mapping Chromosome 3	CATGCAATTTGCATCTGAGG
nga162-R		CTCTGTCACTCTTTTCTCTGG
cer451778-F	Mapping Chromosome 3	ATCCACCAATTCCAGGAGCCA
cer451778-R		GTTATTGTTGACAACCTTTAGCCT
cer470805-F	Mapping Chromosome 3	GAACTCCAAATCAAGCAATACAT
cer470805-R		CAAATATCTCATCGGTTGTCCA
cer476627-F	Mapping Chromosome 3	CCATGCATACCTCATAACTA
cer476627-R		GGAGCTGAAAAGGACTACAAAT
cer479349-F	Mapping Chromosome 3	TTGCCGTCAGGTTCCCAACG
cer479349-R		GTGCCACAACTTCTCCAGG
nga6-F	Mapping Chromosome 3	TGGATTTCTTCTCTCTTCAC
nga6-R		ATGGAGAAGCTTACACTGATC
nga8-F	Mapping Chromosome 3	GAGGGCAAATCTTTATTTTCGG
nga8-R		TGGCTTTCGTTTATAAACATCC
nga76-F	Mapping Chromosome 3	GGAGAAAATGTCACTCTCCACC
nga76-R		AGGCATGGGAGACATTTACG

3.5. Generation and characterization of Arabidopsis double mutants.

To generate double mutants, *sgb10 rbohD*, *sgb10 NahG*, *sgb10 sid2*, *mkp1 rbohD*, *sgb10 35S::RBOHD* and *mkp1 35S::RBOHD*, parental lines were crossed and F1 were identified by PCR genotyping. PCR reactions were performed using standard conditions with a lab made thermostable polymerase (Desai *et al.*, 1995) in a final volume of 20 µl using the oligonucleotides and restriction enzymes detailed in table 3.8. Annealing conditions were optimized according to the melting temperature of oligonucleotides. Finally, PCR products were mixed with bromophenol-blue dye and separated in 1% agarose gel containing ethidium bromide.

3.6. Stomata counting and morphometric analysis.

To count the number of stomata, 4-5 expanded leaves from 25-day-old plants from each genotype grown under short day conditions were selected. First, leaves were completely destained in 95% ethanol and then were transferred into 50 % glycerol. Subsequently, destained leaves were analysed under an optical microscope. Stomatal density refers to the total number of stomata per leaf whereas stomata index represents the number of stomata divided by total number of cells in the abaxial side (i.e. epidermal cells + stomata).

For morphometric analysis, plant height, silique and pedicle length were measured on 45-day-old plants grown under long day conditions. 25 plants were considered for each genotype. For the measurement of plant height, the main inflorescence was taken. For measurement of silique and pedicle length, 5 pedicles along with its siliques were taken per plant.

3.7. Gene expression analysis.

To validate microarray data, quantitative reverse transcriptase PCR amplification and detection was carried out in a LightCycler 480 system (Roche). Tissues were harvested and fast frozen in liquid nitrogen, RNA was extracted, and cDNA was synthesized. PCR was performed with 10 ng cDNA in a 10 μ l reaction, which includes 5 μ l of 2X SYBR green master mix I (ROCHE Applied Science, USA) and 0.5 μ l (10 mM) of each oligonucleotide.

Table 3.8. Oligonucleotide sequences used in this study to genotype double mutants. Mutant-specific forward and reverse oligonucleotide sequences were used for genotyping. *LBa1*, *LB1* and *GABI* are specific primers that anneals with the T-DNA insertion of SALK, SAIL and GABI-Kat lines. These primers were used with reverse oligonucleotide for T-DNA confirmation. * Digestion with MbolI will produce in WT a 720 bp band and in *sgb10* two fragments of 552 and 250 bp.

Gene accession	Mutant /T-DNA insertional mutant	Primer combination (5'-3')	Comment
At5g47910	<i>rbohD</i>	CTTATTTAGTAAGAGTGTGGGGTTTTGG	
		CAAGACTAAGCTTGGTACTGTCG	
		GATCATCTTAACCGTTTGATGC	
At1g74710	<i>sid2</i>	ATGCTTCATTTCTTGATAATAG	
		GGGAGAAAGAGATCAAATAAGC	
At3g55270	<i>mkp1</i>	ACAAGTCTATGGAAGAAGC	
		TGTCTTTCGCCACAGCATC	
At3g55270	<i>sgb10</i>	TGGAATCACGCATATCTGAAC	MbolI*
		TGTTGGATGATGCTAAAGTTGG	
At1g65295	SALK_120534	TTTTGGCAACGCATTTTCTAG	
		AAGGAGAAGGCAAGCAAAGAC	
At2g25510	SALK_132861	TGAGTTCTCTGCAATATCGGG	
		GCCTTTGTGGTTTAGCATGTC	
At2g37750	SALK_016668	TTCCGTAATCGAAGCACAG	
		AGATGTATCGGGTCTGGATC	
At3g48640	GABI_807H09	AATGTGTAGACTTGTATTACACCG	
		AAGAGCATGACATGATCCCTG	
At3g49550	SALK_011318C	ATCAAATACCATTCTCTGCC	
		AATAGCGAAAACAAGAACGTCC	
At4g31130	SALK_143902	ACAATTCCTGTTGCTTTTGG	
		TTGAGATTCACATCCCTGGAG	
At5g20790	SALK_083842	CAAGACAGAAGCATCTTTCGC	
		GGTACGTCGATCAGACCAGAG	
At5g44570	SALK_020569	GAAATCGGATCCTGAAAAAGC	
		AATTCATGATAGAGCGCAGAC	
At1g51890	SALK_200112	TGATAAATCTCAAGGCCGTTG	
		TGATTCAGAACAAACATCCCC	
At1g56120	SALK_043782	TCTTGGCTGTGTGTCAACAAG	
		TGTTAAGGTCCAGAGCTCTGG	
At1g67000	SAIL_842_D05	AACACCTGTTAATGCACCCAG	
		CTTCAAGCTCAACTGTAGCGG	
At1g16030	SAIL_241_G12	ATGGCGTCTGGTTCTGTTATG	
		TTACCTCTTTGTAACGTGCGG	
At1g70690	SAIL_46_E06	TTTGCATAGACGAAAAACATGG	
		TGGATCTTACAGGACAGGTGG	
At1g73500	SAIL_60_H06	GGCTTTAGTACGTGAACGTCG	
		CCCAAACTTATGTACACGATTG	
At2g13800	SALK_147275	GATGAGCTCGAGAAGCATGAC	
		AACATCCACTTGGTTGATGC	
At4g23230	SALK_067729	TCCTACAAAGCCAATGCAATC	
		AACCTTCTAGGACTGCTCGCC	
At5g59660	SAIL_760_G02	TAGTGTCCAAAGTGTGACCCC	
		TCAATACAGCAACGACAATGG	
At5g59680	SALK_001929	ATTGACGATGGGTTTACCTCC	
		AACATGAAATCGCTGTTGGTC	
LBa1.3 (SALK)	internal SALK lines	ATTTTGCCGATTTCGGAAC	
LB1 (SAIL)	internal SAIL lines	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC	
GABI	internal GABI kat lines	ATAATAACGCTGCGGACATCTACATTTT	

Dissociation curves were generated at the end of the cycle (95°C for 15 seconds, 60°C for 30 seconds and 95°C for 15 seconds) to ensure that only one single product was produced in each case. In qRT-PCR, transcript level in each sample was normalised with *UBC21* expression and data was represented as differences in fold change compared to WT plants. Applied Biosystems program Primer Express 2.0 was used for primer design (Table 3.9). This protocol was also used to quantify fungal biomass (Material and Methods 3.4.1) by using 50 ng of genomic DNA extracted according to the protocol described in section 3. 5.2.

Table 3.9. Oligonucleotide sequences used for gene expression analysis through qRT-PCR.

Gene accession	Gene name	Primer combination (5'-3')
At1g35140	<i>PHI 1</i>	TTGGTTTAGACGGGATGGTG
		ACTCCAGTACAAGCCGATCC
At4g23810	<i>WRKY 53</i>	CACCAGAGTCAAACCAGCCATTAC
		CTTTACCATCATCAAGCCCATCGG
At5g47910	<i>RbohD</i>	ACGTGCGTCCAAGAAAAAC
		GTCGTCCCTGATGTCTAGCG
<i>UBC21</i> cDNA	At5g25760	GCTCTTATCAAAGGACCTTCGG
		CGAACTTGAGGAGGTTGCAAAG
At2g25510.1	<i>SSP1</i>	AGGTGCAGAAAAGACTTACCA
		TTCCGGAATAGGCGGATCTT
At2g25510.2	<i>SSP1</i>	GCTGTATAGCCTATATAAATGCAT
		TGCGGAAACACAAGAAGAAG
At4g31130	<i>SSP3</i>	CAAGGTGCAGAAAAGACTTACCA
		GGCGGATCTTTTGCGTAG
At3g48640	<i>SSP4</i>	TCCCTTGTTTCGTCGTAGCG
		TAGAGCTTCTTCGCCACCTC

3.8. MAPK phosphorylation detection.

These experiments were typically performed with 12-days-old seedlings grown *in vitro* (Material and Methods 3.1). 12 to 15 seedlings were treated with the suitable inducers (e.g., 1 μ M flg22 or 1 μ M PEP1) and frozen immediately in liquid nitrogen at different time points (0, 5 and 20 minutes). Tissues were ground at 6200 rpm for 18 s (twice) in a MagNA Lyser homogenizer (Roche Applied Science, USA)

and total protein was extracted with 50-75 µl of Extraction buffer [25 mM TRIS -HCl pH 7.8; 75 mM NaCl; 15 mM EGTA; 10 mM MgCl₂; 15 mM β-glycerophosphate; 15 mM 4-nitrophenylphosphate bis; 1 mM DTT; 1 mM NaF; 0.5 mM activated Na₃VO₄; 0.5 mM PMSF; 1 % (v/v) protease inhibitor cocktail P9599 (Sigma); 0.1 % (v/v) Tween-20]. Samples were centrifuged at 13000 rpm for 20 min at 4°C. Supernatant was transferred to a pre-chilled 1.5 ml Eppendorf tube and total protein was quantified by Bradford quantification (Bradford, 1976). Approximately, 30-40 µg of total protein per time point were separated in a 10% SDS-PAGE and proteins were transferred on nitrocellulose membrane by an iBlot2 (Thermo Fisher scientific) following manufacturer's instructions.

Membranes were blocked with Pierce Protein-Free T20 (TBS) Blocking Buffer (Thermo Scientific) at RT for 1-2 h and were subsequently incubated with anti-p44/p42 (Anti-Erk1-Erk2; Thr202-Tyr204) MAPK rabbit primary antibody at a 1:1000 dilution at 4°C over-night. Next day, membranes were washed for 5 min with 1% TBS-T thrice and then incubated at RT with an anti-rabbit IgG-HRP secondary antibody (Fisher Scientific) at 1:5000 dilution for 2 h. Finally, membranes were developed with Millipore western developing substrate and membranes stained with Ponceau S/ amido black to confirm equal loading.

3.9. Ca²⁺ detection and quantification.

Transgenic Col-0 plants expressing a cytosolic 35S::Apoaequorin were used (*Col-0^{AEQ}*; Knight *et al.*, 1991). 8 days old seedling were separately distributed in 96-well plates (Greiner GmbH) which contain 10 µM coelenterazine (P.J.K., Germany) in dH₂O and incubated in darkness overnight. Next day, luminescence was measured with a Varioskan Flash Multimode Reader (Thermo Scientific) after elicitation (1µM of *flg22*, *PEP1*, PEP-A, PEP-E, PEP-I, PEP-O), as described in Ranf *et al.*, 2011, Afterwards, remaining aequorin was discharged by addition of CaCl₂ (final concentration 1M, 20% ethanol) and Ca²⁺ concentrations were calculated according to Rentel *et al.*, 2004, using the following equation:

$$[Ca^{2+}]_{cyt} = 0.332588 (-\log(L/L_{max})) + 5.5593$$

where $L/L_{\max}$ are luminescence counts per second divided by total luminescence counts. The calibration equation was determined empirically assuming that all aequorin is discharged from all cells and all emitted light is detected (Rentel *et al.*, 2004).

3.10. Reactive oxygen species measurement assay.

12 leaf discs from 5-weeks-old plants were sampled with a 4 mm diameter cork borer and incubated overnight in sterile water. Next day, water was carefully replaced with a solution of 100 μM luminol (Sigma) and 10 $\mu\text{g ml}^{-1}$ horseradish peroxidase (Sigma), along with the appropriate inducer. Luminescence was measured during 30-40 mins with a 1 min interval with a Varioskan Flash Multimode Reader (Thermo Scientific), and expressed as relative light units (RLU).

3.11. Bioinformatic resources and statistical analyses.

TAIR, the Arabidopsis Information Resource Database (<http://arabidopsis.org>), was used to retrieve genomic DNA, cDNA and mRNA sequences and information of all mutants and extensions of TAIR database (e.g. *eFP*; <http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi>; Winter *et al.*, 2007). The browser was used to examine gene expression under different stress conditions.

TAIR Gene Ontology, (<https://www.arabidopsis.org/tools/bulk/go/>; Berardini *et al.*, 2004) was used to perform the functional classification of genes.

ATTED-II, (<http://atted.jp/cgi-bin/locus.cgi>, Aoki *et al.*, 2016) was used to perform co-expression gene analyses.

SUBA, (<http://suba.live/suba-app/factsheet.html>, Hooper *et al.*, 2016) was used to predict the subcellular location of Arabidopsis proteins.

SIGNAL T-DNA Express and the **Arabidopsis Gene Mapping Tools** (<http://signal.salk.edu/cgi-bin/tdnaexpress>) were used to obtain desirable T-DNA mutants and specific primers to genotype the selected T-DNA lines. Apart from this website for primer design, Integrated DNA Technologies.

Oligoanalyzer 3.1 (<https://eu.idtdna.com/calc/analyzer>) and **Primer Express® Software v2.0** (Applied Biosystems, USA) were frequently used for primer design.

MEGA, Molecular Evolutionary Genetics Analysis (<http://www.megasoftware.net/>), was used for the alignment of multiple Protein sequences.

STATGRAPHICS Plus Version 5.1 standard edition software (2006) StatPoint, Inc., was employed for statistical analysis (http://www.statgraphics.com/statgraphics_plus.htm).

SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP>, Nielsen H. 2017) and **ExPASy PeptideCutter** (http://web.expasy.org/peptide_cutter) were used for the prediction of protein cleavage sites.

PubMed, from the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>) was used for literature search.

RESULTS

RESULTS

4.1. Identification of molecular components of YDA mediated immunity pathway.

Plant immune responses can be triggered by recognition of PAMPs or DAMPS by plasma membrane localized PRRs that structurally are RLKs or RLPs (Couto and Zipfel, 2016; Dodds and Rathjen, 2010). Plant PRRs form dynamic complexes at the plasma membrane that can bind proteins or peptides from microbial (such as bacterial flagellin; Sajio *et al.*, 2017) or endogenous origin (E.g. AtPEP peptides; Yamaguchi *et al.*, 2006). The recognition induces recruitment of additional elements to the complex including supplementary RLKs and RLPs. SOMATIC EMBRYOGENESIS RECEPTOR KINASES (SERKs) form also part of these complexes with many RLKs and RLPs and can contribute to transduce the signal by activating CDK and MAPK cascades (Jordá *et al.*, 2016; Santiago *et al.*, 2016; Schwessinger *et al.*, 2015; Ding and Wang, 2015; Liebrand *et al.*, 2014). These latest elements have been described as crucial transducers of extracellular cues into intracellular responses. Multiple biological processes are regulated by specific MAPK modules, as the MAP3K YODA-MKK4/5-MPK3/6 cascade that modulates stomata and embryo development, inflorescence architecture and lateral organ shape (Xu and Zhang, 2015; Shpak, 2013; Lukowitz *et al.*, 2004) or the MEKK1-MKK5-MPK3/6 signalling module that contributes to Arabidopsis immune responses against bacteria (Cheng *et al.*, 2015).

ERECTA (ER) is a RLK that regulates embryo development, inflorescence architecture and lateral organ shape (Shpak *et al.*, 2013). Besides, ER together with the ERECTA-family proteins (ERf) and the receptor like protein TOO MANY MOUTHS (TMM) bind the extracellular Epidermal Patterning Factor Peptides (EPFs) to control stomata development (Lin *et al.*, 2017). However, ER has been hypothesized to act as a PRR, since *er* mutants are impaired in their defence responses against pathogens with different lifestyles, such as *PcBMM*, *Ralstonia solanacearum* and *Pythium irregulare* (Häffner *et al.*, 2014; Adie *et al.*, 2007; Llorente *et al.*, 2005; Godiard *et al.*, 2003). Interestingly, the protein complexes

that regulate stomatal patterning are also involved in ER-mediated immune responses, although EPF1 and EPF2 do not seem to participate in the activation of defence responses (Jordá *et al.*, 2016).

Previously, our laboratory has demonstrated that *Arabidopsis elk2 (erecta like 2)* mutant, which shows a developmental associated phenotype similar to *er* mutants, was compromised in its resistance responses against different types of fungi, as the necrotrophic *PcBMM*, *B. cinerea* or *A. brassicicola* (Llorente *et al.*, 2005). A map based cloning strategy confirmed that *elk2* is a hypomorphic allele of *YDA*, as *elk2* plants had a substitution of a proline to leucine (P619L) in the kinase domain of this MAP3K. Therefore, *elk2* was renamed as *yda11* (Figure 4.1). Previously described alleles of *yda* were embryo-lethal or extremely dwarf (Lukowitz *et al.*, 2004). However, *yda11* is viable, providing a valuable tool to further characterize the role of YDA in plant innate immunity. Genetic crosses with *er*, confirmed that *YDA* and *ER* genetically interact in the control of the defence signalling pathway. Therefore, the described ER-YDA-MKK4/5-MPK3/6 signalling module that operates in stomata development and patterning is common to immunity (Sopeña-Torres *et al.*, 2018).

N-terminal domain of YDA protein, which is conserved among a specific clade of plant MAP3K, is considered a negative regulatory domain. It has been described in other MAP3Ks that the partial or complete deletion of this N-terminal regions results in the constitutive activation of the kinases (Lukowitz *et al.*, 2004). Accordingly, deletion of amino acids 184 to 322 in this domain results in a constitutive active YDA protein (*CA-YDA*), that can be embryo lethal when expressed in homozygous state (Figure 4.1; Sopeña-Torres *et al.*, 2018; Bergmann, 2004; Lukowitz *et al.*, 2004). Resistance analysis of *CA-YDA*^{+/-} (heterozygous state) and *yda11* plants performed with *PcBMM* revealed that *CA-YDA* supports lower growth of this necrotrophic fungus as compared to WT plants (Col-0 and La-0), while *yda11* was more susceptible. Interestingly, *CA-YDA* and *yda11* plants showed high resistance and susceptibility, respectively, towards biotrophic powdery mildew fungi such as *Golovinomyces orontii* and *Erysiphe cruciferarum*, and the oomycete *Hyaloperonospora arabidopsidis*. Remarkably, the immune response of *yda11* and *CA-YDA* plants against the hemibiotrophic bacteria *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto*) was also altered in a similar way. *yda11* plants were susceptible

while *CA-YDA* plants exhibited an enhanced resistance response against this pathogen. Together these data indicate that *YDA* has a relevant function in Arabidopsis immunity and constitutive activation of MAP3K *YDA* results in a broad-spectrum disease resistance to pathogens with different lifestyles (Sopeña-Torres *et al.*, 2018).

Figure 4.1. *YDA* gene scheme. Boxes correspond to exons, and gaps to introns; N-terminal, kinase and C-terminal domains of the *YDA* protein are shown. The amino acids mutated in *yda1* and *yda11* or deleted in *CA-YDA* plants are also indicated (Sopeña-Torres *et al.*, 2018).

4.1.1. Selection of putative signalling mediators.

To understand the molecular basis mediating *CA-YDA* broad spectrum disease resistance, a comparative transcriptomic analysis between *CA-YDA* and WT plants was performed. For that purpose, total RNA was isolated from mock and *PcBMM*-treated plants at 1-day post inoculation (dpi). A set of 586 genes were differentially expressed in *CA-YDA* plants ($p < 0.05$, fold change > 2) and among these, 360 genes were upregulated, and 226 genes were downregulated. Detailed analysis of differentially upregulated genes in *CA-YDA* plants showed a high degree of concordance with genes induced in WT plants after pathogen treatment. However, no overlapping and significant correlation was found with genes induced by PAMPs as flg22, chitin or even treatments with defensive hormones (Sopeña-Torres *et al.*, 2018). The functional categories overrepresented in the set of 360 up-regulated genes showed clusters of genes involved in plant immunity and response to biotic stresses such as *PRR* genes (TIR-NBS-LRRs, RLKs, RLPs) or genes coding for antimicrobial peptides/proteins (THI2.2 and PR5). In addition, genes involved in cell wall biosynthesis and remodelling were included in one of these categories. As shown in Figure 4.2, a significant proportion of genes encoding unknown function

proteins were present (17%). This prompted us to clarify the role of some of these unknown genes in the Arabidopsis immune response. Out of 62 unknown genes, 12 genes could code for putative small molecular weight peptides. Regulatory role of peptides in plant innate immunity has been already established (Ghorbani *et al.*, 2014). Upon pathogen perception, these small peptides are released from the cytoplasm or the cell wall into the extracellular space, where they induce immune responses resembling PTI following perception by PRRs. Thus, PEP1, PIP1 and PIP2 are short peptides released upon pathogen perception that amplify PTI response to enhance resistance against pathogens (Hou *et al.*, 2014).

Figure 4.2. Functional classification of up-regulated genes in mock CA-YDA plants compared to WT plants. Up-regulated genes were functionally classified according to their Gene Ontology annotation for biological process. Gene expression data was obtained from Gene Ontology at TAIR (Berardini *et al.*, 2004).

In this study, 8 putative *SMALL SECRETED PEPTIDES* (*SSP1-8*) were selected to be further characterized (Table 4.1). The selection was based on their significant induction in CA-YDA plants compared to WT plants (>2-fold change; Table 4-1). Other comparisons such as WT *PcBMM* treated vs. WT mock, indicate that *SSP1*, *SSP4*, *SSP7* and *SSP8* are highly induced by *PcBMM* infection. This elevated level of induction in some of these genes further endorses their putative role in immunity. *SSP1*, *SSP6* and

SSP8 display several splicing variants, although their putative biological role is still unknown.

To corroborate the selection of these SSPs, their expression profile in response to different elicitors, pathogens and plant signalling hormones was evaluated (Table 4.2). Based on public expression analyses available, these selected SSPs were significantly induced by at least one pathogen with the exception of SSP2 that has no transcriptomic data available (see Material and Methods). Remarkably, we found that close to 57% (4 out of 7 genes) were highly induced by at least three pathogens, endorsing their possible relevance in Arabidopsis immunity. Induction of SSPs was also assessed in response to the plant signalling hormones, ET, ABA, JA, and SA (Table 4.2). While expression of SSP1, SSP3, SSP4, SSP5 and SSP6 was mainly repressed upon defence-related hormones, SSP7 and SSP8 expression was upregulated after hormone treatment.

Table 4.1. Selection of putative *SMALL SECRETED PEPTIDES (SSPs)*. Among 360 differentially upregulated genes in the mock CA-YDA plants compared to mock WT plants, 62 genes were annotated as unknown function. 8 SSPs, with their respective splice variants, displayed >2-fold change expression in CA-YDA mock vs. WT mock and were selected for further analysis.

Putative SSPs	Gene accession	Signal peptide	Full peptide length (aa)	Fold change expression	
				CA-YDA mock vs. WT mock	WT 1 dpi <i>PcBMM</i> vs. WT mock
SSP1.1	At2g25510.1	NO	103	307.99	5.48
SSP1.2	At2g25510.2	YES	93		
SSP2	At3g49550	NO	147	2.12	
SSP3	At4g31130	YES	195	2.68	
SSP4	At3Gg8640	NO	224	2.88	17.86
SSP5	At1g65295	YES	115	3.55	
SSP6.1	At5g20790.1	NO	193	2.73	
SSP6.2	At5g20790.2	NO	139		
SSP7	At2g37750	NO	82	6.86	4.03
SSP8.1	At5g44570.1	YES	133	2.25	42.66
SSP8.2	At5g44570.2	YES	140		

Table 4.2. Summary of the expression of selected SSPs following treatment with different elicitors, pathogens or immune-related phytohormones. Elicitor and pathogen concentration and response time used were *flg22* (1 μ M; 1 h post treatment-hpt-), *HrpZ* (10 μ M; 1 hpt), *P. syringae* (10^8 cfu; 6 hpt), *B. cinerea* (5×10^5 spores/ml; 18 hpt), *P. infestans* (10^6 spores/ml; 24 hpt), *G. orontii* (750 spores/cm²; 5 dpi), *H. arabidopsidis* (5×10^6 spores/ml; 6 dpi). Same concentration (10 μ M) of plant signalling hormones and response time (3 hpt) were taken into account to compare different data sets. Colour code represents the degree of induction of genes in response to elicitors, pathogens and plant signalling hormones. Red codes for high induction, yellow codes for low induction, black codes for no change and green codes for repression in gene expression. Expression data was mined on TAIR Arabidopsis eFP browser (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>, Winter *et al.*, 2007).

Putative SSPs	Gene expression in response to different pathogens and elicitors							Hormone expression			
	<i>Flg22</i>	<i>HrpZ</i>	<i>P. syringae</i>	<i>B. cinerea</i>	<i>P. infestans</i>	<i>G. orontii</i>	<i>H. Arabidopsidis</i>	ET	ABA	JA	SA
<i>SSP1.1</i>	Yellow	Black	Yellow	Red	Yellow	Red	Red	Green	Black	Black	Black
<i>SSP1.2</i>	Yellow	Black	Yellow	Red	Yellow	Red	Red	Green	Black	Black	Black
<i>SSP2</i>	NO-DATA										
<i>SSP3</i>	Yellow	Yellow	Yellow	Yellow	Red	Yellow	Red	Black	Green	Green	Black
<i>SSP4</i>	Red	Yellow	Black	Black	Yellow	Black	Red	Green	Green	Green	Black
<i>SSP5</i>	Black	Black	Black	Yellow	Black	Black	Black	Green	Green	Green	Green
<i>SSP6.1</i>	Red	Red	Yellow	Black	Black	Red	Yellow	Green	Green	Green	Green
<i>SSP6.2</i>	Red	Red	Yellow	Black	Black	Red	Yellow	Green	Green	Green	Green
<i>SSP7</i>	Yellow	Yellow	Red	Red	Yellow	Yellow	Red	Yellow	Red	Yellow	Green
<i>SSP8.1</i>	Yellow	Yellow	Red	Yellow	Black	Yellow	Yellow	Red	Green	Red	Red
<i>SSP8.2</i>	Yellow	Yellow	Red	Yellow	Black	Yellow	Yellow	Red	Green	Red	Red

Small secreted peptides are usually perceived by membrane-localized receptors. To date, most of the receptors are RLKs (Couto and Zipfel, 2016). Therefore, we searched in the microarray data for genes coding for RLKs whose expression was up-regulated in *CA-YDA* plants compared to Col-0. We found that expression of 17 genes coding for RLKs was upregulated. Based on their significant induction in *CA-YDA* compared to WT plants (>2-fold change expression), 9 genes were selected to further analyse their contribution to Arabidopsis defence responses (Table 4.3). These genes were named as *PUTATIVE RECEPTOR PROTEINS* (*PRP1-9*). Interestingly, six out of nine *PRP* genes were upregulated upon *PcBMM* treatment, further supporting their role in immunity.

Table 4.3. Selection of PUTATIVE RECEPTOR PROTEIN (PRP). 9 PRPs that displayed >2-fold change expression in CA-YDA mock vs WT mock and were selected for further analysis.

RLKs	Gene accession	Fold Change expression	
		CA-YDA mock vs. WT mock	WT 1 dpi <i>PcBMM</i> vs. WT mock
<i>PPR1</i>	At1g67000	3.78	
<i>PPR2</i>	At3g16030	2.43	3.11
<i>PPR3</i>	At1g51890	2.13	33.06
<i>PPR4</i>	At1g56120	2.68	12.33
<i>PPR5</i>	At1g70690	3.54	2.64
<i>PPR6</i>	At2g13800	6.72	2.03
<i>PPR7</i>	At4g23230	6.34	
<i>PPR8</i>	At5g59660	4.28	
<i>PPR9</i>	At5g59680	8.75	2.53

Similarly, to the analysis performed with the genes encoding small peptides (SSPs), public data on gene expression upon pathogen and elicitors treatments of all these selected *PRPs* was analysed (Table 4.4). Expression analysis data indicate that the *PRP* genes selected are induced by one or more pathogens, which suggests that *PRPs* might have a possible role in Arabidopsis immunity. Induction of *PRPs* was also checked in response to the defence related plant signalling hormones: ET, ABA, JA and SA (Table 4.4). While expression of *PRP3*, *PRP4*, *PRP6* and *PRP8* genes was mainly repressed upon defence-related hormones, *PRP2* and *PRP8* showed an upregulation in their expression after ET treatment.

Table 4.4. Expression of selected *PRPs* following treatment with different elicitors, pathogens or immune-related phytohormones.(Next page) The concentration of elicitors and defence hormones as well as the inoculum of pathogen treatments were the following: *flg22* (1 μ M; 1 h post treatment -hpt-), *HrpZ* (10 μ M; 1 hpt), *P. syringae* (10^8 cfu; 6 hpt), *B. cinerea* (5×10^5 spores/ml; 18 hpt), *P. infestans* (10^6 spores/ml; 24 hpt), *G. orontii* (750 spores/cm²; 5 dpi), *H. arabidopsidis* (5×10^6 spores/ml; 6 dpi). Same concentration (10 μ M) of plant signalling hormone and response time (3 hpt) were taken into account to compare results. The colour bar code represents: red for high induction, yellow for low induction, black for no change and green codes for repression in gene expression. Data mining was done using the data base from TAIR Arabidopsis eFP browser (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>, Winter *et al.*, 2007).

RLKs	Gene expression in response to different pathogens and elicitors							Hormone expression			
	<i>Flg22</i>	<i>HrpZ</i>	<i>P. syringae</i>	<i>B. cinerea</i>	<i>P. infestans</i>	<i>G. Orontii</i>	<i>H. arabidopsidis</i>	ET	ABA	JA	SA
<i>PRP1</i>											
<i>PRP2</i>											
<i>PRP3</i>											
<i>PRP4</i>											
<i>PRP5</i>											
<i>PRP6</i>											
<i>PRP7</i>								NO-DATA			
<i>PRP8</i>											
<i>PRP9</i>

Microarray data were validated by analysing the expression of some of the selected *SSPs* in Col-0, *yda11* and CA-YDA untreated plants by qRT-PCR. As shown in Figure 4.3, expression levels of selected *SSPs* were significantly higher in CA-YDA compared to Col-0 and *yda11* plants, further confirming that these *SSPs* are upregulated in CA-YDA plants displaying constitutive defences on.

Figure 4.3. Validation of microarray data. Gene expression was determined by qRT-PCR in tissues of Col-0, *yda11* and CA-YDA eighteen-days-old plants. Transcript levels of analysed genes were represented as absolute values ($\pm$ SE). This is a representative experiment from the two experiments performed. Both gave similar results. Letters indicate values statistically different among genotypes (ANOVA; $p < 0.05$).

4.1.2. Identification of insertion mutants in the *SMALL SECRETED PEPTIDES* and *PUTATIVE RECEPTOR PROTEINS*.

To evaluate the contribution of the *SSPs* and *PRPs* to Arabidopsis defence responses, T-DNA insertion lines in the selected genes were identified and ordered from the Nottingham Arabidopsis Stock Centre (NASC). Plants were grown, and identification of homozygous mutant lines was performed by PCR as described in material and methods. Two different sets of oligonucleotides were used for the selection of homozygous lines: first a pair of flanking primers that amplifies in WT plants and second the combination of an internal (T-DNA specific) oligonucleotide with a flanking one that amplifies only in T-DNA insertional mutants (Figure 4.4.A and Figure 4.5.A). In addition, to confirm that all the T-DNA insertional lines were null mutants, an RT-PCR was performed to analyse gene expression in each of the insertion mutants. Thus, these expression studies confirmed that all T-DNA insertion lines used in this study were null mutants or presented a very low transcript accumulation (Figure 4.4.B and Figure 4.5.B).

Figure 4.4. Identification and validation of homozygous insertion mutants in the selected *SMALL SECRETED PEPTIDES*. (Previous page, A) PCR was performed on genomic DNA from the genotypes stated with one flanking and a T-DNA primer to confirm the T-DNA insertion in the selected *SSP* genes (right) and with flanking primer combinations to validate that they are homozygous (left). A negative control is also included. (B) Detection of mRNA accumulation by RT-PCR in WT and the different mutant lines, demonstrated that all selected lines were null mutants or displayed a considerably reduction of their expression. (C) Diagram depicting the gene structure and the position of the T-DNA insertion of the selected *SSPs*. Black boxes correspond to exons and gaps to introns. T-DNA insertions are represented as red boxes. T-DNA line name is also indicated. Primers used for genotyping and expression studies are described in Material and Methods.

Figure 4.5. Selection of homozygous *Putative Receptor Protein* mutants. PCR was performed on genomic DNA from the genotypes stated to confirm the T-DNA insertion in the selected *PPR* genes (right) and with flanking primer combinations to confirm that they are homozygous (left). (A) PCR negative control is also included. (B) Detection of mRNA accumulation by RT-PCR in WT and the different mutant lines, demonstrated that all selected lines were null mutants or displayed a considerably reduction of their expression. (C) Diagram depicting the gene structure and the position of the T-DNA insertion of the selected *PPRs*. Black boxes correspond to exons and gaps to introns. T-DNA insertions are represented as red boxes. T-DNA line name is also indicated. Primers used for genotyping and expression studies are described in Material and Methods.

4.1.3. Disease resistance response analyses in selected *ssp* mutants.

It has been reported that mutations in genes coding for secreted peptides result in compromised resistance to pathogens, as is the case of *PIP1* whose mutants are highly susceptible to *Pseudomonas syringae* and *Fusarium oxysporum* (Hou *et al.*, 2014). Therefore, to evaluate the contribution of the selected peptides to Arabidopsis immune responses, mutants in these *SSPs* were analysed against a range of three different pathogens with different mechanism of colonization: the necrotrophic fungus *PcBMM*; the biotrophic oomycete *Hpa* isolate Noco2 and the hemibiotrophic bacteria *Pto* DC3000.

First resistance response of selected *ssp* mutants to *PcBMM* was analysed. Nineteen-day-old plants of the genotypes Col-0, hyper-susceptible *agb1-2* and resistant *irx1-6* mutants, along with *ssp* mutants, were sprayed with a spore suspension of the fungus (4×10^6 spores/ml). At 5 dpi, genomic DNA was extracted from the plants and fungal biomass was determined by quantifying *PcBMM* β -*tubulin* gene by qPCR. Remarkably, mutations in three of the genes coding for small peptides led to increased susceptibility to *PcBMM*. *ssp1*, *ssp7* and *ssp8* mutants showed higher fungal biomass than that of the corresponding WT plants (Figure 4.6.A). Also, *ssp2*, *ssp4* and *ssp6* showed a slightly enhanced susceptibility to this fungus, as biomass accumulation was moderately higher compared to WT plants in three independent experiments, although these differences were not statistically significant. By contrast, *ssp3* and *ssp5* mutants showed no alteration in the defence responses against *PcBMM*. Fungal biomass accumulation data was also supported by the disease rating recorded and the macroscopic disease symptoms as shown in Figure 4.6.B, and 4.6.C, respectively. Thus, some of the small secreted peptides, particularly *SSP1*, *SSP7* and *SSP8* may play a relevant role in the resistance response of Arabidopsis to *PcBMM*, as mutations in these *SSPs* led to an enhanced susceptibility to this fungus.

Figure 4.6. Resistance analysis of *ssp* mutants to the necrotrophic fungus *PcBMM*. 18-days-old seedlings of the mentioned genotypes were spray inoculated with 4×10^6 spores/ml. (A) *PcBMM* biomass quantification by qPCR at 5 dpi. Values represented are the average $\pm$ SE (n=4) of the n-fold increase compared to WT plants (Col-0). (B) Average disease ratings $\pm$ SE (n=16) of the indicated genotypes at 8 dpi: 1, no symptoms; 2, one chlorotic or necrotic leaf; 3, two to three necrotic leaves; 4, more than three necrotic leaves; 5, dead plant. (C) Macroscopic disease symptoms of the indicated genotypes at 8 dpi. Letters indicate values statistically different among genotypes (ANOVA; $p < 0.05$). These analyses were performed three times with similar results.

To test whether the increased susceptibility of some *ssp* mutants to *PcBMM* was also extended towards different lifestyle pathogens, we analysed the defence responses against the hemibiotrophic

bacterial pathogen *Pto* and the biotrophic oomycete *Hpa* isolate Noco2 in these lines. To analyse resistance/susceptibility to *Pto*, twenty-four-days-old plants were inoculated with a bacterial suspension (O.D.600 0.2) and high humidity conditions were maintained throughout the experiment.

Figure 4.7. Resistance analysis of *ssp* mutants to the hemibiotrophic bacteria *P. syringae* pv. *tomato* DC3000.

(A) Quantification of bacterial growth on 4-week-old plants after spray inoculation with the virulent bacterial strain *Pto* DC3000. Bacterial colony forming units (cfu) per cm² of leaf were determined at 2 and 4 dpi. Values are means $\pm$ SE (n=3). Letters indicate genotypes with significant differences in their level of resistance (ANOVA; $p < 0.05$). (B) Macroscopic disease symptoms of *Pto* DC3000-inoculated plants at 5 dpi. These analyses were performed three times with similar results.

Along with the selected *ssp* mutants, *agb1-2* and *cpr5* plants were included as susceptible and resistant controls, respectively. Leaf samples of the inoculated genotypes were collected at 2 and 4 dpi, and colony forming units per cm² of infected leaf were determined. As shown in Figure 4.7.A, at

2 dpi only *ssp6*, *ssp7* and *ssp8* were slightly more susceptible to the *Pto* DC3000 than Col-0 while other *ssp* mutants showed a bacterial growth similar to WT plants. However, when colony counts were performed at 4 dpi, *ssp1*, *ssp2*, *ssp6*, *ssp7* and *ssp8* were more susceptible than Col-0 plants. Bacterial growth in the *ssp3*, *ssp4* and *ssp5* mutants were similar to that of Col-0 plants, indicating that these SSPs have minor or no contribution to resistance to this pathogen (Figure 4.7.A). Visualization of macroscopic disease symptoms of inoculated plants correlated with the bacterial growth progression (Figure 4.7.B). Thus, these experiments suggest that *SSP1*, *SSP2*, *SSP6*, *SSP7* and *SSP8* may have an important role in the resistance response to *P. syringae*, while *SSP3*, *SSP4*, and *SSP5* may have no contribution.

Another pathogen with a different lifestyle, the biotrophic oomycete *Hpa* isolate Noco2 was employed to evaluate the defence response of the selected *ssps*. Noco2 isolate is virulent on Arabidopsis ecotype Col-0, as it lacks the *RPP5* gene conferring resistance to this isolate that is present in La-0 ecotype (Parker *et al.*, 1993). Two-weeks-old plants of the different *ssp* mutants were inoculated with a conidia suspension (2×10^4 spores/ml). *NahG* transgenic plants (expressing a salicylic acid degrading enzyme; Delaney *et al.*, 1994) and La-0 plants were included in the assay as susceptible and resistant controls. *Hpa* colonization was evaluated after 7 days post inoculation by harvesting the plants from the different genotypes and counting the number of conidiospores per milligram of fresh weight (FW). As shown in Figure 4.8, *ssp1* and *ssp8* mutants displayed an enhanced susceptibility phenotype compared to Col-0 plants. *ssp2* and *ssp6* mutants exhibited a slight increase in the number of conidia per FW in the different assays performed, although this increase was in the limit of the statistical significance. On the other hand, the resistance response of *ssp3*, *ssp4*, *ssp5* and *ssp7* against Noco2 was not compromised, as the number of conidiospores per FW was similar to that observed in the Col-0 plants. These data suggest that *SSP1* and *SSP8* play a major role in the defence mechanisms against *Hpa*, while *SSP2*, *SSP5* and *SSP6* seem to have a minor contribution.

Figure 4.8. Defence response analysis of *ssp* mutants to the oomycete *H. arabidopsidis* Noco2. 4-weeks-old plants of the indicated genotypes were spray inoculated with a 1×10^5 spores/ml from the virulent isolate Noco2. Numbers of conidiospores per milligram (mg) of leaf fresh weight (FW) were measured at 7 dpi. Letters represents statistically different values for each genotype (ANOVA; $p < 0.05$). These assays were performed at least three times with similar results.

All together, these data highlight the relevant role of some of the selected *SSPs* in Arabidopsis immunity, as T-DNA insertional mutants in these *SSPs* showed enhanced susceptibility towards at least one of the pathogens tested, with the exception of *ssp3* and *ssp5*. Remarkably, *ssp1* and *ssp8* were more susceptible than WT to all three pathogens with different lifestyle analysed. Other *ssp* mutants, as *ssp2*, *ssp6* and *ssp7*, also showed enhanced susceptibility to at least two of the tested pathogens.

4.1.4. Disease resistance response analyses of selected *prp* mutants.

To unravel the contribution of the selected RLKs to Arabidopsis immunity, the defence response to the same pathogens employed in the previous chapter was evaluated in the insertion mutants identified in the *PRP* genes. In these analyses, the experimental conditions were the same used in the analysis of the *ssp* mutants.

Homozygous T-DNA insertional mutants in the *PRP* genes were analysed for their contribution to Arabidopsis defence response and resistance to *PcBMM*. Interestingly, analysis of biomass quantification revealed that *prp1*, *prp4*, *prp5* and *prp7* plants showed enhanced growth of the necrotrophic fungus as compared to Col-0 plants at 5 dpi, while mutants in other *prps* did not show any significant difference in their resistance responses (Figure 4.9.A). Disease rating and macroscopic symptoms of inoculated plants corroborated these data (Figure 4.9.B and C). These analyses indicate that *PRP1*, *PRP4*, *PRP5* and *PRP7* might contribute to Arabidopsis disease resistance against *PcBMM*.

To further determine how general is the contribution of the selected *PRP* genes to immunity, the resistance response to the hemibiotrophic *Pto* was evaluated in the *prp* mutants. For this analysis, *cpr5* and *agb1-2* were included as the resistant and susceptible controls, respectively. Twenty-four-days-old plants were inoculated with a bacterial suspension of *Pto* DC3000 (OD₆₀₀ 0.2). Plants were subsequently kept under high humidity and, at 2 and 4 dpi, leaf samples of the inoculated genotypes were collected and colony forming units per cm² of infected leaf were determined. As displayed in Figure 4.10.A, only *prp1* plants showed increased bacterial growth. None of the remaining *prps* analysed showed a statistically relevant alteration of the defence responses against this pathogen. Bacterial growth correlated to the macroscopic visualization of the disease progression (Figure 4.10.B).

Figure 4.9. Resistance of *prp* mutants to the necrotrophic fungus *PcBMM*. 18-days-old seedlings of the mentioned genotypes were spray inoculated with 4×10^6 spores/ml. (A) *PcBMM* biomass quantification by qPCR at 5 dpi. Values are represented as the average $\pm$ SE (n=4) of the n-fold increase compared to WT plants (Col-0). Letters indicate values statistically different among genotypes (ANOVA; $p < 0.05$). (B) Average of disease ratings (DR) $\pm$ SE (n=15) of the indicated genotypes at 6 dpi: 1, no symptoms; 2, one chlorotic or necrotic leaf; 3, two to three necrotic leaves; 4, more than three necrotic leaves; 5, dead plant. (C) Macroscopic disease symptoms of the indicated genotypes at 6 dpi.

Figure 4.10. Resistance analysis of *prp* mutants to the hemibiotrophic bacterium *P. syringae* pv. *tomato* DC3000. (A) Quantification of bacterial growth on plants after spray inoculation with the virulent strain *Pto* DC3000. Bacterial colony forming units (cfu) per cm² of leaf were determined at 2 and 4 dpi. Values are means $\pm$ SE (n=3). Letters indicate genotypes with significant differences in their level of resistance (ANOVA; $p < 0.05$). (B) Disease symptoms of *Pto* DC3000-inoculated plants at 5dpi.

In addition, the resistance response of *prp* mutants was evaluated against the biotrophic oomycete *Hpa*. We could observe that *prp1* and *prp7* mutants showed an enhanced number of conidiospores per milligram of FW that was statistically significant (Figure 4.11.A). However, mutations in the remaining *PRPs* had slight or no effect on *Hpa* conidiospores counting. Trypan blue staining of inoculated leaves at 7 dpi also confirmed the data obtained with the spore counting, as more growth of *Hpa* could be observed on *prp1* and *prp7* mutants compared to WT plants (Figure 4.11.B).

Figure 4.11. Defence response analysis of the selected *prps* to *H. arabidopsidis* (*Hpa*) isolate Noco2. (A). Number of conidiospores per milligram (mg) of leaf fresh weight (FW) $\pm$ SE (n=4) were measured at 7 dpi on the indicated genotypes. *NahG* transgenic lines and *La-0* plants were included as susceptible and resistant controls, respectively. Letters indicate genotypes with significant differences in their level of resistance (ANOVA; $p < 0.05$). (B) Trypan blue staining of Noco2 inoculated leaves at 7 dpi. Bar represents 100 μ m.

Resistance response analysis of selected *prp* mutants against three different pathogens suggests that PRP1 have a relevant contribution to plant immunity, as mutants impaired in this RLK were more

susceptible to the fungi *PcBMM*, bacteria *Pto* and the oomycete *Hpa*. However, other *PRP* genes also might display a favourable contribution to *Arabidopsis* defence response, as *prp4*, *prp5* and *prp7* mutants showed a compromised disease resistance against *PcBMM*.

4.1.5. Resistance response of selected *ssps* and *prps* against *Cucumber mosaic virus*.

Viruses are important plant pathogens that are frequently omitted in the resistant/susceptibility studies. To broaden the knowledge about the role of *SSPs* and *PRPs* in immunity, the contribution of selected *SSPs* and *PRPs* to the defence response of *Arabidopsis* against *Cucumber mosaic virus* (CMV) strain LS was evaluated (Jacquemond *et al.*, 2012). CMV, an economically important virus, is found to be the most prevalent virus among the five monitored viruses in natural populations of *Arabidopsis* in Central Spain (Pagán *et al.*, 2010). For these assays only, a reduced group of mutants was selected based to the previous pathogen analyses. These include *ssp1*, *ssp2*, *ssp6*, *ssp7* and *prp1*, which showed enhanced susceptibility to some of the three tested pathogens. Also *prp5* mutants were analysed, as this *PRP* is co-expressed with *SSP7* (based on ATTED II data; Aoki *et al.*, 2016). *agb1-2* and *NahG* plants were used as susceptible controls and *yda11* mutants were also included for the resistance analysis against CMV. After mechanical inoculation of LS-CMV at 4-5 rosette leaves (stage 1.04; Boyes *et al.*, 2001), whole plant shoot-up was collected at 15 dpi and fresh weight was measured for each plant. Viral accumulation was quantified by qRT-PCR as described in Materials and Methods (3.5.4). Generalized linear model (GzLM) analysis suggested that accumulation of viral RNA is dependent on host genotype (Wald $X^2_{(9,45)} = 35.771$, $p \leq 10^{-3}$). *Arabidopsis* genotypes ranked differently according to viral RNA accumulation, indicating that they differ in resistance to LS-CMV (Figure 4.12.A). Heterotrimeric G protein mutant *agb1-2*, and T-DNA insertional mutant *ssp1* showed a statistically significant enhanced susceptibility to CMV, suggesting that the product of these genes plays an important part in controlling virus accumulation. *Nah-G*, *yda11* and *prp1* also showed higher viral accumulation compared to Col-0 WT, although these differences were not statistically significant. On

the other hand, *ssp7* plants showed a reduced viral accumulation compared to Col-0 plants. Mutations in *SSP2*, *SSP6* and *PRP5* showed no differences in accumulation of this virus compared with the control, suggesting that these genes may not have any contribution in resistance to CMV.

Defence mechanisms are essential for survival in case of infection. However, based on assumption that plants possess a limited resource pool that can be invested in developmental programs or in defence, the energy deployed on defence activation often comes with the cost of plant growth (Huot *et al.*, 2014; Herms and Mattson, 1992). Therefore, the effect of virus infection on fresh biomass was also quantified. For that purpose, the ratio of fresh biomass of infected plants (BM_i) relative to mock-inoculated controls (BM_m) was obtained. General linear model (GLM) analysis suggested that biomass reduction in infected plants is also dependent on host genotype ($F_{(9,45)} = 3.4$, $p = 0.003$). As shown in Figure 4.12.B, *ssp7* was the only genotype that showed differences statistically significant in biomass reduction compared to Col-0. *ssp7* showed lower viral accumulation and biomass increase, which suggests that mutations in *SSP7* appears to confer resistance to LS-CMV or that *ssp7* plants may have other compensatory mechanisms different than resistance. On the contrary, *yda11* plants resulted to be highly susceptible to CMV, as the viral accumulation was higher than WT plants. This is particularly interesting because this line displays susceptibility against a broad range of bacteria, oomycetes and fungal pathogens. However, no statistically significant biomass reduction due to the virus infection was observed in this line. Similarly, to *yda11*, although *NahG* and *ssp1* plants had higher number of virus particles, the reduction in the biomass was no so dramatic and it was comparable to Col-0 plants.

Figure 4.12. Resistance analysis of selected *ssps* and *prps* against *Cucumber mosaic virus*. (A) Average $\pm$ SE viral accumulation expressed in ng of viral RNA/ μ g of total plant RNA for each genotype, from at least 4 replicates (B) Comparison of infected/mock inoculated fresh biomass at 15 days post inoculation ($n \geq 4$). Letters represent statistically different values for each genotype according to LSD test ($p < 0.05$). (C) Pictures of mock control or LS-CMV infected plants from the indicated genotypes at 15 dpi.

4.1.6. Morphological parametric analysis in *ssps* and *prp1*.

Cell to cell communication is becoming integral part to many plant processes. Arabidopsis genome encodes thousands of putative secreted peptides and many of these peptides transport the information required for cell differentiation and proliferation and have been established as important regulators for different developmental patterns (Coninck and Smet, 2016). *yda11* plants display some *er-like* phenotypes such as compact inflorescences and reduced plant height and silique and petioles

length. Therefore, in order to clarify whether any of these *SSPs* may have a role in some developmental processes mediated by YDA we performed a morphometric analysis on the T-DNA insertional mutant lines in the *SSPs*. The mutant in the receptor like kinase *PRP1* was also included in these analyses.

4.1.6.1. Minor developmental phenotypes observed in some *ssp* mutants and in *prp1*.

To assess the contribution of small secreted peptides to plant developmental associated phenotypes, plants mutated in the *SSPs* and in *PRP1* together with Col-0 and *er105*, null mutant that displays strong developmental phenotypes (Torii *et al.*, 1996), were grown on soil under long day conditions (14 h light/10 h dark). Subsequently, a quantitative assessment of morphometric parameters was performed. Plant height and silique and its associated pedicel length from the main stem were measured in 45-days old plant. As shown in Figure 4.13.A and B, *ssps* did not show any significant difference in silique and pedicel length as compared to Col-0 plants. Same results were obtained with the mutant *prp1* (Figure 4.14.A-B). When plant height was measured (Figure 4.13.C and Figure 4.14.C), only *ssp7* mutants showed a little reduction in height compared to WT Col-0, while *prp1* plants were slightly taller. However, plant height changes were not comparable to the phenotype observed in the *er105* null mutants. Also, mutations in *ER* dramatically modify inflorescence architecture and leaf morphology (Torii *et al.*, 1996). Compared to WT, *er* flower buds are tightly clustered at the top and the leaves blades are reduced. However, none of the *ssps* analysed or *prp1* showed any developmental alteration affecting neither floral architecture nor elongation of leaves (Figure 4.13.D and 4.14.D). Therefore, our analysis indicates that neither the *SSPs* genes nor *PRP1* are required for plant morphogenesis, as no evident changes in organ development could be observed in the null mutants.

4.1.6.2 Stomatal patterning remains unaffected in most *ssp* mutants.

A precisely coordinated cell-to-cell communication network defines asymmetric cell divisions that determine stomata development. Multiple cysteine-rich signalling peptides belonging to the EPF family, e.g. EPF1, EPF2, EPF-LIKE6/CHALLAH and EPF-LIKE9/STOMAGEN, are associated with the

regulation of stomatal density and positioning (Lau and Bergmann, 2012; Shimada *et al.*, 2011; Abrash and Bergmann, 2010; Wang *et al.*, 2007). And TMM (Nadeau and Sack, 2002; Yang and Sack, 1995), YDA (Bergmann, 2004) and ER family members (Shpak *et al.*, 2005) are well characterized regulators of stomata development.

Figure 4.13. Analysis of developmental phenotypes performed in the *ssp* mutants. Length of pedicel (A) and respective silique (B) from the main stem of 45-days-old plants. Values are means $\pm$ SE ($n = 72$). (C) Plant height determined in 45-days-old plants. Values are means $\pm$ SE ($n = 15$). (D) Phenotype of adult plants (4 weeks-old plants; bottom) and inflorescence apices (7 weeks-old plants; top) from the indicated genotypes. Letters in A, B and C indicate different statistically significant groups (ANOVA; $p < 0.05$).

Figure 4.14. Analysis of developmental phenotypes in the *prp1* mutant. Length of pedicel (A) and respective silique (B) from the main stem of 45-days-old plants. Values are means $\pm$ SE (n = 72). (C) Plant height of 45-days-old plants. Values are means, $\pm$ SE (n = 15). (D) Phenotype of adult plants (4 weeks-old plants; bottom) and inflorescence apices (7 weeks-old plants; top) from Col-0, *er105* and *prp1* plants. Letters in A, B and C indicate different statistically significant groups (ANOVA; $p < 0.05$).

Since mutations in the first two genes and a triple mutant of *ERECTA*, *ERECTA-LIKE1* and *ERECTA-LIKE2* result in plants with excess and clustered stomata. TMM and proteins of the ER family form constitutive complexes at the plasma membrane, where TMM endows the ER family proteins with ligand specificity, allowing different tissue-dependent phenotypes (Lin *et al.*, 2017). To analyse the contribution of the selected *SSPs* to stomata development and patterning, mutant plants in the *SSPs*, together with Col-0 and *er-105* mutant (Shpak *et al.*, 2005), were grown on soil for 25 days at short

day conditions (10 h light/ 14 h dark) and stomata were counted on fully expanded leaves. Selected leaves were destained and mounted for stomata counting as described in Material and Methods section. As shown in Figure 4.15.A, stomatal density (SD; Material and Methods 3.3) in *ssp1* plants was similar to Col-0 plants. However, all other *ssps* mutants showed statistically significant differences in terms of SD from WT plants, although these differences were not comparable to the level observed in *er105* plants. When stomata index (SI; Material and Methods 3.3) was analysed, only *ssp3* plants displayed an increased SI compared to WT plants (Figure 4.15.B). SI is a more accurate parameter as it normalizes for the effects of cell expansion, taking into account the number of epidermal cells within a unit leaf area. Therefore, although in the previous counting the number of stomata was higher in all the *ssps* except for *ssp1*, SI estimation indicate that only *ssp3* mutation had an impact on stomata development. Mutations in *TMM* lead to the development of stomatal clusters in leaves and siliques (Shpak *et al.*, 2005; Serna, 2001). None of the mutants analysed in this study showed this type of phenotype. Therefore, all these data suggest that, with exception of *SSP3*, these genes seem not to have a role in stomata development and patterning.

Figure 4.15. Stomata patterning of *ssp* (1-8) mutants. Analysis of stomata was performed on fully expanded leaves from 25-days-old plants of the above-mentioned genotypes. (A) Stomata density in the different genotypes. (B) Stomatal index for each genotype, which represents the number of stomata divided by total number of cells in the abaxial side. Values are means $\pm$ SE ($n = 10$). (C) Representative pictures of epidermal patterning in the abaxial surface of 25-days-old rosette leaves from the indicated genotypes.

4.1.7. Assessment of PTI induced responses to synthetic peptides.

Defence response analyses performed showed that null mutants *ssp1* and *ssp8* displayed enhanced susceptibility towards the necrotrophic *PcBMM*, the oomycete *Hpa* isolate Noco2 and to the hemibiotrophic bacteria *Pto* DC3000. Therefore, we hypothesized that these SSPs could be important mediators of constitutive immunity in Arabidopsis *CA-YDA* plants, which display enhanced resistance against these same pathogens. Moreover, *ssp7* was also susceptible to *PcBMM* and *Pto*, while *ssp2* and *ssp6* were affected in the defence response against *Pto*. All these data indicate that 5 out of 8

SSPs evaluated in this work that are up-regulated in *CA-YDA* plants may have a relevant contribution to plant immunity.

The role of extracellular peptides in plant immunity have been previously described (Coninck and Smet, 2016; Masachis *et al.*, 2016). Many of these peptides suffer post-translational modifications. After cleavage of the extracellular signal peptide, some more cleavages by subtilases or other extracellular peptidases are needed to render a mature and biologically active peptide. According to the literature, only the C-terminal region of these peptides is the active region (Zhang *et al.*, 2016; Ghorbani *et al.*, 2014; Liu *et al.*, 2013). To determine whether some of the selected SSPs could act as activators of immune responses such as PTI, synthetic peptides corresponding to the C-terminal region of SSP1, SSP2 and SSP7 were generated (Table 4.3). Presence of proline, glycine, asparagine and histidine residues was taken into account during peptide design as structural studies have highlighted the relevance of these residues to form extensive interactions with the corresponding PRRs (Zhang *et al.*, 2016; Tang *et al.*, 2015; Lin *et al.*, 2017; Huffaker *et al.*, 2006). In the case of *SSP1*, with alternative splicing, two possible peptide variants V1 and V2 were contemplated. Subsequently, early-activated PTI defence responses such as Ca^{2+} fluxes, oxidative burst, MAPK cascade activation or induction of defence-gene expression were measured on WT plants after treatment with these synthetic peptides.

Table 4.3. Chemically synthesized C-terminal sequence of some SSPs.

Peptide	Shorten Peptide	Peptide sequence N→C	Length (aa)
SSP1 (V1)	PEP-E	LCLPMHVFVIFYSLSNGRYNK	21
SSP1 (V2)	PEP-A	LHLSPPPPQRSFKGTNEPGH	20
SSP2	PEP-O	SPFERSFAAVSPFCSQAGGDGMEIN	25
SSP7	PEP-I	NRASSSTSTTTFAVYAGVQSPVSRQVVT	28

4.1.7.1. A shorten version of SSP7 induces early Ca²⁺ uptake.

Uptake of Ca²⁺ in response to any pathogenic attack is one of the earliest responses generated by plant cells. Detection of cytoplasmic Ca²⁺ can be done by the bioluminescent Ca²⁺-binding protein aequorin (Knight *et al.*, 1991). Aequorin is composed of an apoprotein (apoaequorin) and a prosthetic group, the luminophore coelenterazine that, in the presence of Ca²⁺, is converted into coelenteramide followed by light emission that can be easily quantified in a luminometer (Mithöfer and Mazars, 2002). Eight-days-old seedlings of Col-0^{AEQ} (35S::*Apoaequorin*; Knight *et al.*, 1991) grown on MS medium were incubated overnight in coelenterazine in a 96-well plate. Next day, a titration assay was performed using increasing concentrations of the synthetic peptides PEP-A, PEP-E, PEP-I and PEP-O from 0.5 µM till 10 µM and measurements were compared with those produced with the control peptides flg22 (1 µM) and PEP1 (1 µM) (Ranf *et al.*, 2011; Figure 4.16 and data not shown). Luminescence was recorded for 20 minutes after elicitor treatments and [Ca²⁺] was calculated (Section 3.9, Materials and Methods). High elevation in cytosolic Ca²⁺ was observed in response to the well characterized elicitors flg22 and PEP1. However, only a lower and later rise in cytosolic Ca²⁺ levels were observed after PEP-E and PEP-O treatments (Figure 4.16). Intriguingly, always a very rapid Ca²⁺ elevation (with a peak earlier than 1 minute, even faster than the peaks observed after flg22 and PEP1 treatments) was observed after PEP-I treatment. However, the total amount of Ca²⁺ quantified in response to PEP-I was smaller than to the control elicitors employed. Finally, PEP-A treatment was unable to induce any response in Arabidopsis seedlings with the two concentrations evaluated (Figure 4.16).

4.1.7.2. SSP-derived peptides cannot trigger reactive oxygen species production.

Production of ROS is another classic early defence response of host in response to pathogen or DAMP recognition. Therefore, we aimed to evaluate whether the shorten versions of the selected SSPs could trigger ROS production in WT plants. For that purpose, a luminol based assay was performed on leaf discs from four-weeks-old plants. As shown in Figure 4.17.A, flg22 and PEP1 are able to induce a strong and transient accumulation of hydrogen peroxide, whereas all the synthetic peptides analysed, PEP-

A, PEP-E, PEP-I and PEP-O were unable to trigger the production of ROS to a range of different concentrations (1-5 μM ; Figure 4.17.A and data not shown).

Figure 4.16. Ca^{2+} measurements after treatment with SSP-derived peptides. Measurement of cytosolic $[\text{Ca}^{2+}]$ in Col-0^{AEQ} 8-days-old plants upon treatment with different peptides at the concentrations stated over a period of 20 min. (A) Comparison of cytoplasmic $[\text{Ca}^{2+}]$ after treatment with 5 μM PEP-E, PEP-A, PEP-I and PEP-O and 1 μM of the controls flg22 and PEP1. (B) Visualization of cytoplasmic $[\text{Ca}^{2+}]$ for each individual peptide applied at 1 μM (flg22 and PEP1) and 1 and 5 μM (PEP-E, PEP-A, PEP-I and PEP-O). Data represented are the average of 8 plants per genotype. Data presented comes from one of three individual experiments performed with similar results.

This ROS detection assay was performed with standard luminol (Sigma, A8511). Given these negative results, production of ROS in response to these synthetic peptides was also analysed using a new and more sensitive luminol (Luminol L-012, Wako; Melcher and Moerschbacher, 2016) . With this new luminol, production of ROS in response to flg22 and PEP1 reaches peaks 40 times more elevated than with standard luminol (Figure 4.17.B), However, with Luminol L-012, although a small amount of ROS production was detected in response to the synthetic peptides (Figure 4.17.B), quantification of RLU produced indicate that ROS produced by synthetic peptides does not show any statistically significant difference from H₂O control (Figure 4.17.C).

Figure 4.17. ROS production after treatment with SSP-derived peptides. H₂O₂ production after treatment with the indicated peptides measured in a luminol-assay using leaf discs from 4-week-old Col-0 plants. (A) Total ROS production over a period of 30 minutes in a luminol based-assay. (B) Total ROS production over a period of 40 minutes using luminol L-012. (C) Total LRU / plant in a luminol L-012 assay. Values are means ± SE (n = 6). Letters indicate values statistically different among genotypes (ANOVA; *p* < 0.05). This assay has been performed three times with similar results.

4.1.7.3. Some PEPs can slightly induce the activation of the MAPK cascade.

Different PAMP- and DAMP-induced signalling converges at very early stages on the activation of MAPKs or CPKs, which play a central part in signal transduction from PRRs to intracellular compartments (Meng and Zhang, 2013a). In PTI, MPK3, MPK4, MPK6 and MPK11, which are the best characterized components of MAPK cascades, have been shown to be phosphorylated upon PAMP/DAMP treatment and plant infection by pathogens (Bethke *et al.*, 2012; Asai *et al.*, 2002).

Given the not very consistent results obtained with the $[Ca^{2+}]_{cyt}$ assays, and ROS production, next, we examined the activation of MAPK cascades upon treatment with these SSP-derived peptides. With this aim we assessed the level of phosphorylation of MPK3 and MPK6 in WT. *In vitro* grown 18 days plants were analysed in a time course after treatment with different inducers: flg22 (1 μ M), PEP-1 (1 μ M) and the peptides PEP-A, PEP-E, PEP-I and PEP-O (5 μ M). Tissues were harvested at different time points (0, 5 and 20 minutes) and the phosphorylation status of MPKs was determined by western blot, using the anti-pTEpY antibody (see Material and Methods 3.8). As it has been described previously, flg22 and PEP1 treatments were able to induce a rapid and strong activation of the MAPK signalling cascade (Bigeard *et al.*, 2015). Remarkably, the chemically synthesized peptide PEP-I led to MAPK activation at 5 and 20 minutes post treatment, although reduced compared to the obtained after flg22 or Pep1 perception. Treatments with PEP-E and PEP-A produced a slight activation of MPK6, while elicitation of Arabidopsis seedlings with PEP-O almost had no effect on MAPK activation.

Figure 4.18. MAPK cascade activation analysis after treatment with shorten versions of SSPs. Immunoblot analysis of phosphorylated MAPKs (MPK6 and MPK3) after treatment of Col-0 plants with flg22 (1 μ M), PEP1 (1 μ M) and the peptides PEP-A, PEP-E, PEP-I and PEP-O (5 μ M). Phosphorylation was determined at the indicated time points by using the anti-pTEpY antibody (top panel). Amido black-stained membranes show equal loading of samples (bottom panel).

4.1.7.4. Defence gene expression was not altered in response to synthetic peptides derived from SSPs.

After pathogen recognition, a dramatic transcriptome reprogramming takes place in the plant cell and expression of many defence related genes is activated. To determine whether exogenous application of PEP-E and PEP-I can modulate the expression of some PTI-related genes, Col-0 plants were treated with 5 μ M concentration of PEP-E and PEP-I. Expression of immunity markers *RBOHD* and *WRKY53* was evaluated at three different time points. Treatment with 1 μ M flg22 was also included as a defence inducer control.

As shown in Figure 4.19.A, in response to flg22 transcript accumulation of defence marker genes *WRKY53* and *RBOHD* reached to a maximum level of expression in Col-0 at 30 minutes. Treatment of Arabidopsis seedlings with PEP-E (5 μ M) do not induce the expression levels on the PTI responsive gene *WRKY53*. However, a slight transient induction of *RBOHD* was observed at 30 minutes (Figure 4.19B). As shown in Figure 4.19C a statistically significant induction for *WRKY53* was observed in response to PEP-I treatment at 60 minutes. *RBOHD* also showed a slight upregulation at 30 minutes and 60 minutes, respectively, although this induction was not statistically significant. Although these are very preliminary results, these data suggest that application of PEP-I could produce a slight

induction of defence related genes and PTI responses, although the induction is minor compared with the flg22 response.

Figure 4.19. Expression of PAMP-induced genes in response to flg22, PEP-E and PEP-I. (A) Expression levels of the PAMP-inducible genes *WRKY53* and *RBOHD* determined by qRT-PCR in 12-days-old WT seedlings after treatment with 1 μM flg22 (A) and 5 μM PEP-E (B) and PEP-I (C) at three different time points. *UBC21* (*At5g25760*) expression was used to normalize the transcript level in each sample. Values are represented as average $\pm$ SE relative expression to *UBC21* ($\pm$ SE) to mock treated plants ($n = 2$). Inverted triangles indicate statistically significant differences with time 0 min based on Student's *t*-test ($p < 0.05$). Data was obtained from one out of two experiments performed with similar results.

4.2. Identification and characterization of new components of the heterotrimeric G protein defence signalling.

Arabidopsis heterotrimeric G protein complex modulates PTI and regulates disease resistance to fungi and bacteria. Resistance analysis performed with mutants in the signalling components of this complex revealed, in most cases, an increased susceptibility to different pathogens in the single *agb1-2* and in the double mutant *agg1 agg2*, whereas the mutant *gpa1* presented values close to WT Col-0 (Torres *et al.*, 2013; Delgado-Cerezo *et al.*, 2012; Trusov *et al.*, 2006). Thus, the G $\beta\gamma$ seems to play a preeminent part in immunity. Pathways such as the salicylic acid, jasmonic acid, ethylene, abscisic acid and tryptophan-derived metabolites signalling were not impaired in *agb1-2* and *agg1 agg2* mutants, suggesting that G-protein-mediated resistance works independently of the classical defensive pathways (Delgado-Cerezo *et al.*, 2012). Moreover, mutants in the G β and G γ subunits display alterations in cell wall structure suggesting that cell wall remodelling mediated by heterotrimeric G protein could play an important part in this immune response (Delgado-Cerezo *et al.*, 2012).

Several extragenic dominant *suppressor of G-protein beta1* (*sgb*) alleles (*sgb1-sgb8*) that were able to rescue the hypocotyl length and apical hook opening phenotypes of *agb1-2* were identified (Friedman *et al.*, 2011). However, none of them suppressed *agb1-2* susceptibility to *PcBMM*. Therefore, we set to identify new molecular components involved in heterotrimeric G-protein-mediated defence signalling. A screening was performed to identify suppressors of *agb1-2* susceptibility to the necrotrophic fungus *PcBMM* (Escudero *et al.*, 2017). Four additional *sgb* mutants, *sgb10*, *sgb11*, *sgb12* and *sgb13* were identified based on their ability to fully rescue *agb1-2* susceptibility. However, as shown in Figure 4.20, none of the selected *sgb* mutants fully restored to WT levels the developmental phenotypes associated with the *agb1-2* mutation such as leaf size and morphology, plant height or flower architecture. These data suggest that the newly identified 4 *sgb* suppressors have minor or no role in developmental processes mediated by *AGB1*, while have a direct impact on the immune responses regulated by heterotrimeric G proteins.

Figure 4.20. Identification of *sgb* (*suppressor of G protein β 1*) mutants. Picture showing rosette of four-week-old plants (top) and six-week-old plants architecture (bottom) of Col-0, *agb1-2* and four *sgb10-13* mutants. All four *sgb* mutants were unable to restore the *agb1-2* developmental phenotypes to WT levels. (Escudero *et al.*, 2017).

In this thesis, we will focus on the identification of *sgb10* and *sgb11* and the characterization of the latter. To determine the genetic interaction between *sgb11* and *agb1-2*, *sgb11* single mutant was obtained by crossing *sgb11 agb1-2* with Col-0 WT plants. We found that the enhanced resistance of the *sgb11* mutant was linked to its characteristic developmental phenotype: dwarf and greener plants with reduced height and branching, and was independent of *AGB1*. In order to map the mutation, *sgb11* plants were crossed with La-0 to obtain a segregating F2 population. After a fine mapping, *sgb11* mutation was found on a region of 200 kb on chromosome 3, between the genetic markers *AthCDC2BG* (76,14 cM) and *nga707* (78,25 cM; Figure 4.20.A). Full genome sequencing revealed a G to A transition in the coding sequence of *ESKIMO1* (*ESK1*; *At3g55990*) gene. This mutation resulted in a premature stop codon (W261U) leading to a truncated 261 aa protein that lacked the DUF231 domain (domain of unknown function 231; Figure 4.20.B). DUF231 is present in the 46 members of a large protein family called TRICHOME BIREFRINGENCE LIKE (TBL) to which *ESK1* belongs (Bischoff *et al.*, 2010). *ESK1* has been described as key negative regulator of freezing, salt tolerance and water

economy (Bouchabke-Coussa *et al.*, 2008; Xin *et al.*, 2007) and encodes a plant specific polysaccharide O-acetyltransferase that is involved in xylan acetylation (Urbanowicz *et al.*, 2014).

To confirm that *sgb11* was a novel *ESK1* allele, *sgb11* mutants were crossed with *esk1-5* plants (SALK 089531; Bouchabke-Coussa *et al.*, 2008). As shown in Figure 4.20.C, fungal biomass quantification of F1 plants (*esk1-5*^{+/-} *sgb11*^{-/+}) and the *esk1-5* mutant showed a reduction in fungal biomass as compared to WT plants. Therefore, *sgb11* was renamed as *esk1-7* allele after these results. The resistance to *PcBMM* of *esk1-7* single allele was similar to that of *esk1-5* and *irx1-6* plants (Escudero *et al.*, 2017).

Next, we sought to identify *sgb10*, which fully rescued the *agb1-2* enhanced susceptibility to *PcBMM*. With that purpose, a F2 population of 1980 individuals was generated by crossing *sgb10* (Col-0) with La-0 plants. F2 population was identified over the lesion based phenotype. As shown in Figure 4.20.D, *sgb10* mutation was mapped to chromosome 3 between markers *cer451778* (BAC F28P10, 2 recombinants) and *cer479349* (BAC T5P19, 1 recombinant). Genomic DNA sequencing of *sgb10* plants led to identify the mutation responsible for the phenotype in a single G to A exchange in gene *At3g55270* (Figure 4.20.E). This gene codes for a MAP Kinase Phosphatase (MKP1) of 784 aa. The point mutation in *sgb10* introduces an early stop codon leading to the formation of a truncated protein of 252 aa. To confirm that *sgb10* is a novel *MKP1* allele, we crossed *sgb10* with the *mkp1-1* allele (Bartels *et al.*, 2009) and found that F1 and F2 plants were identical to the parental ones showing the characteristic lesion phenotype (Bartels *et al.*, 2009; data not shown). In addition, a resistance response analysis of *sgb10*, *mkp1-1* and the F1 plants (*mkp1*^{+/-} *sgb10*^{-/+}) was performed with the necrotrophic fungi *PcBMM*. As shown in Figure 4.20.F, F1 (*mkp1*^{+/-} *sgb10*^{-/+}) and the parental *sgb10* and *mkp1* mutants showed a significant reduction in fungal biomass as compared to WT plants. The resistance to *PcBMM* of these mutants was comparable to that of the resistant control *irx1-6*. Therefore, these results confirmed that *SGB10* is the kinase phosphatase *MKP1*.

Figure 4.20. Identification of *sgb10* and *sgb11* mutants. (A) and (D) Fine mapping of *sgb11* and *sgb10* mutations to chromosome 3, between the indicated markers. The number of genetic recombinants are indicated below the markers. (B) and (E) Gene model of *ESKIMO1* (locus *At3g55990*) and *MKP1* (*At3g55270*) showing the gene structure and different mutant alleles. (C) and (F) Quantification of *PcBMM* biomass at 5 dpi. Fungal Biomass was determined by qPCR quantification. Values are represented as the average $\pm$ SE (n=4) of the n-fold increase compared to WT plants (Col-0). Letters indicate values statistically different among genotypes (ANOVA; $p < 0.05$). *irx1-6* was included as resistant control. Letters represents statistically different values for each genotype (ANOVA; $p < 0.05$).

Protein phosphatases counteract the action of protein kinases in the reversible phosphorylation that regulate the activity of many proteins and contribute to the adequate

phosphorylation/dephosphorylation balance required for the optimal regulation of cellular processes (Luan *et al.*, 2003; Smith and Walker, 1996). Arabidopsis has three major classes of protein phosphatases, namely, Tyr-specific phosphatases (PTPs), Ser/Thr-specific phosphatases and dual-specificity (Ser, Thr/Tyr) protein phosphatases (DSPs; Keyse *et al.*, 2008). Dedicated MKPs belong to the subgroup of the DSP class and act as negative regulators of MAPKs signalling cascades (Bartels *et al.*, 2010; Camps *et al.*, 2000).

In *Arabidopsis thaliana*, five functional MPKs dephosphorylate the 20 MAPKs present and counterbalance their action (Bartels *et al.*, 2010). Thus, MPKs are pleiotropic and regulate many responses to biotic and abiotic stress. Particularly, MKP1 have been implicated in genotoxicity conferred by DNA-damaging agents and in the response to UV light, salinity, wounding and also pathogens (Bartels *et al.*, 2010). In the pathogen response, MKP1 interacts with MPK3, MPK4 and MPK6 and acts as a negative regulator of PTI suppressing ROS production, SA biosynthesis and the accumulation of defence marker genes (Anderson *et al.*, 2011; Bartels *et al.*, 2009). Accordingly, *mkp1* mutant plants present elevated SA levels, constitutive activated defences and enhanced resistance against virulent *P. syringae* (Anderson *et al.*, 2011; Bartels *et al.*, 2009). Although the initial *mkp1* mutant identified in *Arabidopsis Wassilewskija* (Ws) accession did not show any abnormal phenotypes under standard conditions (Ulm, R. *et al.*, 2001), the introgressed allele into Columbia accession displayed a slight dwarf phenotype with aberrant leaf development, long pedicels, early senescence, misshapen inflorescences and reduced fertility (Bartels *et al.*, 2009). Interestingly, these aberrant phenotypes are suppressed by *mpk3* and *mpk6* mutants and SA deficient lines (Bartels *et al.*, 2009).

Previous work in our laboratory have demonstrated that ROS production was impaired in *agb1-2* mutant plants in response to PAMPs (Torres *et al.*, 2013). Therefore, we assessed if *sgb10* mutation restored ROS production in *agb1-2*. ROS production in response to flg22 was evaluated in *sgb10* mutant plants and in *sgb10* in *agb1-2* background. Together with the controls, the already characterized *mkp1-1* mutant (thereafter *mkp1*) and the overexpressor line *35S::Pyo-MKP1* in *mkp1-1* background were also included in this analysis. Levels of ROS in *agb1-2* mutant is considerably

reduced compared to WT and ROS production is restored to levels similar to WT in *sgb10 agb1-2*. Moreover, both *sgb10* and *mkp1* show higher levels of ROS as compared to Col-0 plants whereas the complemented line 35S::Pyo-MKP1 (in *mkp1* background) also behaves similarly to Col-0 plants. Mutant line *rbohD*, lacking the functional NADPH oxidase responsible for most ROS produced in response to pathogens, are unable to produce ROS in response to flg22 (Figure 16; Viviana Escudero PhD thesis, 2014). Due to the enhanced ROS production observed in *mkp1/sgb10* mutants, we hypothesized that RBOHD could be a target of MKP1 phosphatase activity and that, in this way, this enzyme would regulate ROS production.

4.2.1. *rbohD* partially complements the small size displayed by *sgb10*.

To get insight into the possible regulation of RBOHD mediated by MKP1 phosphatase, we crossed *rbohD* with *sgb10* plants. Double mutants *sgb10 rbohD* were identified by PCR genotyping in the F2 generation and were further confirmed in the F3. During the identification process, we observed that the double mutant *sgb10 rbohD* plants displayed a slight increase in size compared to *sgb10* individual mutant. Therefore, we grew in parallel plants of the double mutant with Col-0 WT and the parental lines and we compared their size after 3, 4 and 5 weeks (Figure 4.21.A and B). *sgb10* plants were smaller than Col-0 throughout the period analysed whereas *rbohD* plants were comparable to the WT. Interestingly the double mutant plants *sgb10 rbohD* showed significant differences in size compared to *sgb10* mutants at 3, 4 and 5 weeks. This result suggests that the small size of *sgb10* is partially dependent on ROS produced by RBOHD.

Mutations in the SA pathway or the expression of *NahG* (a bacterial salicylate hydroxylase that degrades SA in plants, Delaney *et al.*, 1994) were shown to diminish the *mkp1* aberrant phenotypes (Bartels *et al.*, 2009). We wanted to test if, similarly to *mkp1*, alterations of the SA pathway also suppressed the *sgb10* aberrant phenotype. Therefore, we crossed *sgb10* to *NahG* and *sid2* (mutant in the *Isochorismate Synthase 1* gene -*ICS1*- required for SA biosynthesis in plant defence; Nawrath *et al.*, 1999). Combinatory lines *sgb10 NahG* and *sgb10 sid2* were identified by PCR genotyping in the F2

generation and further confirmed in the F3. Plants of the individual *sgb10*, *NahG* and *sid2* and double *sgb10 NahG* and *sgb10 sid2* together with Col-0 were grown in parallel and their size was assessed after 3, 4 and 5 weeks (Figure 4.22.A and B). Again, *sgb10* plants displayed a reduced size compared to Col-0 whereas *NahG* and *sid2* displayed a size comparable to the WT. Interestingly, both the double *sgb10 NahG* and *sgb10 sid2* displayed a very similar size to the individual *NahG* and *sid2*. This data confirm that abrogation of the SA pathway largely suppresses the small size phenotype displayed by *sgb10*, further confirming that *sgb10* is an allele of *mkp1*.

Figure 4.21. Partial suppression of *sgb10* small size phenotype by *rbohD*. (A) Image of 4-week-old plants of the listed genotypes. (B) Size (diameter) of plants of the listed genotypes at 3, 4 and 5 weeks after sowing. Data was obtained from one of the three independent experiments with similar results ($n=15$ for each experiment). Letters represents statistically different values for each genotype (ANOVA; $p < 0.05$).

A

B

Figure

4.22. Partial

suppression of *sgb10* small size phenotype lines defective in SA signalling. (A) Image of 4-week-old plants of the listed genotypes. (B) Size (diameter) of plants of the listed genotypes measured at 3, 4 and 5 weeks after sowing. Data was obtained from one of the three independent experiments with similar results (n=15 for each experiment). Letters represents statistically different values for each genotype (ANOVA; $p < 0.05$).

4.2.2. RBOHD is responsible for ROS in *sgb10* and *mkp1*.

To determine whether the enhanced ROS produced in *sgb10* and *mkp1* mutants in response to PAMPs is dependent on *RbohD*, a luminol assay in response to flg22 was performed with *sgb10 rbohD* and *mkp1 rbohD* plants identified and results were compared to the WT Col-0 and the individual parental

mutants. As shown in Figure 4.23.A, both *sgb10* and *mkp1* produced elevated levels of ROS in response to flg22 as compared to Col-0 plants. However, double mutants *sgb10 rbohD* and *mkp1 rbohD* are unable to produce ROS, similarly to the individual *rbohD*. To confirm this result, production of ROS was analysed in response to the fungal PAMP chitin in the same set of mutants. Again, *mkp1* and *sgb10* displayed higher levels ROS compared to Col-0, and *rbohD* suppressed this enhanced ROS production in *sgb10 rbohD* and *mkp1 rbohD* double mutants (Figure 4.23.B). These results indicate that the enhanced ROS production observed in *sgb10/mkp1* mutants is produced by the NADPH oxidase RBOHD.

To confirm the origin of ROS observed in *sgb10/mkp1* mutants, *sgb10* and *mkp1* were crossed to a line over-expressing *RbohD* (*35S::RBOHD*; Kadota *et al.*, 2014). Similarly, to the procedure described above for the crosses to *rbohD*, the combinatory lines were selected in the F2 by PCR genotyping and further confirmed in the F3. Once the homozygous lines were identified, a similar luminol-based assays in response to flg22 and chitin were performed with these combinatory lines *sgb10 35S::RBOHD* and *mkp1 35S::RBOHD* as well as with the control lines (Figure 4.23.C and D). *35S::RBOHD* plants produced ROS faster and to a higher level than WT compared to *sgb10* and *mkp1* mutants. Interestingly, *sgb10 35S::RBOHD* and *mkp1 35S::RBOHD* produced higher levels of ROS in comparison to the parental lines. These results further confirm that the higher levels of ROS produced in *sgb10* and *mkp1* mutant plants in response to PAMPs is *RbohD*-dependent.

Figure 4.23. Enhanced ROS production observed in *sgb10/mkp1* is *RbohD* dependent. H₂O₂ production after PAMP treatment measured in a luminol-based assay using leaf discs from 4-week-old plants of the listed genotypes. In (A) and (C) discs were treated with 100 nM flg22, whereas in (B) and (D) the elicitor was 1 mg/ml of chitin. Relative light units (RLU) were measured over a period of 40 minutes. Values are means $\pm$ SE (n = 12). The experiment was repeated 3 times with similar results.

4.2.3. *sgb10/mkp1* is epistatic over *rbohD* and *35S::RBOHD* on the response to *P. cucumerina*.

sgb10 and *mkp1* are more resistant to the necrotrophic *PcBMM* than Col-0 (Viviana Escudero, PhD Thesis, 2014), indicating that *MKP1* is a negative regulator of resistance. In the other hand, *rbohD* is slightly more susceptible to this fungus than Col-0 (phenotype that is enhanced in the double mutant

rbohD rbohF), which suggests that ROS produced by RBOHD contribute to resistance to this necrotroph (Torres *et al.*, 2013). We performed an epistasis assay by analysing *PcBMM* growth and symptoms in the *sgb10 rbohD* and *mkp1 rbohD* double mutants. We also included in this analysis the combinatory lines *sgb10 35S::RBOHD* and *mkp1 35S::RBOHD*. Apart of Col-0 and the parental lines, we used as a control the hyper susceptible *agb1-2*. We inoculated spores of the fungus and we quantified fungal biomass at 5 dpi by qPCR. As shown in Figure 4.24.A, biomass quantification revealed reduced fungal growth in *sgb10* and *mkp1*, whereas *rbohD* displayed enhanced growth compared to the WT Col-0 plants. However, susceptibility of the double mutant *sgb10 rbohD* and *mkp1 rbohD* to *PcBMM* was comparable to that of the parental lines *sgb10* and *mkp1*, supporting less growth than Col-0. Intriguingly, *35S::RBOHD* showed even more fungal growth than *rbohD*. And again the combinatory lines *sgb10 35S::RBOHD* and *mkp1 35S::RBOHD* displayed reduced fungal growth than Col-0 and comparable to the levels of *mkp1* and *sgb10* parental lines. Thus, quantification of fungal biomass suggests that *mkp1/sgb10* is epistatic over *rbohD* and *35S::RBOHD* in the control of Arabidopsis resistance response to *PcBMM*. However, a careful analysis of the disease rating does not fully back up the results obtained in the quantification of biomass. At 10 dpi *sgb10* and *mkp1* displayed comparable disease rating than Col-0 (Figure 4.24.B). However, the presence of these mutants in the combinatory lines reduced the disease rating displayed by the individual *rbohD* and *35S::RBOHD*. Macroscopic disease symptoms observed at 10 dpi corroborate these results (Figure 4.24.C and D). The fact that *sgb10* and *mkp1* display leaf lesions, probably due to SA accumulation (Bartels *et al.*, 2009) , we speculate that interferes with the visual evaluation of the disease symptoms, which are undifferentiable from necrotrophic spots caused by the fungus *PcBMM*.

Taken all together, these results indicate that there is an epistatic, genetic interaction between *mkp1/sgb10* and *rbohD* in the control of Arabidopsis resistance response to *PcBMM*. This data suggests that MKP1 and RBOHD are components of the same immune pathway. And this fact supports the idea that RBOHD could be dephosphorylated by MKP1.

Figure 4.24. *sgb10/mkp1* are epistatic over *rbohD* and *35S::RBOHD* on the response to *P. cucumerina*. (A) *PcBMM* biomass quantification on the listed genotypes by qPCR at 5 dpi. Values are represented as the average ($\pm$ SE) of the n-fold increased expression compared to WT plants (Col-0). Letters indicate values statistically different among genotypes (ANOVA; $p < 0.05$). (B) Average of disease ratings (DR $\pm$ SE) of the indicated genotypes at 8 dpi. DR varies between 0 (no symptoms) and 5 (dead plant). (C) and (D) Macroscopic disease symptoms of the indicated genotypes at 10 dpi.

DISCUSSION

DISCUSSION

The Arabidopsis MAP3K YDA has been described to work together with the RLK ER in a signalling module to regulate several crucial plant developmental processes including stomatal formation and patterning, embryo polarity and floral organ identity (Lukowitz *et al.*, 2004; Bergmann *et al.*, 2004). YDA N-terminal domain acts as a negative regulatory domain, as deletions in that region result in constitutive activation of the MAP3K activity (*CA-YDA*), complementing the *yda1* lethal-associated defective developmental phenotypes (Bemis *et al.*, 2013; Meng *et al.*, 2012; Lukowitz *et al.*, 2004). Remarkably, we found that *YDA* also modulates plant immunity (Sopeña-Torres *et al.*, 2018). We identified a viable allele of *YDA* (*elk2/yda11*), whose defense responses against fungal pathogens as *PcBMM*, *B. cinerea* and *F. oxysporum* were abolished. Furthermore, constitutive activation of *YDA* resulted in a broad-spectrum disease resistance phenotype to pathogens with different lifestyles, as fungi, bacteria and oomycetes. These data suggest that plants have co-opted this developmental signalling ER-YDA module to generate a new surveillance mechanism to endow immunity, as stomata are natural pathogen entries.

The nature of the cues activating the ER-YDA immune responses remains to be determined. Thus, a transcriptomic analysis of *CA-YDA* and WT plants was performed to unravel the molecular mechanism underlying *CA-YDA* broad spectrum disease resistance. *CA-YDA* triggers the transcriptomic reprogramming of more than 500 genes. Remarkably, among the 360 upregulated genes, a significant percentage overlap with sets of defence genes induced in WT plants upon infection with *PcBMM* (116 genes, $p < 9.84 \times 10^{-48}$), *G. cichoracearum* (21 genes, $p < 4.47 \times 10^{-15}$) or *G. orontii* (58 genes, $p < 3.68 \times 10^{-36}$; Sopeña-Torres *et al.*, 2018). However, no significant enrichment of genes regulated by the canonical immune responses mediated by the phytohormones salicylic acid, jasmonic acid or ethylene was identified. Furthermore, a significant core of genes coding for proteins associated with cell wall biosynthesis and remodelling, such as *FUT6*, *FUT8*, *EXT3*, *CESA9* and pectin methylesterase inhibitors were also upregulated in *CA-YDA* plants. All these data indicate that a novel immune mechanism could

be mediating the broad enhanced resistance displayed by the *CA-YDA* plants in which plant cell wall has a relevant contribution. Consistent with this hypothesis, a significant percentage of genes upregulated in *CA-YDA* plants were classified as unknown (17%).

In this work we have focused on the study of 8 genes with unknown function coding for putative *SMALL SECRETED PEPTIDES* (*SSPs*). Recent studies have provided strong evidences that plant small secreted signalling peptides play a crucial role in short-distance signalling as peptide hormones, mediating developmental processes and responses to biotic stresses (Zhang *et al.*, 2016). Indeed, some extracellular peptides secreted upon pathogen recognition, such as PROPEP or PIP peptides, act amplifying the immune responses (Hou *et al.*, 2014; Huffaker *et al.*, 2006). In fact, similarly to genes encoding PROPEP and its corresponding receptor AtPEP RECEPTOR 1 (*PEPR1*), *SSPs* expression was induced by a variety of pathogens and PAMP treatments such as flg22 and HrpZ, suggesting a putative role in immunity (Table 4.1 and Table 4.2; Huffaker and Ryan, 2006; Zipfel *et al.*, 2004). Using a reverse genetic approach, we could assess the contribution of the *SSPs* to the resistance response to the necrotrophic fungus *PcBMM*, the bacteria *Pto* DC3000 and the oomycete *H. arabidopsidis*. Our results showed that impairment of *SSP1*, *SSP2*, *SSP6*, *SSP7* or *SSP8* leads to an enhanced susceptibility to all three pathogens evaluated. Even more, the contribution of *SSP1* and *YDA* to the defence responses of *Arabidopsis* against *Cucumber mosaic virus* strain LS seems also to be critical. Single mutants *ssp1* and *yda11* exhibited higher viral RNA accumulation than WT plants, and *yda11* plants showed a significant biomass reduction due to the virus infection. This is the first time that involvement of *YDA*–mediated immune pathway in *Arabidopsis* virus defence responses is described. Therefore, these data strengthen the relevant role of this previously uncharacterized immune pathway regulated by ER-*YDA*. By contrast, *ssp7* mutants were more resistant to CMV, as these plants showed lower viral accumulation and biomass increase than WT plants. However, the mechanism underlying this resistance is unknown and more studies should be performed to unveil whether the resistance is due to the lack of a docking recognition site for CMV or that *ssp7* plants may have other compensatory mechanisms different than resistance.

Thus, based on these data, it is plausible that constitutive expression of some of these extracellular peptides would contribute to the enhanced disease resistance displayed by *CA-YDA* plants. Although all *SSP* genes were induced after pathogen inoculation or elicitor treatments, only *SSP1*, *SSP2*, *SSP6*, *SSP7* and *SSP8* had an impact on *Arabidopsis* defence responses against the pathogens evaluated. Therefore, we can conclude that, of the putative small peptides identified in this work, *SSP1*, *SSP2*, *SSP6*, *SSP7* and *SSP8* have a contribution to the plant defence mechanisms, while *SSP3* seems to be not involved. We cannot rule out that *SSP4* or *SSP5* are also required for immunity, as these two genes have paralogous in *Arabidopsis* genome (*SSP5* with At5g01015 and *SSP4* belongs to a family of 10 members; pATsi; Ambrosino *et al.*, 2015) and functional redundancy could mask the single mutant phenotypes. To address this question, combinatory mutants should be analysed in further studies.

We speculate that enhanced disease resistance phenotype of *CA-YDA* plants could be due to the role of these *SSPs* as secondary endogenous danger signals (Gust *et al.*, 2017). These types of molecules are produced as larger pro-proteins that are usually processed by specific peptidases rendering small biologically active peptides derived from its C-terminal region, as PROPEP1 and PEP1 (Huffaker *et al.*, 2006). Synthetic peptides corresponding to the C-terminal domains of *SSP1* (PEP-A and PEP-E), *SSP2* (PEP-O) and *SSP7* (PEP-I) were generated based on the sizes of different characterized families of peptide elicitors (Zhang *et al.*, 2016). However, none of the predicted C-terminal sequences of the synthetic *SSPs* aligned with any of the known small signalling peptide families involved in immunity (PEP, PIP) or even development (IDA), suggesting that we may be dealing with novel unknown peptides (Figure 5.1). Only PEP-A had at the C-terminal two amino acids that are highly conserved in the PIP family (GH), but the other conserved signatures are not present in this peptide. Interestingly, all the synthetic peptides designed in this work display some amino acids such as cysteine and proline, which are frequently present in mature small signalling peptides. However, additional work that includes the overexpression of these putative peptides and strategies to define its active size need to be performed to confirm the role of these putative small peptides in immunity.

	1	10		1	10	20	
PIP1	F	QRLA	SGPS	PRGR	GH		
PIP2	R	FKH	SGPS	SPGP	GH		
PIP3	E	YKHS	SGPS	TSGP	GH		
PIPL1	F	GRAND	DSGPS	SPGV	GH		
PIPL2	G	RKLA	SGPS	RRGC	GH		
PIPL3	M	HMTMA	SGPS	RRGA	GH		
PIPL4	M	RILAS	SGPN	KRGR	GH		
PIPL5	A	YRLAS	SGPS	SRGR	GH		
PIPL6	A	FRLAS	SGPS	RRGR	GH		
PIPL7	G	RKLV	SGPS	RSSC	GH		
PIPL8	A	FRLAS	SGSS	RRGR	GH		
PEP-A	L	HLSPP	PPQ	RSFK	GTNEP	GH	
PEP-E	L	CLPMH	VFVI	FYSLS	NGRYNK		
PEP-I	N	RASS	STST	TTFAV	YAGVQ	SPVSR	QVVT
PEP-O	...	SPFER	SFAAV	SPFCS	QAGGD	GMEI	N
PEP1	A	TKVKAK	QRGKE	KVSS	GRPG	QH	HN★
PEP2	D	NKAKS	KRDKE	KPSS	GRPG	QT	HN
PEP3	E	I KARG	KNKT	KTPSS	GKGG	KH	HN
PEP4	G	LPGKK	NVLK	KSRESS	GKPG	GT	HN
PEP5	S	LNVMR	KGI	RKQP	VSS	GKRG	GVN
PEP6	I	TAVL	RRRPR	PPPYSS	GRPG	QN	HN
PEP7	V	SGNVA	AARKG	KQQTSS	GKGG	GT	HN
IDA	P	I PPS	A	PSKR	HN	HN	HN★
IDL1	L	VPPS	G	PSMR	HN	HN	HN★
IDL2	P	VPAS	G	PSRK	HN	HN	HN
IDL3	P	VPT	S	PSRK	HN	HN	HN
IDL4	P	VPAS	A	PSRK	HN	HN	HN
IDL5	P	I PHS	A	PSRK	HN	HN	HN

Figure 5.1. Mature small signalling peptides have conserved amino acids in the C-terminal. Sequence alignment of mature small signalling peptides involved in immunity (PIP and PEP family), development (IDA family members) and the synthetic mature peptides from the SSPs (PEP-A, PEP-E, PEP-I, PEP-O). Red colour and squares indicate conserved amino acids in the different families. Peptides with known structures bound by receptors are depicted by green stars following their last residues (Adapted from Zhang *et al.*, 2016).

Arabidopsis genome has been proposed to contain more than 1000 extracellular peptides (The Arabidopsis Genome Initiative, 2000). However, very few peptides have been described as ligands regulating PTI (Hou *et al.*, 201; Huffaker *et al.*, 2006). To demonstrate the role of the selected SSPs as putative signalling DAMPs or elicitors, the ability of PEP-A, PEP-E, PEP-I and PEP-O to induce PTI was assessed. Plants elicited with PEP-A (derived from SSP1) or PEP-O (derived from SSP2) had no response at all, even at higher concentrations, indicating that these peptides are not able to trigger PTI-responses or that the size is not adequate. Only PEP-I (derived from the C-terminal of SSP7) induced consistently an early influx of Ca^{2+} . Remarkably, PEP-I also induced the transient activation of the MAPK cascade. Thus, a processed peptide from SSP7 could work as a DAMP and activate immunity. However, the level of induction of these responses is lower than the level observed with the control peptides flg22 and PEP1. This fact could be related to a less efficient recognition because PEP-I would not have the optimum size given that the structure of elicitors is critically important (Tang *et al.*, 2015; Felix *et al.*, 1999). Minor alterations in the features of the signalling peptides have a big impact on its detection by the corresponding receptor. For example, deletion of only the last residue of AtPEP1

(Asparagine) significantly compromised AtPEP1 interaction with its receptor PEPR1 (Tang *et al.*, 2015). Also, binding of flg22 to FLS2 showed a strong drop in activity on shortening the amino acids from N-terminal or C-terminal ends (Felix *et al.*, 1999). PEP-E (variant one of SSP1) was also able to induce the Ca^{2+} influx, although the response was inconsistent. The treatment with PEP-E seemed to slightly activate MAPK phosphorylation at 5 and 20 minutes compared to mock treated plant, but the impact on gene expression was not conclusive and has to be reevaluated. Alternatively, to the proteolytic cleavage some small secreted peptides require other posttranslational modifications, as proline hydroxylation, that has a clear impact on their biological activity. Therefore, a more accurate characterization of the SSP peptides would be necessary in order to identify the proper mature sequences and the putative post-translational processing.

Of note, none of the chemically synthesized peptides could trigger a consistent ROS production in WT plants. The fast accumulation of ROS is recognized as one of the hallmarks of plant defence activation (Torres *et al.*, 2006). However, this feature varies between treatments. Whereas flg22 treatment produces high level of ROS, other PAMPs such as PEP1, chitin and OGs produce a reduced oxidative burst (Korner *et al.*, 2013; Galletti *et al.*, 2008). This could be the case for the SSPs, which trigger some early PTI events as Ca^{2+} efflux (PEP-I), or MAKP activation (PEP-E and PEP-I) but no a consistent oxidative burst.

As peptide hormones are usually perceived by membrane-localized receptors, we also selected some genes upregulated in CA-YDA plants coding for *PUTATIVE RECEPTOR PROTEINS (PRP1-9)*, in an effort to identify the SSPs-receptor kinase pairs. All selected *PRPs* modulated their expression upon pathogen or elicitor treatments (Table 4.4). We have demonstrated that *prp1*, *prp4*, *prp5* and *prp7* mutants are highly susceptible to some of the evaluated pathogens. Notably, *prp1* plants exhibited an enhanced susceptible phenotype to fungus, bacteria, oomycetes and CMV, highlighting the relevant role of this putative receptor in immunity. As these pathogens are so different it is difficult to think in a common PAMP to be recognized by PRP1. In fact, it would be plausible that PRP1 would act as receptor of an endogenous danger signal released by plant cells upon cell damage caused by any of

these pathogens. Indeed, PRP1 belongs to the RLK superfamily protein that includes WALL-ASSOCIATED (RECEPTOR) KINASE 1 (WAK1) and LRK10L2 (Sakamoto *et al.*, 2012). WAK1 transcripts accumulate to high levels upon pathogen infection or following treatment with SA (He *et al.*, 1998). A more direct role in pathogen resistance has been shown for WAKL22/RFO1 (RESISTANCE TO FUSARIUM OXYSPORUM 1), which confers broad spectrum resistance against *Fusarium* species (Diener and Ausubel, 2005). PRP1 has two WAK-like domains in its extracellular region. These domains have been implicated in sensing DAMPs derived from the cell wall, specifically pectin fragments termed OGs (Brutus *et al.*, 2010; Decreux and Messiaen, 2005). All these results are highly indicative of a role of PRP1 as a cell wall sensor, whose activation would lead to trigger new immune responses. Likewise, PRP5 and PRP7 have ginkbilobin2 (Gnk2) domains in their extracellular region. These cysteine-rich motifs have been involved in monosaccharide recognition and function as mannose-binding lectins (Miyakawa *et al.*, 2014). Interestingly, *SSP7* was found to be co-expressed with *PRP5* (based on ATTED II data; Aoki *et al.*, 2016), indicating that both could work in the same signalling pathway. Supporting this idea both single mutants are more susceptible to *PcBMM*. PRP5 seems to be located in plasmodesmata (Lee *et al.*, 2011), however more assays need to be performed to demonstrate that *SSP7* and PRP5 co-localize. Altogether, this data suggests that some of these PRPs, as PRP1, PRP5 or PRP7, might act as cell wall sensors, while others, as PRP4, would perceive secreted signalling peptides. Recognition of putative DAMPs could activate a positive feedback loop that would be responsible for the enhanced disease resistance displayed by *CA-YDA* plants.

YDA-ER signalling pathway also regulates stomatal patterning and organ development. Moreover, ER pathway has been recently demonstrated to be the target of bacterial effector proteins to favour stomatal development and bacterial colonization (Meng *et al.*, 2015). Stomatal density and patterning is governed by members of the EPF family (Lau and Bergmann, 2012; Shimada *et al.*, 2011; Abrash and Bergmann, 2010; Wang *et al.*, 2007). However, the small secreted peptides EPF1 and EPF2 are only required to trigger the ER-YDA developmental pathway, as these peptides have no impact on the activation of ER-YDA –mediated immune responses (Jordá *et al.*, 2016). Our analyses indicate that

only SSP3 had an impact on stomatal development, as *ssp3* plants had higher stomata index than WT plants. This points to a specific role in development of this peptide.

As it has been previously described, constitutive activation of the MAP3K YDA results in alterations of the cell wall integrity and composition (Sopeña-Torres *et al.*, 2018). Indeed, cell wall extracts derived from CA-YDA plants are able to trigger responses to damage in WT plants, suggesting the presence of novel or enriched putative DAMPs in these fractions (Sopeña-Torres *et al.*, 2018). This in line with the emerging functions of wall integrity and plant wall-derived DAMPs in the regulation of immune responses (Bethke *et al.*, 2016; Hamann *et al.*, 2014). In fact, we hypothesized that changes in the cell wall of CA-YDA plants could be sensed by PRP1 leading to the transcriptional upregulation of secondary endogenous signals. The same thing could happen with other SSPs and their corresponding PRP-pairs. The released and processed peptides, in response to a primary endogenous danger signal, would act as modulators of plant immune responses (Figure 5.2).

Besides the transcriptomic analysis on plants displaying enhanced resistance, an alternative approach to dig for new components in immunity is the identification of suppressors of an enhanced susceptibility phenotype. *agb1-2* displays enhanced susceptibility to many pathogens tested (Torres *et al.*, 2013; Klopffleisch *et al.*, 2011; Llorente *et al.*, 2005). Thus, we performed a suppressor screening to revert its enhanced susceptibility to *PcBMM* and thus identifying new signalling elements of the heterotrimeric G protein defence signalling. In this line of work, four new components *sgb10-sgb13* (*suppressor of G protein beta1*) were identified based on their ability to rescue susceptibility of the G β subunit mutant, *agb1-2*, to *PcBMM*. Previous suppressors *sgb1-sgb9* were identified based on their capacity of suppressing sugar hypersensitivity of the *agb1-2* mutant.

Interestingly, these previous identified mutants were not related to the plant defence response, which indicates that the role of AGB1 in developmental pathways and in immunity is mediated by different elements. The previous analysis of the *ssps* and *prp1* mutants, which did not display altered developmental features compared to Col-0 WT (with the only exception of *ssp3*), also suggests that

ER-YDA pathways mediating development and immunity involve different signalling components. This data is in line with recently established role of *CLAVATA2* (*CLV2*) in distinct physiological programs, including plant development and innate immunity to microbe and nematode infections (Pan *et al.*, 2016).

Figure 5.2. Model of the non-canonical immune responses activated by the ER-YDA signalling pathway. Model depicting ER-YDA function in stomatal patterning, immunity response. The immune pathway regulated by ER-YDA controls resistance to pathogens by release of antimicrobial compound and modification of cell wall. Putative DAMPs released from cell wall are perceived through ER or other PRPs which leads to the activation of an immunity amplification loop. Overexpression of SSPs and corresponding PRPs would contribute to amplify plant immune responses. Adapted from Jordá *et al.*, 2016 and Sopeña-Torres *et al.*, 2018.

One of the suppression mutants, *sgb11*, is a new mutant allele of *ESKIMO1*, which encodes a plant specific polysaccharide *O*-acetyltransferase that is involved in xylan acetylation (Escudero *et al.*, 2017; Urbanowicz *et al.*, 2014). Arabidopsis G protein mutant *agb1-2*, as well as double mutant *agg1 agg2*, showed minor alterations in cell wall composition, i.e., putative modifications of xyloglucan or xylan structures compared with WT, that lead to enhanced susceptibility (Delgado-Cerezo *et al.*, 2012; Sánchez-Rodríguez *et al.*, 2009). *ESK1* allele *sgb11* showed enhanced resistance to *PcBMM*. This is in agreement with previously described *irx1-6* (mutant impaired in the biosynthesis of cellulose in

secondary cell walls) that shows enhanced resistance to *PcBMM*, *B. cinerea*, *G. cichoracearum* and *H. arabidopsidis* (Delgado-Cerezo *et al.*, 2012; Hernandez-Blanco *et al.*, 2007). However, *ESK1* null allele *sgb11/esk1-7* restores to WT levels the enhanced susceptibility of *agb1-2* to the necrotrophic fungus *PcBMM* but not to the bacterium *Pto* DC3000 or to the oomycete *H. arabidopsidis*, suggesting that this may be a specific function of this allele (Escudero *et al.*, 2017). The *esk1-7* allele seems to specifically affect disease resistance and seedling development responses, but it does not have any impact on other developmental phenotypes modulated by AGB1, which is indicative of the diverse signalling requirements for different functions of this protein (Escudero *et al.*, 2017).

Other suppression mutant, *sgb10*, was also identified and characterized as a novel allele of MAP kinase phosphatase *MKP1*. Under pathogen attack, *MKP1* interacts with different elements of the MAPK cascade, including *MPK3*, *MPK4* and *MPK6*, and acts as negative regulator of *PTI*, by suppressing ROS production, SA biosynthesis and the accumulation of defence marker genes (Anderson *et al.*, 2011; Bartels *et al.*, 2009; Katou *et al.*, 2007). However, *MKP1* acts as positive regulator in DNA damage response since *mkp1* is hypersensitive to genotoxic stress conferred by several DNA-damaging agents, but it is more resistant to abiotic (salinity) and biotic (*P. syringae*) stresses (Bartels *et al.*, 2009; Ulm *et al.*, 2001). We observed that *mkp1/sgb10* showed elevated levels of ROS. RBOHD is the main NADPH oxidase responsible for most ROS in Arabidopsis (Torres *et al.*, 2005). We speculate that RBOHD might be a target of *MKP1* under pathogen attack and that this phosphatase could contribute to the regulation of ROS production. In line with this hypothesis, we demonstrated that elevated levels of ROS in *sgb10/mkp1* is dependent on ROS produced by RBOHD, as double mutant *sgb10 rbohD* and *mkp1 rbohD* showed impairment in ROS production (Figure 4.24). Furthermore, we observed that the characteristic small size phenotype of *sgb10* is partially complemented by absence of ROS production in *rbohD*, as double mutants *sgb10 rbohD* showed significant increase in size compared to parental *sgb10/mkp1*. This data is in line with the idea that RBOHD could be a direct target of *MKP1*. Alternatively, MKPs such as *MPK3/MPK6* that have been proposed to work upstream of RBOHD (Zhang *et al.*, 2007) could be the direct target for dephosphorylation. However, the fact that *flg22*-

induced activation of these MKPs was not affected in *rbohD* mutant seem to suggest that they may belong to different pathway (Xu *et al.*, 2014). We have shown that removal of SA or alteration of SA signalling, that were shown to lessen the *mkp1* aberrant phenotype (Bartels *et al.*, 2009), restored almost to WT levels the small size phenotype of *sgb10*. This suggests that MKP1 may be targeting a wide array of developmental and defence pathways. However, SA signalling has also been often related to ROS signalling, particularly in defence response and cell death (Pogany *et al.*, 2009; Torres and Dangl, 2005). Therefore, these apparently broad phenotypes could be related to the same signalling events.

The hypothesis of MKP1 and RBOHD functioning in the same pathway was further corroborated by the demonstration that *sgb10/mkp1* showed epistatic interaction over the *rbohD* mutant in the resistance response against *PcBMM*, where *sgb10/mkp1* suppressed the enhanced susceptibility displayed by *rbohD* (Figure 4.25). Interestingly, *35S::RBOHD* plants also displayed an enhanced *PcBMM* growth that is suppressed by *sgb10/mkp1*. The fact that both, suppression of ROS production in the *rbohD* and enhanced ROS in its overexpression line, increase susceptibility is counterintuitive and seems to indicate that alteration of ROS balance is affecting resistance. The explanation could be in the fact that these reactive species interact with many signals that could counterbalance their alteration (Torres, 2010). In fact, *rbohD* (and *rbohF*) contribute to resistance to some pathogens but promote susceptibility to other (Marino *et al.*, 2012). Also, *RbohD* and *RbohF* act as positive or negative regulators of cell death in different contexts (Marino *et al.*, 2012; Torres *et al.*, 2002). And it has been proposed that RBOHD triggers death in cells that are damaged by infection but simultaneously inhibits death in neighbouring cells through the suppression of free salicylic acid and ethylene levels (Pogany *et al.*, 2009). All these data support the idea that unbalancing of ROS levels have a great impact on the immune responses. Together, these results suggest that MKP1 could play a role in regulation of ROS production by RBOHD, although this protein could be either directly modified by MKP1 or a downstream target of other regulatory proteins dephosphorylated by MKP1 (Figure 5.3).

Figure 5.3. Model of MKP1 mediated regulation of RBOHD under pathogen attack. Proposed model depicting activation of RBOHD by phosphorylation through MAPK cascade and BIK1 upon pathogen perception and negative regulation through MKP1. Several components of MKP1 mediated regulation of RBOHD, MAPK, and other cellular events, remain to be elucidated.

In summary, we have developed two approaches to advance in knowledge of the elements leading to plant immunity. In the first approach, through a transcriptomic analysis of the *CA-YDA* plants we have identified elements that are upregulated and that could explain the enhanced resistance deployed by these plants. Among the up-regulated genes at higher level we identified several with short coding sequence and we speculate that some of them may code for peptides that could act as DAMPs. In addition, some genes coding for putative receptors are among the up-regulated genes. The characterization of mutant plants in these *SSPs* and *PRPs* indicate that some of these peptides and receptors have an important contribution to immunity, contribution that remains unique and applicable to the *CA-YDA* enhanced resistance. *SSPs* might be working as elicitors or immunity amplifier, as for example *AtPEP1*, being perceived by upregulated *PRPs*. This back up the hypothesis

that they could conform a positive feedback loop working through the ER/YDA pathway with the release of DAMPs upon pathogen perception that would be detected by surface receptors that activate signalling leading to enhanced immune responses. These new elements could comprise a novel immune surveillance system at the cell surface distinct from the canonical immune pathways mediated by previously described PRRs and plant defence hormones. In the second approach, the characterization of suppressors of *agb1-2* enhanced disease susceptibility points also to additional elements contributing to heterotrimeric G protein mediated immune signalling. In particular, MKP1 could act as a negative regulator of plant immune responses, and we speculate that RBOHD would be a downstream target of this phosphatase to regulate ROS signalling. The characterization of SSPs, PRPs and MKP1 would contribute to further understanding the immunity pathways and expand our current knowledge on plant immunity.

CONCLUSIONS

CONCLUSIONS

1. SSP1, SSP2, SSP6, SSP7 and SSP8 play a relevant role in Arabidopsis immune response against pathogens with different lifestyles, based on the resistance response analyses on *ssp* mutants.
2. PRP1, PRP4 and PRP7 are important mediators of Arabidopsis immune response against different pathogens according to resistance response analyses on *prp* mutants.
3. With the exception of SSP3 that has a clear role on stomata patterning, neither these putative peptides nor receptor like kinase PRP1 seem to have an important regulatory function in plant architecture and stomatal development.
4. Among the synthetic peptides derived from these SSPs, PEP-I can trigger early Ca^{2+} influx and slight MAPK activation, therefore this peptide could act as a DAMP initiating early immune responses
5. The allelism tests confirm that *SGB10* and *SGB11* genes correspond to *MKP1* (At3g55270) and *ESKIMO1* (At3g55990), respectively.
6. MKP1 1 is a general component of innate immunity, as *sgb10/mkp1* not only suppress *agb1-2* susceptibility to *PcBMM* but also displays enhanced disease resistance to the bacteria *Pto*.
7. RBOHD could be a potential downstream target of MKP1.
8. Developmental phenotype of *sgb10/mkp1* is dependent on SA accumulation and ROS production.

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