University of São Paulo
"Luiz de Queiroz" College of Agriculture

Subcellular dynamics of the endogenous elicitor peptide *At*Pep1 and its receptors in *Arabidopsis*: implications for the plant immunity

Fausto Andres Ortiz-Morea

Thesis presented to obtain the degree of Doctor in Science.
Area: Genetics and Plant Breeding

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“You never know what is around the corner. It could be everything. Or it could be nothing. You keep putting one foot in front of the other, and then one day you look back and you have climbed a mountain”

Tom Hiddleston

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RESUMO

Dinâmica subcelular do peptídeo endógeno *At*Pep1 e seus receptores em *Arabidopsis*: implicações na imunidade de plantas

Neste trabalho, foi investigada a dinâmica subcelular do peptídeo elicitor de planta *At*Pep1 e suas implicações nas respostas de defesa. Primeiramente, é fornecida uma introdução do sistema imune inato de plantas com ênfase na imunidade ativada por moléculas elicitoras derivadas de organismos invasores ou da mesma planta, após seu reconhecimento por receptores localizados na membrana plasmática (PTI responses). Peptídeos endógenos que têm sido reportados em *Arabidopsis* como ativadores de PTI são descritos, dando especial destaque para o peptídeo *At*Pep1 e seus receptores PEPRs. O tráfego de endomembranas em plantas é introduzido, abrangendo as vias de internalização, endocitose mediada por proteínas clathrinas (CME) e endocitose mediada por receptor (RME). No capítulo seguinte, foram avaliadas estratégias para o estudo *in vivo* da dinâmica subcelular do *At*Pep1. Para isso a proteína precursora do *At*Pep1 (PROPEP1) foi fusionada a GFP e sua localização visualizada, encontrando que PROPEP1 é associado com o tonoplasto e acumula dentro do vacúolo, fato que sugere uma função de armazenamento do PROPEP1 para esta organela, desde onde é liberado em caso de uma situação de perigo dando origem ao *At*Pep1. Adicionalmente, foram produzidas versões biologicamente ativas do *At*Pep1 marcado com fluróforos. No capítulo três foram combinados genética clássica e genética química com visualizações *in vivo* para estudar o comportamento de um *At*Pep1 bioativo e marcado fluorescentemente na células meristemática da ponta da raiz de *Arabidopsis*, sendo encontrado que *At*Pep1 se liga rapidamente na membrana plasmática numa forma dependente de receptor. Em seguida, o complexo *At*Pep1-PEPR foi internalizado via CME e transportado para o vacúolo, passando através do endossomo primário e secundário. Quando o funcionamento da CME foi comprometido, as respostas ao *At*Pep1 também foram afetadas. Estes resultados fornecem a primeira visualização *in vivo* de um peptídeo de sinalização em plantas, mostrando sua dinâmica e destino intracelular. O papel regulatório durante as respostas induzidas pelo *At*Pep1 do co-receptor BRI1-associated kinase 1 (BAK1) foram investigadas (Capítulo quatro). Nossos resultados confirmaram que BAK1 interage com PEPRs numa forma dependente do ligante e indicam que BAK1 modula sinalização e endocitose do *At*Pep1, no entanto quando ausente, BAK1 pode ser substituído por seus homólogos SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE os quais poderiam ter funções adicionais durante as repostas induzidas pelo *At*Pep1. Eventos de fosforilação após a formação do complexo PEPR-BAK1 parecem ditar as bases moleculares da internalização e sinalização do *At*Pep1. Finalmente, são discutidos os resultados encontrados nesta pesquisa numa perspectiva geral, destacando a relevância destas descobertas na área de pesquisa em que estão inseridos, o potencial que representa o uso de ligantes marcados fluorescentemente como ferramenta para o estudo de complexos entre ligante-receptor, a disponibilidade do sistema *At*Pep1-PEPRs como modelo de estudo da endocitose em plantas e sua relação com sinalização, e os futuros desafios na área.

Palavras-chave: Peptídeos de sinalização; Receptores reconhecedores de padrões; PTI; *At*Pep1 – PEPR; Endocitose

ABSTRACT

Subcellular dynamics of the endogenous elicitor peptide *At*Pep1 and its receptors in *Arabidopsis*: implications for the plant immunity

This work investigated the subcellular dynamics of the plant elicitor peptide *At*Pep1 and its interplay with plant defense responses. First, an introduction of the plant innate immunity system is provided with emphasis on pattern trigger immunity (PTI), which is based on the recognition of "non-self" and "self" elicitor molecules by surface-localized pattern-recognition receptors (PRRs). Then, the *Arabidopsis* endogenous peptides that act as self-elicitor molecules are presented, with details on *At*Pep1 and its PEPR receptors. Plant endomembrane trafficking is described, encompassing endocytic pathways, clathrin mediated endocytosis (CME) and receptor-mediated endocytosis (RME). In the next chapter, we explored strategies for the *in vivo* study of the subcellular behavior of *At*Pep1; to this end, we fused the precursor protein of *At*Pep1 (PROPEP1) to GFP and assessed its localization. We found that PROPEP1 was associated with the tonoplast and accumulated in the vacuole, suggesting that this organelle could work as the station where PROPEP1 is stored and later released, only in a danger situation, hence initiating *At*Pep1. Moreover, we generated *At*Pep1 versions labeled with fluorescent dyes and demonstrated that this peptide could be fluorescently tagged without loss of its biological activity. In chapter 3, we combined classical and chemical genetics with life imaging to study the behavior of a bioactive fluorescently labeled *At*Pep1 in the *Arabidopsis* root meristem. We discovered that the labeled *At*Pep1 was able to bind the plasma membrane very quickly in a receptor-dependent manner. Subsequently, the PEPR-*At*Pep1complex was internalized via CME and transported to the lytic vacuole, passing through early and late endosomal compartments. Impairment of CME compromised the *At*Pep1 responses. Our findings provide for the first time an *in vivo* visualization of a signaling peptide in plant cells, thus giving insights into its intracellular fate and dynamics. The role of the coregulatory receptor BRI1-associated kinase 1 (BAK1) in *At*Pep1-responses was also investigated (chapter 4). Our results confirmed that BAK1 interacts with PEPRs in a ligand-dependent manner and indicate that BAK1 modulates *At*Pep1 signaling and endocytosis, but that, when absent, it might be replaced by homologous SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE *(*SERK) proteins that could have additional functions during the *At*Pep1 signaling. Furthermore, phosphorylation events after the formation of PEPR-BAK1 complexes seem to dictate the molecular bases of *At*Pep1 internalization and signaling. Finally, we discussed our findings in a more general perspective, highlighting the important findings for the plant endomembrane trafficking field, the potential use of fluorescently labeled ligands as a tool to study ligand-receptors pairs, the availability of *At*Pep1-PEPRs as an excellent model to study endocytosis and its interplay with signaling, and the future challenges in the field.

Keywords: Plant signaling peptides; PTI; Pattern –recognition receptors; *At*Pep1-PEPR; Endocytosis

SAMENVATTING

De Subcellular dynamiek van de endogene elicitor peptide *At*Pep1 and zijn receptor in *Arabidopsis*: implications voor de immuniteit van de plant

Dit werk onderzoekt de subcellulaire dynamica van de "plant elicitor peptide" *At*Pep1 en de wisselwerking met de verdedigingsresponsen van planten. In de inleiding wordt het immuunsysteem van planten beschreven met de nadruk op de patronen die de immuniteit activeren, en die gebaseerd zijn op de herkenning van "niet-eigen" en "eigen" moleculen door de aan de oppervlakte-gelocaliseerde herkenningsreceptoren. De endogene peptiden van *Arabidopsis* worden beschreven die zich als zelf-elicitoren of auto-immuniteitsopwekkers gedragen met een focus op *At*Pept1 en zijn receptoren. Verder wordt het endomembraantransport in planten besproken, waarbij dieper wordt ingegaan op clathrine gemedieerde endocytose (CME). In het volgend hoofdstuk bestuderen wij de strategieën voor het onderzoek naar de *in vivo* subcellulair localisatie van *At*Pep1. Hiervoor maakten wij een fusie tussen zijn precursorproteïne (PROPEP1) en GFP, en bepaalden zijn lokalisatie. We vonden dat PROPEP1 was geassocieerd met de tonoplast en accumuleerde in de vacuole, wat erop wijst dat dit organel zou kunnen fungeren als opslagplaats voor PROPEP1. In het geval van een immuun response wordt het precursor peptide vrijgegeven, en ontstaat *At*Pep1. Verder genereerden we versies van *At*Pep1 die verbonden zijn met fluorescente kleurstoffen, zonder het verlies van zijn biologische activiteit. In hoofdstuk 3 combineerden we klassieke en chemische genetica met "life"-beeldvorming om het gedrag te bestuderen van een bioactieve en fluorescente vorm van *At*Pept1 in het wortelmeristeem van *Arabidopsis*. We ontdekten dat het fluorescente *At*Pep1 in staat was zeer snel de plasmamembraan te binden in een receptor-afhankelijke wijze. Vervolgens werd de receptor (PEPR) in complex met *At*Pep1 geïnternaliseerd via CME en naar de lytische vacuole getransporteerd doorheen de vroege en late endosomale compartimenten. Inhibitie van CME bracht de *At*Pep1 geïnduceerde responsen in het gedrang. Dankzij onze resultaten werd voor de eerste keer een signaalpeptide gevisualiseerd *in vivo* in plantencellen, waardoor inzicht werd verschaft in zijn intracellulair lot en dynamica. De rol van de co-regulatorische receptor BRI1-associated kinase 1 (BAK1) in de responsen van *At*Pep1 werd ook onderzocht (hoofdstuk 4). Onze resultaten bevestigden dat BAK1 interageert met PEPRs in een ligand-afhankelijke wijze; tevens duiden ze aan dat BAK1 AtPep1 geïnduceerde signalisatie en endocytose moduleert. BAK1 kan, bij afwezigheid, vervangen worden door de homologe proteïnen SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK), die bijkomende functies zouden kunnen hebben tijdens *At*Pep1 geïnduceerde signalisatie. Daarenboven, fosforylatie na de vorming van de complexen PERP-BAK1 kan de moleculaire basis van de internalisering en signalering van *At*Pep1 te dicteren. Tenslotte bespreken wij onze resultaten in een meer algemeen perspectief. We leggen de nadruk op de voorname bevindingen betreffende het endomembraantransport van het PEPR- *At*Pep1 receptor-ligand complex, we wijzen op het potentieel gebruik van fluorescente liganden als middel om receptor-ligand complexen te bestuderen en op de geschiktheid van *At*Pep1 en PEPRs als model om endocytosis in relatie tot immuun signalisatie te onderzoeken. Finaal vermelden we de toekomstige uitdagingen in het veld.

Keywords: Planten signalisatie peptides; Patroonherkenning receptor; PTI; *At*Pep1-PEPR; Endocytose

LIST OF ABBREVIATIONS

***At*Pep1**	*Arabidopsis thaliana* Plant elicitor peptide1
AFCS	Alexa Fluor 647–castasterone
ARF-GEF	ADP-ribosylation factor–GTP exchange factor
BAK1	BRI1-associated kinase 1
BFA	Brefeldin A
BIK1	Botrytis-induced kinase 1
BIR2	BAK1-interacting receptor2
BR	Brassinosteroid
BRI1	BRASSINOSTEROID INSENSITIVE 1
CCV	Clathrin coated vesicles
CHCs	Clathrin heavy chain
CHX	Cycloheximide
CIE	Clathrin-independent endocytosis
CLCs	Clathrin light chain ~~proteins~~
CME	Clathrin-mediated endocytosis
ConcA	Concanamycin A
Cy5	Cyanine 5
DAMPS	Damage-associated molecular patterns
DMSO	Dimethyl sulfoxide
EFR	EF-Tu receptor
ETI	Effector-triggered immunity
flg	flagellin
FLS2	FLAGELLIN SENSING2
FM4-64	N-(3-triethylammoniumpropyl)-4-(4-diethylaminophenylhexatrienyl) pyridinium dibromide
GFP	Green fluorescent protein
GUS	β-glucuronidase
HPLC	High-performance liquid chromatography
LRR	Leucine-rich repeats
LRRRK	Leucine-reach repeat–receptor kinase
MAMPs	Microbe-associated molecular patterns
MAPKs	Mitogen-activated protein kinases

MS	Murashige and Skoog
MVB	Multivesicular body
PAMPs	Pathogen-associated molecular patterns
PDF1	Plant DEFENSIN 1
PEPR	Peptides Peps receptor
PRR	Pattern-recognition receptors
PTI	Pattern-triggered immunity
RD	Arginine-aspartic acid motif
RLP	Receptor-like protein
RLCKs	Receptor-like cytoplasmic kinases
RME	Receptor-mediated endocytosis
ROS	Reactive oxygen species
SERKs	Somatic embryogenesis-related kinases
TAMRA	5-Carboxytetramethylrhodamine
TGN/EE	*Trans*-Golgi network/early endosome
VHA-a1	Vacuolar H+-ATPase subunit a1

1 INTRODUCTION

1.1 Plant defense

Plants have the ability to use sunlight energy to transform CO_2, water and other elements, into organic molecules that become the primary food source of many organisms. These organisms can be beneficial to plant growth, but many of them have detrimental effects on plant development and long-term survival (JONES; DANGL, 2006). Moreover, because of their sessile life style, plants cannot avoid danger organisms by simply running away and have, therefore, evolved stunning defense strategies to stop invading organisms, thus allowing plants to successfully colonize different environments on earth. The first obstacle that an organism must overcome before it succeeds in invading the plants is the presence of structural, enzymatic or chemical preformed barriers, such as waxy cuticles, lignified cell walls, thorns, trichomes, antimicrobial enzymes and secondary metabolites (BOYAJYAN et al., 2014; THORDAL-CHRISTENSEN, 2003). Once these barriers are breached, the invaders are confronted with a refined plant immune system designed to perceive elicitor molecules derived from the invading organisms and from the already attacked plant cells (BOLLER; FELIX, 2009; MACHO; ZIPFEL, 2014).

1.1.1 The plant immune system

Plants, unlike mammals, lack a somatic adaptive immune system and mobile defender cells that access most part of the organism. Instead, plants rely on the innate immunity of each cell and on systemic signals emanating from infection sites (JONES; DANGL, 2006). The plant innate immunity is based on the recognition of potentially invading organisms and subsequent induction of protective responses, comprising a two-tier perception system (DODDS; RATHJEN, 2010). The first layer is mediated by the recognition of specific microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) by surface-localized pattern-recognition receptors (PRRs) that induce basal responses termed PRR-triggered immunity (PTI) (BOYAJYAN et al., 2014; MACHO; ZIPFEL, 2014). As exogenous elicitors are conserved and widely distributed within pathogens and non-pathogens from invading organisms (MEDZHITOV; JANEWAY, 1997), we chose to use the term MAMPs to refer to elicitors from invading organisms throughout this thesis. Additionally, PTI can be activated by endogenous host-derived elicitor molecules (damage-associated molecular patterns [DAMPs]) that are released upon pathogen perception or pathogen-induced cell damage, and recognized as danger/alarm signals (BOLLER; FELIX, 2009; MACHO; ZIPFEL, 2014). As most of the

MAMPS are essential for the life style of the pathogens, they cannot easily be amended to evade recognition; however, the existence of PTI has instigated the necessity for plant pathogens to evolve a suite of diverse effector molecules that are secreted into host cells to interfere with specific steps of pathogen detection or subsequent downstream signaling responses (PUMPLIN; VOINNET, 2013). In most cases, these effectors are virulence factors because they promote microbial growth and disease (BOLLER; FELIX, 2009; DODDS; RATHJEN, 2010; PUMPLIN; VOINNET, 2013). As a counter defense to the action of virulence effectors, the second layer of plant immunity appears, based on a large family of mostly intracellular plant receptors of the nucleotide-binding leucine-rich repeat domain class (R proteins) that recognize virulence effectors directly or by monitoring the integrity of their endogenous targets; as a consequence, responses, called effector-triggered immunity (ETI) that is stronger than PTI, are induced and lead, in many cases, to hypersensitive responses characterized by rapid apoptotic cell death and local necrosis (BOLLER; FELIX, 2009; BOYAJYAN et al., 2014; DODDS; RATHJEN, 2010; MACHO; ZIPFEL, 2014; PUMPLIN; VOINNET, 2013).

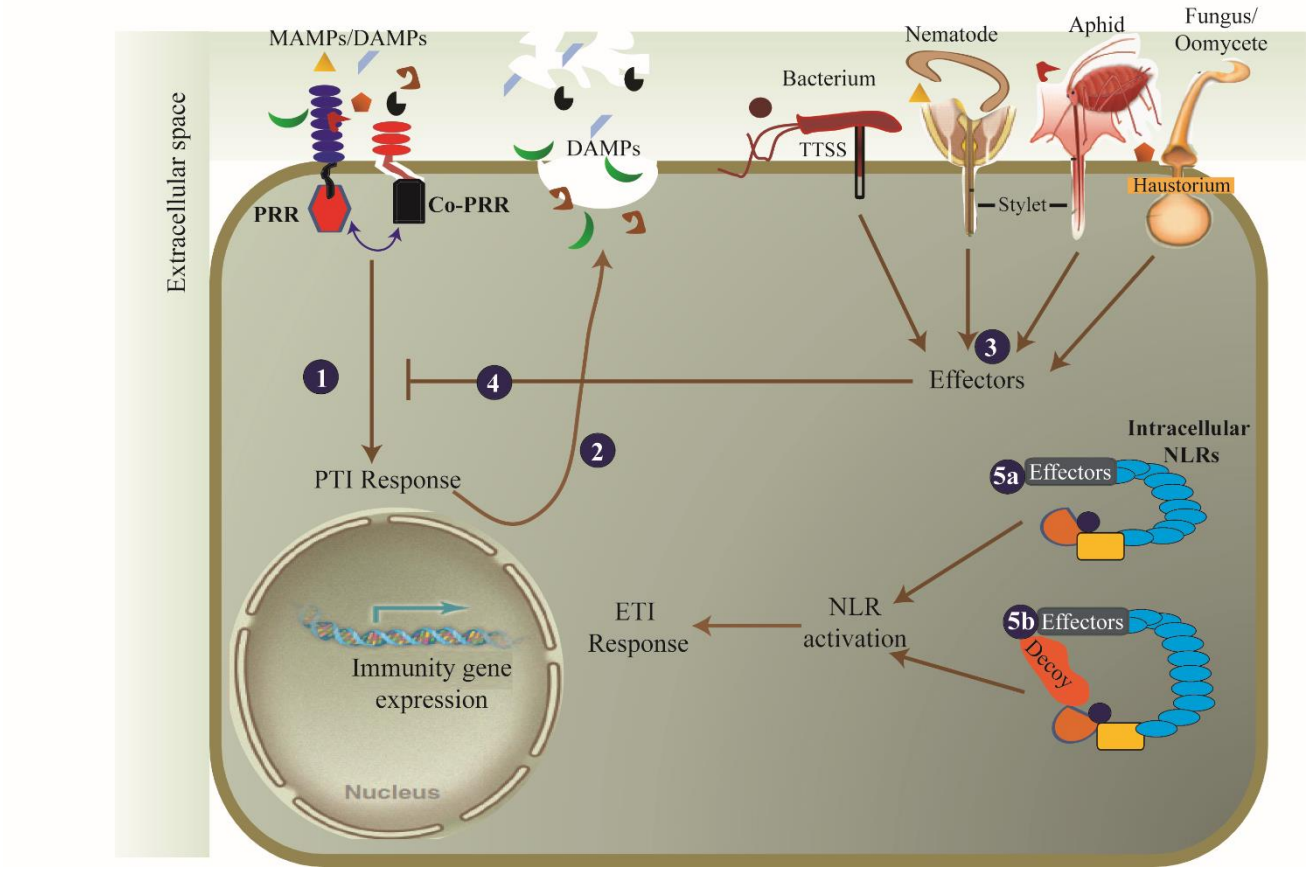

Figure 1 – Schematic of the plant immune system. Pathogens of different life styles release MAMPs (Shapes are color coded to the pathogens) into the extracellular space as they colonize plants. (1) MAMPs are recognized by the extracellular domain of PRR and initiate PTI. Many PRRs interact with coregulatory receptors to initiate PTI signaling pathway. (2) Host-derived elicitor molecules DAMPs are released upon pathogen perception or pathogen-induced cell damage, and they are recognized by PRRs to amplify PTI. (3) Pathogens deliver a suite of effector proteins into host cells through specialized structures. (4) These effector are addressed to specific subcellular locations where they can interfere with PTI and facilitate virulence. (5) However, intracellular nucleotide-binding (NB)-LRR receptors NLRs can recognize virulence effectors directly (5a) or by monitoring the integrity of their endogenous targets (5b), triggering ETI. (TTSS) type III secretion system. Modified from Dangl et al. (2013). Decoy represent an alteration of a host virulence target, like the cytosolic domain of a PRR.

1.1.2 Pattern recognition receptors

Pattern recognition receptors are localized in the plasma membrane and perceive MAMPs or DAMPs of “non-self” and “self” origin, respectively. They are either receptor kinases (RKs) that have a ligand-binding ectodomain, a single-pass transmembrane domain, and an intracellular kinase domain, or receptor-like proteins (RLPs), which share the same overall structure but lack an intracellular kinase domain (ZIPFEL, 2014). As RLPs do not possess an intracellular signaling domain, they are believed to rely on the interaction with one

or several RKs to propagate the signal induced by the receptor-ligand binding (TOR; LOTZE; HOLTON, 2009; ZIPFEL, 2014)

As evidence of their biological relevance, the *Arabidopsis thaliana* genome is predicted to encode more than 600 RKs and 56 RLPs and the rice (*Oryza sativa*) genome encodes more than 1000 RKs and 90 RLPs (DARDICK et al., 2007; FRITZ-LAYLIN et al., 2005; SHIU; BLEECKER, 2003), although not all RKs and RLPs are PRRs, some of which being related to different physiological processes (SHIU; BLEECKER, 2003). According to their sequence analysis, RKs can be classified into 21 structural classes depending on the structure of their extracellular domain, with the leucine-rich repeats (LRR) RKs, being the largest family with at least 233 members divided into 16 subfamilies (LEHTI-SHIU et al., 2009; SHIU; BLEECKER, 2001; SHIU et al., 2004). Furthermore, RKs can also be grouped into RD and non-RD kinases, according to the presence or absence of the so-called RD motif, which refers to a conserved arginine (R) located in front of an aspartate (D) in the catalytic loop that facilitates phosphotransfer (DARDICK; SCHWESSINGER; RONALD, 2012; SHIU; BLEECKER, 2003). Currently, all characterized plant RKs that carry the non-RD kinase motif are involved in the recognition of MAMPs, suggesting that it might be a hallmark of PRRs, but this characteristic cannot be extended to all PRRs because RKPRRs that recognize DAMPs belong to the RD kinases (BOLLER; FELIX, 2009; DARDICK; SCHWESSINGER; RONALD, 2012; SCHWESSINGER; RONALD, 2012).

Presently, in spite of the large number of genes predicted to encode RKs and RLPs, only a minority of PRRs has been characterized and few ligands have been identified. The first successfully characterized ligand-PRR pair was the bacterial flagellin (or the derived peptide flg22) recognized by the LRRRK FLAGELLING SENSIN2 (FLS2) (GOMEZ-GOMEZ; BOLLER, 2000). Initially, FLS2 was identified in the model plant *Arabidopsis*, but functional orthologs have already been found in a wild relative of tobacco (*Nicotiana benthamiana*), rice, tomato (*Solanum lycopersicum*), and grapevine (*Vitis vinifera*) (HANN; RATHJEN, 2007; ROBATZEK et al., 2007; TAKAI et al., 2008; TRDA et al., 2014). Another well-characterized PRR, is the EF-Tu RECEPTOR (EFR) that recognizes the conserved N-acetylated epitope elf18 of the first 18 amino acids of the bacterial elongation factor Tu (ZIPFEL et al., 2006). Nevertheless, whereas flg22 seems to be recognized by most higher plants, the ability to perceive elf18 seems restricted to the plant family *Brassicaceae* (BOLLER; FELIX, 2009).

Table 1 – *Arabidopsis* PRRs and their respective ligands

Receptor	**Receptor type**	**Extracellular domain**	**ligand**	**Ligand Type**	**References**
PEPR1/2	RK	LRR	*At*Peps	DAMPS	(HUFFAKER; PEARCE; RYAN, 2006; YAMAGUCHI et al., 2010)
RLK7	RK	LRR	*At*PIPs		(HOU et al., 2014)
WAK1	RK	EGF	OGs		(BRUTUS et al., 2010)
DORN1/ LecRK-I.9	RK	Lec	eATP		(CHOI et al., 2014)
FLS2	RK	LRR	flg22	MAMPs	(CHINCHILLA et al., 2006; GOMEZ-GOMEZ ; BOLLER, 2000)
EFR	RK	LRR	elf18		(ZIPFEL et al., 2006)
CERK1	RK	LysM	Chitin		(MIYA et al., 2007)
LYK4/5	RK	LysM	Chitin		(PETUTSCHNIG et al., 2010; WAN et al., 2012)
LYM1/3	RLP	LysM	PGN		(WILLMANN et al., 2011)
LYM2	RLP	LysM	Chitin		(FAULKNER et al., 2013; PETUTSCHNIG et al., 2010)
ReMAX	RLP	LRR	eMAX		(JEHLE et al., 2013)
RBGP1	RLP	LRR	PGs		(ZHANG et al., 2014)

RK, receptor kinase; RLP, receptor-like protein; LRR, leucine-rich repeats domain; EGF, epidermal growth factor (EGF)-like domain; Lec, lectin-domain; LysM, lysine motif domain; *At*Peps, *Arabidopsis* elicitor peptides; *At*PIPs, *Arabidopsis* PIP peptides; OGs, oligogalacturonides; eATP, extracellular ATP; flg22, bacterial flagellin peptide; elf18, epitope of the bacterial elongation factor Tu; PGN, Peptidoglycans; eMAX, enigmatic bacterial PAMP partially purified from *Xanthomonas axonopodis pv. citri;* PGs endopolygalacturonases

As an example of RLPPRRs, in *Arabidopsis*, there are two RLPs with lysine motif (LysM)-containing ectodomains, *At*LYM1 and *At*LYM3, that specifically bind peptidoglycans (PGNs) that are the major components of the cell walls of both Gram-positive and Gram-negative bacteria (ERBS; NEWMAN, 2012; WILLMANN et al., 2011). Interestingly, the PGN-induced responses are impaired in the absence of the LysMRK CERK1 that does not bind PGN itself (WILLMANN et al., 2011), implying that PGN perception in *Arabidopsis* employs a multimeric receptor system, comprising PGN-binding LysMRLPs and signaling-transducing LysMRKs, such as CERK1 (ZIPFEL, 2014). Moreover, CERK1 has also been shown to be

required for chitin perception, the major constituent of fungal cell walls. However, in contrast to PGNs, the extracellular domains of CERK1 specifically bind chitin oligomers that act as bivalent ligands, leading to the homodimerization of CERK1 and generation of chitin-induced signaling (LIU et al., 2012; MIYA et al., 2007).

As stated above, PRRs can also sense DAMPs that are self-molecules available for recognition only after cell/tissue damage or pathogen recognition (ZIPFEL, 2014). To date, in *Arabidopsis*, the best-characterized DAMP-PRR pairs correspond to the *At*Pep peptides and the LRRRKs PEPR1 and PEPR2 (BARTELS; BOLLER, 2015) that will be presented further in detail. Recently, the PIP1 and PIP2 peptides have been shown also to bind the receptor-like kinase 7 (RLK7) (HOU et al., 2014). Besides peptides, in *Arabidopsis*, DAMPs can also be lytic plant cell wall fragments, such as oligogalacturonides (OGs) that are perceived by the epidermal growth factor (EGF) motif-containing the RK wall-associated kinase 1 (WAK1) (BRUTUS et al., 2010), and extracellular adenosine 5'-triphosphate (eATP) that is recognized by the receptor DORN1/LecRK-I.9 (CHOI et al., 2014).

1.1.3 Coregulatory receptor kinases

RKs also can act as regulatory proteins that do not necessarily interact with a ligand, but are important facilitators or suppressors of signaling activation and allow signaling cross-talk at the plasma membrane, coordinating the different signals perceived in the apoplast to ensure the proper downstream signaling (SCHWESSINGER; RONALD, 2012). For instance, members of the SERK family have been shown to redundantly hetero-oligomerize with other RKs, albeit with different affinities, and to regulate multiple physiological programs (AAN DEN TOORN; ALBRECHT; DE VRIES, 2015; POSTEL et al., 2010; ROUX et al., 2011). In *Arabidopsis*, the SERK family consists of five close homologs (SERK1 to SERK5) that have arisen through gene duplications, are all characterized by a small extracellular domain consisting of 4.5-5 LRRs and by the presence of a typical serine and proline-rich motif after a truncated extracellular LRR domain, and belong to the LRRRK subclass II (AAN DEN TOORN; ALBRECHT; DE VRIES, 2015; HECHT et al., 2001).

Among the SERK members, SERK3/BAK1 (hereafter referred as BAK1) is the most extensively studied and, because it has been shown to be required for the proper functionality of numerous RKs, it has been proposed as a multifunctional adaptor molecule implicated in plant development, cell death control, and innate immunity (HE et al., 2007; POSTEL et al., 2010; ROUX et al., 2011; SCHWESSINGER et al., 2011). BAK1 was originally discovered to dimerize with the main brassinosteroid (BR) receptor BRASSINOSTEROID-INSENSITIVE1 (BRI1) (LI et al., 2002;

WANG et al., 2001). Later on, its rapid ligand-dependent interaction with PRRs (FLS2 and EFR) and PEPRs has been documented as well (CHINCHILLA et al., 2007; POSTEL et al., 2010; SCHULZE et al., 2010; SCHWESSINGER et al., 2011). This interaction appears to be functionally important because null mutants of BAK1 displayed impaired defense responses upon perception of a plethora of fungal, bacterial, and oomycete-derived conserved microbial signatures, indicating that it is a positive regulator of PRRs. However, this impairing differs between MAMPs and residual responses might still be found (CHINCHILLA et al., 2009; CHINCHILLA et al., 2007; RANF et al., 2011; SCHWESSINGER et al., 2011), possibly due to the BAK1 substitution in the receptor complex by other members of the SERK family (BOLLER; FELIX, 2009). Indeed, the closest BAK1 homolog, SERK4/BKK1 (hereafter referred as BKK1), plays a partially redundant role during PTI signaling and forms ligand-dependent complexes with EFR and FLS2 (ROUX et al., 2011). Partially redundant roles among other SERK family members have been observed with BRI1 that interacts with four out of five members of the SERK family (SERK1 to SERK4) (GOU et al., 2012; HE et al., 2007; LI et al., 2002). Recently, the identification of a novel mutant allele, *bak1-5*, which is specifically impaired in PTI signaling without displaying any other pleiotropic defects in BR signaling or cell death control, confirmed that BAK1 contributes significantly to disease resistance against biotrophic and hemibiotrophyc pathogens (ROUX et al., 2011; SCHWESSINGER; RONALD, 2012; SCHWESSINGER et al., 2011).

The specificity of *bak1-5* to affect immunity responses is determined by a single amino acid substitution (Cys to Tyr at position 408) in the kinase domain that reduces the phosphorylation status, probably by affecting the recruitment or activation of downstream signaling components (SCHWESSINGER et al., 2011). Phosphorylation events are the earliest responses after formation of ligand-induced complexes and they have been proposed to modulate the downstream responses of BAK1 and its interacting partners (SCHULZE et al., 2010; SCHWESSINGER et al., 2011). In supporting of this hypothesis, the recently mapped phosphorylation patterns of BAK1 associated with different RK partners (BRI1, FLS2, and EFR) revealed that differential phosphorylation patterns of RKs resulted from the altered BAK1 phosphorylation status, suggesting that these phosphorylation events could be the molecular basis for selective regulation of multiple BAK1-dependent pathways (WANG et al., 2014).

Additionally, BAK1 can also associate with members of the BAK1-interacting receptor (BIR) of the RK family, predicted to be very similar in structure as BAK1 (GAO et al., 2009; HALTER, et al., 2014b). Interaction between BIR2 and BAK1 has recently been found to occur in the absence of MAMP perception, preventing interaction with the ligand-binding FLS2; however, perception of MAMPs leads to BIR2 release from the BAK1 complex and enables

the recruitment of BAK1 into the FLS2 complex. These findings imply that BIR2 act as a negative regulator of PTI by limiting the BAK1-receptor complex formation in the absence of ligands (HALTER, et al., 2014a; HALTER, et al., 2014b).

1.1.4 Receptor-like cytoplasmic kinases as direct substrates of PRR complexes

In addition to coregulatory receptor kinases, cytoplasmic partners are also required to link the PRR activation with the downstream intracellular signaling. In recent years, receptor-like cytoplasmic kinases (RLCKs) have emerged as direct substrates of PRR complexes and key positive regulators of PTI signaling (MACHO; ZIPFEL, 2014). Currently, the RLCK *Botrytis*-induced kinase 1 (BIK1) that is part of the large multigenic RLCKVII subfamily, has been shown to be an important regulator of the activation of PRR complexes that mediate the innate immunity in *Arabidopsis* and interact constitutively with FLS2, EFR, CERK, PEPRs and BAK1 (LIU et al., 2013; LU et al., 2010; ZHANG et al., 2010). The ligand-binding activation of PRR complexes results in a rapid BIK1 phosphorylation that dissociates from the receptors to trigger the downstream signaling (LU et al., 2010; ZHANG et al., 2010). BIK1 and its closely related proteins (PBLs) seem to have partially redundant functions during PTI, but specific roles have been suggested because PBL1, PBL2 and PBL5 can also regulate flg22-induced responses, whereas only PBL1 additionally interacted with PEPR1 (LIU et al., 2013; ZHANG et al., 2010). The requirement of different RLCKs for specific PTI responses suggests that the choice of specific RLCKs as PRR substrates constitutes another layer in the signaling branching regulation from PRR complexes (MACHO; ZIPFEL, 2014).

1.1.5 Pattern-triggered immunity responses

Most of the known PRRs heteromerize with coregulatory receptor kinases (BAK1 or other RK) almost instantaneously after ligand perception, followed by phosphorylation and activation of the intracellular kinase domain of both receptors and subsequent activation of a sequential set of early and long-term responses as well as downstream signaling cascades to adapt to the imminent attack (BOLLER; FELIX, 2009; MACHO; ZIPFEL, 2014; SCHULZE et al., 2010; SCHWESSINGER et al., 2011). Some of these responses are routinely used as read-outs to study PRRs and their ligands.

In the first minutes after MAMPs/DAMPS perception, changes in the ion fluxes can be detected, including H^+ and Ca^{2+}, leading to membrane depolarization (MITHÖFER; EBEL; FELLE, 2005). The effects on H^+ fluxes can be clearly observed in suspensions of cultured cells in which they lead to alkalinization of the liquid growth medium (BOLLER, 1995). Recently, calcium-dependent protein kinases (CDPKs), acting as Ca^{2+} sensor protein kinases, have been

reported to be major mediators of the early PTI immune signaling (BOUDSOCQ et al., 2010). Another very early response is the production of reactive oxygen species (ROS) that acts as antibiotic agents directly and as secondary signals to induce intracellular and systemic signaling events (APEL; HIRT, 2004). The initial downstream signaling of PTI includes also the activation of so-called mitogen-activated protein (MAP) kinases (MAPKs), considered key regulatory elements in the early transduction of MAMPs/PAMPs-PRRs signaling (HETTENHAUSEN; SCHUMAN; WU, 2014). In *Arabidopsis*, triggered PRRs induce activation of MPK3 and MPK6, starting with a lag phase of approximately 1–2 min and peaking after 5–10 min (RANF et al., 2011). MAPK activation leads to induction of some early defense-related genes, such as some members of the WRKY transcription factor family (ASAI et al., 2002). Interestingly, MAPKs and Ca^{2+}-dependent protein kinases seem to function independently and the transcriptional reprogramming that results from the MAPK activity differs from the modulations achieved through the CDPK activity (BOUDSOCQ et al., 2010).

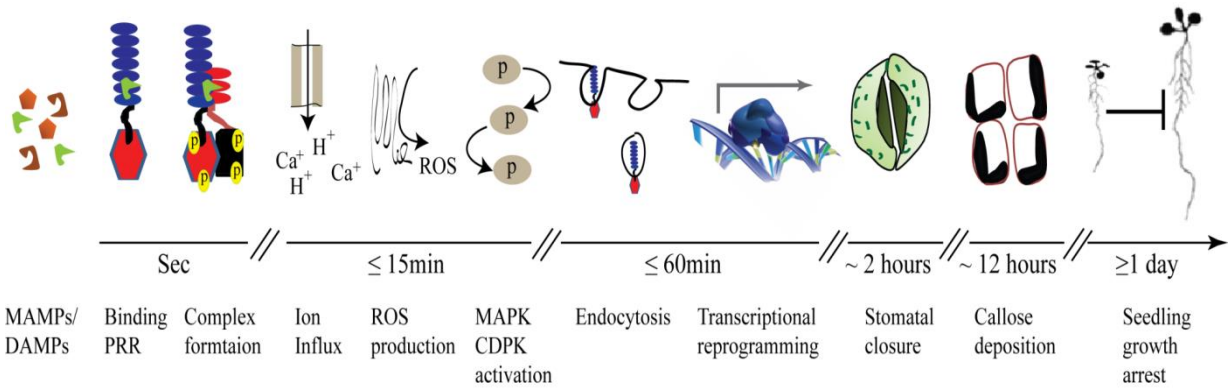

Figure 2 - Proposed model of pattern trigger immunity responses based on studies of known ligand-PRRs pairs. MAMPs/DAMPs are recognized by the respective PRR and almost instantaneously the activated PRR interact with a coregulatory receptor kinase followed by phosphorylation and activation of the intracellular kinase domain of both receptors. Then, a set of very early responses including increased of intracellular calcium, extracellular alkalinization, ROS production, and MAPK and CDPK activation by phosphorylation occur. Subsequently, the activated receptors are removed from the plasma membrane by endocytosis and a massive transcriptional reprogramming is detected. Finally, late responses are found hours or days after the MAMP/DAMP stimuli, including stomata closure, callose deposition and seedling growth inhibition

Following the initial responses, pattern recognition results in a massive transcriptional reprogramming. Transcriptomic studies in *Arabidopsis* indicated that as early as 30 min after

flg22 treatment, almost 1000 genes were up-regulated and approximately 200 genes were down-regulated (ZIPFEL et al., 2004). Additional studies also revealed a similar pattern of gene regulation upon elf26 and chitin application, suggesting a stereotypical response to MAMPs and DAMPs (NAVARRO et al., 2004; RAMONELL et al., 2002; ZIPFEL et al., 2006). Most up-regulated genes encode enzymes involved in the synthesis of antimicrobial compounds and proteins involved in signal perception and transduction, including RKs, transcription-regulatory factors and phosphatases (NAVARRO et al., 2004; RAMONELL et al., 2002; ZIPFEL et al., 2006). The fact that several RKs are present implies that the early gene induction plays a role as positive feedback to increase the PRR perception capabilities (BOLLER; FELIX, 2009). Although transcriptomic studies have shown an overlap in genes induced by different MAMPs and DAMPs, assessment of transcriptional changes in long-term patterns resulting from exposure to flg22 and oligosaccharides (OGs) derived from the plant cell walls over an extended time course revealed that the transcriptional regulation can differ not only in timing and amplitude, but also in gene activation during flg22 elicitation and not during OG responses (DENOUX et al., 2008). Thus, although different elicitor molecules may trigger conserved transcriptional responses, specific aspects depend on the sensed elicitor.

Another interesting aspect of PTI is the endocytosis of the PRRs that has been first demonstrated by using GFP-labeled FLS2 that, after flg22 stimulation (30-60 min) is translocated from the plasma membrane via vesicle-mediated endocytosis and targeted to endosomal sorting (BECK et al., 2012; ROBATZEK; CHINCHILLA; BOLLER, 2006). Similar observations have also been documented for the LeEIX2 receptor in tobacco after stimulation with the fungal protein elicitor ethylene-inducing xylanase (EIX) (BAR; AVNI, 2009; SHARFMAN et al., 2011), suggesting that this process could be a hallmark of triggered PRR activation. Although an increasing amount of data indicates that endocytosis is part of the PRR response, its role during PTI signaling, trafficking machinery and subcellular dynamics of the process is still poorly understood.

Subsequently to early PTI events, late responses are detected hours or days after the MAMP/DAMP stimuli, including stomata closure, callose deposition and seedling growth inhibition (BOLLER; FELIX, 2009; MELOTTO et al., 2006). Stomata are considered a constitutively natural access for a significant number of microbes; therefore, plants can restrict pathogen entry by closing stomata or by inhibiting stomata opening (MELOTTO et al., 2006). Callose deposition is also a hallmark of PTI responses of which the biological relevance is not clear, but that can be detected approximately 16 h after MAMP treatment (GOMEZ-GOMEZ; FELIX; BOLLER, 1999). If MAMP/DAMP perception continues for days, a seedling growth

inhibition in a concentration-dependent manner can be detected, suggesting a switch of resource allocation from growth to a defense program; nevertheless, the physiological role and molecular details of this response is under debate (BOLLER; FELIX, 2009; KROL et al., 2010; LOZANO-DURAN; ZIPFEL, 2015). Downstream of PTI, plant hormones can be involved, mainly salicylic acid (SA), ethylene and jasmonic acid (JA), that further finely modulate immune responses, spreading the message of imminent danger to yet unaffected tissues (PIETERSE et al., 2012).

1.2 Endogenous plant elicitor peptides

As indicated above, in addition to elicitor molecules derived from invading organisms, plants also recognize host-derived molecules, referred as DAMPs, to activate PTI. Known DAMPs are, for instance, cell wall fragments, such as OGs and cellulose fragments, cutin monomers, eATP and peptides (CHOI et al., 2014; FERRARI et al., 2013; KAUSS et al., 1999; YAMAGUCHI; HUFFAKER, 2011). Significant progress has been achieved in identifying new endogenous peptide elicitors among the different classes of DAMPs and in delineating their downstream signaling mechanism (YAMAGUCHI; HUFFAKER, 2011). Generally, these small (5-100 amino acids in length) peptides are categorized as plant signaling peptides; within this category, the endogenous plant peptides are grouped that coordinate multiple and very diverse biological and physiological plant responses (GHORBANI et al., 2014). Because of the aims of this thesis, I will present only the signaling peptides related to plant immunity.

Plant elicitor peptides are released from large precursor proteins and have been classified with respect to their precursor protein structure in three major groups: (i) peptides derived from precursor proteins without an N-terminal secretion signal, (ii) peptides derived from precursor with an N-terminal secretion signal, and (iii) peptides derived from proteins with distinct primary functions (ALBERT, 2013; YAMAGUCHI; HUFFAKER, 2011).

1.2.1 Peptides derived from precursor proteins without an N-terminal secretion signal

This category includes the 18-amino-acid peptide systemin that was the first isolated peptide with a hormone characteristic and that had been shown to be involved in systemic responses to wounding in tomato (PEARCE et al., 1991). Systemin derives from the C-terminus of a 200-amino-acid precursor protein, prosystemin, that mainly accumulates in the cytosol of vascular phloem parenchyma cells (MCGURL et al., 1992) via a still unknown cleavage mechanism. Systemin induces JA biosynthesis in the neighboring cells, leading to induction of proteinase inhibitors, anti-nutritive proteins, and plant volatiles to deter plant herbivores

(DEGENHARDT et al., 2010; OROZCO-CARDENAS; MCGURL; RYAN, 1993). Systemin homologs have only been found in the *Solanoideae* subfamily of the *Solanaceae* family (CONSTABEL; YIP; RYAN, 1998) and so far the systemin receptor has not been identified.

In *Arabidopsis*, the first isolated DAMP peptide was the plant elicitor peptide1 (Pep1) that binds two RK PRRs with high affinity PEPRs (YAMAGUCHI et al., 2010). *At*Pep1 that is a representative member of a protein family composed by eight members derives from the C-terminus of a 92-amino-acid precursor protein *At*PROPEP1 (BARTELS et al., 2013; HUFFAKER; PEARCE; RYAN, 2006). The *At*Pep-PEPR system will be presented in detail (see below).

Another peptide that could fit within this category is the recently identified 25-amino-acid peptide Kiss of death (KOD) that has been proposed as an early regulator of programmed cell death (PCD) (BLANVILLAIN et al., 2011). Although no direct link with plant immunity has been demonstrated yet, the *KOD* gene is induced upon biotic and abiotic stresses, suggesting that this peptide could also act as a potential DAMP, but further clarifying studies are needed (ALBERT, 2013; BLANVILLAIN et al., 2011). Interestingly, in contrast to systemin and *At*Pep1, KOD seems to be directly translated as the active form, because the corresponding gene only encodes the active 25-amino-acid sequence, avoiding the cleaving-off from a precursor protein (BLANVILLAIN et al., 2011).

1.2.2 Peptides derived from precursor with an N-terminal secretion signal

This group comprises the secreted peptides, including the hydroxyproline-rich systemin (HypSys) peptides that are derived from a precursor protein with an N-terminal secretion signal for the cell wall matrix localization (PEARCE et al., 2001). Two distinct 18-amino-acid HypSys were first isolated from tobacco leaves (*Nt*HypSysI and *Nt*HypSysII), derived from one single precursor protein encoded by one single gene (PEARCE et al., 2001). Orthologs of *Nt*HypSys have only been isolated in *Solanaceae* and *Convolvulaceae* (NARVÁEZ-VÁSQUEZ; OROZCO-CÁRDENAS; RYAN, 2007). The amino acid sequence of HypSys resembles that of systemin, but, due to their passage through the secretory system, the polyprolines are hydroxylated and then glycosylated with pentose sugar chains (PEARCE et al., 2001). HypSys peptides have been described as important plant immunity amplifiers and triggers, especially during herbivore attack, but also during interaction with other plant pathogens (BHATTACHARYA et al., 2013). The HypSys receptor still remains to be identified.

Recently, in *Arabidopsis*, members of a new peptide family, termed PAMP-induced peptides (*At*PIP) have been shown to activate immune responses and to enhance resistance

against *Pseudomonas syringae* and *Fusarium oxysporum* (HOU et al., 2014). These peptides are derived from the C-terminus of precursor proteins (prePIPs) with an N-terminal signal peptide, localized in the pericellular apoplastic space, of which the transcripts are up-regulated in plants exposed to MAMPs (HOU et al., 2014). Interestingly, transgenic plants expressing the GFP gene under control of the *prePIP1* promoter exhibited fluorescent tissues corresponding either with potential entry points or proliferation routes for invading organisms (HOU et al., 2014). Genetic and biochemical evidence suggested that Receptor Kinase 7 (RLK7) functions as PIP1 receptor (HOU et al., 2014).

1.2.3 Peptides derived from proteins with distinct primary functions

The first peptide that had been discovered to regulate immunity in plants derives from a protein with other primary functions and belongs to the family of inceptin peptides. These acidic 11-13-amino-acid peptides originate from the chloroplastic ATP synthase that is broken down in the gut of fall armyworm larvae (SCHMELZ et al., 2006, 2007). Inceptin treatments of cowpea (*Vigna unguiculata*) leaves was shown to trigger plant defense responses, such as production of SA, JA, and metabolites with defensive roles that together reduce fall armyworm growth (SCHMELZ et al., 2006, 2007). The elicitor activity of inceptins seems to be specific for *Phaseolus* and *Vigna genera* (SCHMELZ et al., 2007).

The *Glycine max* (soybean) subtilase peptide (*Gm*SubPep) is another member of this peptide category. *Gm*SubPep is a 12-amino-acid-long peptide that was discovered embedded in the protein-associated domain of a putative extracellular subtilase and, like other DAMPs, is able to trigger extracellular alkalinization and to induce the expression of defense- and stress-related genes (PEARCE et al., 2010). The mechanism of the *Gm*SubPep release from the subtilase and its receptor has not yet been identified.

1.3 The system of plant elicitor peptides (Peps) and PEPRs

1.3.1 Plant elicitor peptides (Peps)

The Peps are a family of defense–inducible peptides. The first member has been isolated in *Arabidopsis* (*At*Pep1) from an extract of wounded leaves by means of an elicitor-induced alkalinization activity assay with *Arabidopsis* suspension–cultured cells (HUFFAKER; PEARCE; RYAN, 2006). *At*Pep1 is a 23-amino-acid-long peptide derived from the C-terminus of a 92-amino-acid precursor protein PROPEP1 that lacks a secretion N-terminal signal (HUFFAKER; PEARCE; RYAN, 2006). Originally, the PROPEP family in *Arabidopsis* has been described as consisting of seven members, but an additional PROPEP has recently been

identified by more sensitive bioinformatic tools (BARTELS et al., 2013; HUFFAKER; PEARCE; RYAN, 2006). Based on sequence homology of the SSGR/KxGxxN motif, all eight PROPEPs are predicted to contain a putative *At*Pep (*At*Pep1 to *At*Pep8) of 23 to 29 amino acids in length at the C-terminus (BARTELS et al., 2013). Although only *At*Pep1 and *At*Pep5 have been biochemically isolated from *Arabidopsis* leaves, the other peptides have been synthetized and their activity confirmed (BARTELS et al., 2013; HUFFAKER; PEARCE; RYAN, 2006). The presence of PROPEPs has been expected in numerous species, including important crops (HUFFAKER; PEARCE; RYAN, 2006), and has been functionally validated in maize (*Zea mays*). The participation of *Zm*Pep1 and *Zm*Pep3 has been demonstrated during maize immunity responses, suggesting that the PROPEP family is largely conserved across the plant kingdom and that such Peps might probably play a role as general defense mediators (HUFFAKER; DAFOE; SCHMELZ, 2011; HUFFAKER et al., 2013).

Exogenous applications of *At*Peps induce a set of similar responses, hinting at functional redundancy (BARTELS et al., 2013; YAMAGUCHI et al., 2010). However, bioinformatic analysis and expression localization assessment revealed that the expression pattern of PROPEPs differs temporally and spatially under normal conditions and in response to various stresses (BARTELS et al., 2013), thus implying differential physiological roles within the *At*Pep members (BARTELS; BOLLER, 2015; BARTELS et al., 2013).

1.3.2 Perception of Peps by PEPRs

The perception of *At*Peps is mediated by binding to the extracellular LRR domain of two RKsPRR, designated PEPRs. PEPR1 is able to detect all eight *At*Peps, whereas PEPR2 can only detect *At*Pep1 and *At*Pep2 (BARTELS et al., 2013). PEPR1 was identified by photoaffinity labeling and further purification from *Arabidopsis* extracts. As the T-DNA mutants of *pepr1* were only partially compromised in *At*Pep1-induced responses (YAMAGUCHI; PEARCE; RYAN, 2006), additional receptors were looked for. PEPR2 was subsequently identified by phylogenetic analysis, and its role as *At*Pep1 receptor was experimentally demonstrated (KROL et al., 2010; YAMAGUCHI et al., 2010). The double *pepr1 pepr2* mutant completely abolished the *At*Peps immune responses, indicating that they are the only receptors able to perceive this family of peptides (KROL et al., 2010; YAMAGUCHI et al., 2010). PEPRs belong to the XI subgroup of LRRRKs and are classified as kinases with an arginine-aspartic acid (RD) motif in the catalytic site that are different from FLS2 and EFR that are non-RD kinases (YAMAGUCHI et al., 2010). Non-RD kinases generally show weak autophosphorylation activity, and there is

a significant correlation between the absence of this motif and a role in the early events of innate immune signaling (DARDICK; SCHWESSINGER; RONALD, 2012).

Recently, the crystal structure of the extracellular LRR domain of PEPR1 (PEPR1LRR) in complex with *At*Pep1 has been revealed, demonstrating that the conserved C-terminal portion of *At*Pep1 dominates the *At*Pep1 binding to PEPR1LRR; moreover, the non-conserved N-terminal sides of *At*Peps might possibly contribute to the preferential recognition of *At*Pep1 and *At*Pep2 by PEPR2 over the other *At*Peps (TANG et al., 2015).

1.3.3 Signaling mediated by Peps-PEPRs

After recognition of *At*Peps by PEPRs, different signaling responses are activated that are greatly similar to those triggered by the receptors FLS2 and EFR, when the bacterial MAMPs flg22 and elf18 are perceived, respectively (BARTELS; BOLLER, 2015). Upon ligand binding, PEPRs interact with the coreceptor BAK1 followed by phosphorylation of both BAK1 and PEPRs (SCHULZE et al., 2010; TANG et al., 2015). Additionally, the RLCK BIK1 that constitutively interacts with PEPR1 and probably with PEPR2 also gets phosphorylated (LIU et al., 2013). Then, induction of ion fluxes across the plasma membrane, ROS and ethylene production, and MPK3 and MPK4 activation are quickly triggered (KROL et al., 2010; RANF et al., 2011; YAMAGUCHI; PEARCE; RYAN, 2006). *At*Peps also lead to a transcriptional reprogramming, inducing expression of pathogen defense genes, such as *PDF1.2*, *MPK3*, *PR-1* and *WRKY* (HUFFAKER; RYAN, 2007; YAMAGUCHI et al., 2010). Interestingly, exogenous *At*Peps also induce the expression of their own precursor genes (except *At*Pep6) and their receptors, potentially indicating a positive feedback loop in the signaling of *At*Peps-PEPRs (HUFFAKER; RYAN, 2007; YAMAGUCHI et al., 2010). Callose deposition and seedling growth inhibition occur when seedlings are maintained in the presence of *At*Peps (KROL et al., 2010; LIU et al., 2013; RANF et al., 2011). Curiously, in contrast to flg22 that affects the whole seedling, the inhibitory effect of *At*Pep perception impairs mainly root growth (KROL et al., 2010; RANF et al., 2011).

1.3.3 Peps as amplifiers of innate immunity

*At*Peps have proposed to act mainly as amplifiers of innate immunity. This role is proposed because they mediate immunity responses, and the expression of their precursor proteins and receptors are induced upon biotic stresses, such as microbial infection, bacterial elicitors, wounding and JA and ethylene application (BARTELS; BOLLER, 2015; YAMAGUCHI; HUFFAKER, 2011). However, additional role in different physiological processes have been considered as well (BARTELS et al., 2013; GULLY et al., 2015).

As amplifiers, in a danger situation, *At*Peps are believed to be released from their precursor proteins (PROPEPs) into the extracellular spaces, where they subsequently bind their receptors located at the plasma membrane of neighboring cells, thus triggering defense responses and, hence, spreading the message **(**BARTELS; BOLLER, 2015; YAMAGUCHI; HUFFAKER, 2011). However, this assumption is based mostly on data generated from genetic and biochemistry experiments. The subcellular dynamics of the *At*Pep1-PEPR complex remains largely unknown.

1.4 Plant endomembrane system and trafficking

Plant cells possess a sophisticated endomembrane system that physically and functionally interconnects membranous compartments, allowing exchange of materials, such as proteins, polysaccharides, and lipids to their suitable cell locations (MORITA; SHIMADA, 2014). This system needs to be well-coordinated to maintain cellular homeostasis and to generate accurate responses toward different environmental stimuli such as challenges by microorganisms (INADA; UEDA, 2014). The plant endomembrane system includes the plasma membrane, the *trans*-Golgi network/early endosome (TGN/EE), multivesicular body/prevacuolar compartment (MVB/PVC), vacuoles, the Golgi apparatus and the endoplasmic reticulum (PIZARRO; NORAMBUENA, 2014). Material is transported within the plant endomembrane system through sequential steps including budding, movement and vesicle fusion (BONIFACINO; GLICK, 2004). Recently, the plant membrane trafficking pathways have been proposed to be classified into several major categories: (i) the secretory pathway that transports newly synthesized proteins from the endoplasmic reticulum to the plasma membrane and/or the extracellular space; (ii) the vacuolar transport pathway that delivers newly synthesized or internalized proteins to the vacuole; (iii) the endocytic pathway that transports proteins localized at the plasma membrane or extracellular cargos to an intracellular compartment, and (iv) the recycling pathway when proteins are sorted back to the plasma membrane (INADA; UEDA, 2014). Nevertheless, in view of the aims of this thesis, I will describe in detail the endocytic pathway only.

1.4.1 Plant endocytosis

Endocytosis can be defined as a dynamic process by which cells take up extracellular material and cell surface proteins via vesicle compartments and that is controlled by a network of regulatory proteins (FAN et al., 2015). In the last decade, endocytosis in plant cells has received considerable attention, demonstrating its pivotal role in a plethora of cellular

processes, including nutrient uptake, hormonal regulation, signaling transduction, and pathogen defense (DU et al., 2013; IRANI et al., 2012; LUSCHNIG; VERT, 2014; ROBATZEK; CHINCHILLA; BOLLER, 2006; TAKANO et al., 2005). Endocytosis has been more extensively studied in animals than in plants. Endocytosis in animals occurs through multiple mechanisms that fall into two main groups: phagocytosis that is related with the uptake of large particles and pinocytosis that is associated with the uptake of fluids and solutes. Pinocytosis can be further divided into macropinocytosis, clathrin-mediated endocytosis (CME), and clathrin-independent endocytosis (CIE) (CONNER; SCHMID, 2003). In plants, CME is accepted as the predominant endocytosis route and has been well characterized (BAISA; MAYERS; BEDNAREK, 2013; GADEYNE et al., 2014), whereas other mechanisms have only been suggested (BANDMANN; HOMANN, 2012; BARAL et al., 2015; LI et al., 2012).

1.4.2 Clathrin-mediated endocytosis

Presently, CME has been proposed to require a series of sequential and highly coordinated steps, including (i) nucleation that defines the sites on the plasma membrane where clathrin will be recruited and vesicles will bud, (ii) cargo recognition/selection, when cargo-specific adaptor proteins are mobilized, (iii) vesicle coat assembly, where clathrin triskelia are recruited from the cytosol to the adaptor proteins to help organize the formation of clathrin-coated pits (CCPs), (iv) scission, when CCPs maturate and are separated from the plasma membrane to form clathrin-coated vesicles (CCVs), and finally (v) uncoating, when the coat falls off from the CCVs, the uncoated vesicles fuse with an intracellular compartment for further processing and the clathrin machinery goes back into the cytoplasm to be reused in another round of CCV formation (BAISA; MAYERS; BEDNAREK, 2013; CHEN; IRANI; FRIML, 2011; FAN et al., 2015; MCMAHON; BOUCROT, 2011). These steps have been considered mainly based on research carried out in animals and yeast, but the CME machinery in plants is still poorly defined, despite the recent discoveries toward understanding CME in plants. However, CME seems to be evolutionarily conserved, because of the presence of several homologous CME effectors in plants, animals and yeast, but important kingdom-specific modifications have been evidenced (GADEYNE et al., 2014).

Clathrin consists of three heavy chain subunits and three light chain molecules that interact to self-assemble into a triskelion structure (FAN et al., 2015). The *Arabidopsis* genome encodes two functionally redundant clathrin heavy chain (CHC1 and CHC2) and three clathrin light chain (CLC1 to CLC3). Genetic studies of all five subunits support the conserved mechanism of CME in plants and its importance for growth and development (CHEN; IRANI;

FRIML, 2011; KITAKURA et al., 2011; WANG et al., 2013). The c*hc2* single mutants and dominant-negative CHC transgenic lines have been shown to be defective in bulk endocytosis and in internalization of plasma membrane proteins (KITAKURA et al., 2011).

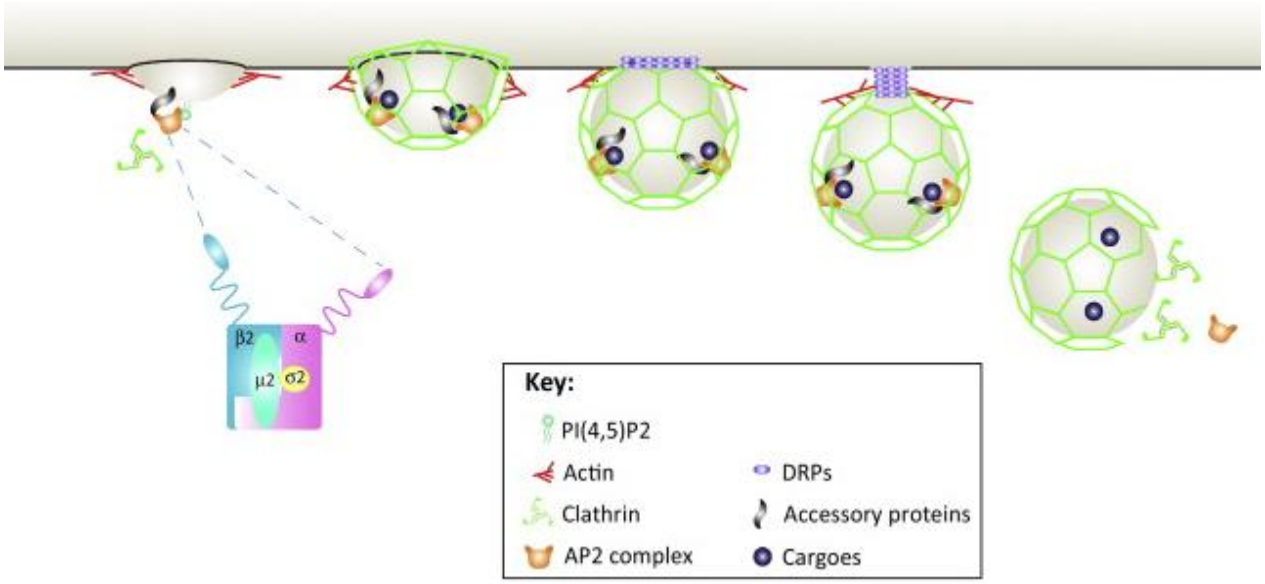

Figure 3 - Proposed model of clathrin-mediated endocytosis (CME) in plants (FAN et al., 2015). CME can be divided into five steps. (1) Clathrin-coated endocytic vesicle formation starts with the association of adaptor protein complex-2 (AP2)) with the plasma membrane (PM) via binding to phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2]. (2) The membrane-associated AP-2 recruits clathrin and the unidentified accessory proteins. After initiation, AP-2 binds to various cargo proteins through its σ2 and μ2 subunits. With the aid of accessory proteins, AP-2 continues to recruit clathrin, which polymerizes and forms a clathrin coat around the newly formed membrane invagination. (3) When the clathrin-coated vesicles (CCVs) mature, the GTPase dynamin-related protein (DRP) is recruited at the neck of the vesicle and (4) is responsible for the detachment of the vesicle from the PM. (5) Once the vesicles have been pinched off, the coated components are disassembled and release the cargo-containing endocytic vesicles into the cytoplasm. In plants, the cortical actin cytoskeleton has been implicated in the regulation of clathrin-coated pit (CCP) dynamics at the PM

Besides clathrin, other components of the CME machinery have been reported, including components of the adaptor protein complex 2 (AP2) that represents the core complex during the cargo recognition/selection of the CME in animals (COCUCCI et al., 2012). The AP2 complex of *Arabidopsis* that has been shown to be similar to its mammalian counterpart consists of four subunits (DI RUBBO et al., 2013) and the *ap-2* mutants of *Arabidopsis* have been found to be defective in BR responses and reproductive organ development (DI RUBBO et al., 2013; YAMAOKA et al., 2013). In support of the idea that CME presents evolutionary

modifications in plants, a unique multisubunit protein complex, designated the TPLATE complex (TPC), has been reported to be mobilized early on to endocytic plant foci during CME, without clear yeast and animal homologs (GADEYNE et al., 2014). The dynamin-related proteins DRP2A and DRP2B have also been described in *Arabidopsis* as players during the scission of CCPs (TAYLOR, 2011).

1.4.3 Intracellular destination of endocytic cargos

Following internalization from the plasma membrane, endocytosed material is delivered to the EEs. In plants, the TGN has been suggested to possess the EE function and to be the first site for endocytic cargo delivery (DETTMER et al., 2006; VIOTTI et al., 2010). From the TGN/EE the cargo may be recycled back to the plasma membrane or continues along the endocytic pathway for possible degradation (VIOTTI et al., 2010). Currently, the best characterized recycling pathway is that of the ADP-ribosylation factor guanine-nucleotide exchange factor (ARF-GEF) that is involved in the constitutive recycling of PIN-FORMED (PIN) proteins and BR receptor BRI1 (GELDNER et al., 2001; GELDNER et al., 2007; TEH; MOORE, 2007). In *Arabidopsis* the sorting functions of the TGN/EEs are associated with the V-ATPase activity (DETTMER et al., 2006) that can modulate the pH homeostasis within this organelle (LUO et al., 2015). Recently, it was shown that inhibition of V-ATPase activity causes substantial alkalinisation of the TGN/EE that in turn negatively affects recycling, providing evidence that acidification of this organelle is indispensable for this process in plants (LUO et al., 2015).

Whether the cargo is directed to be degraded instead of recycling, it is transported to the MVBs/late endosome with intraluminal vesicles that finally fuse to the vacuole (SINGH et al., 2014). In *Arabidopsis*, the endosomal maturation from the TGN/EE to MVB has been proposed to originate in a subdomain of the TGN/EE that recruits Rab5-like ARA7 and subsequently matures into an MVB (SCHEURING et al., 2011; SINGH et al., 2014). The intraluminal vesicles at the MVBs are responsible for the isolation of cargos from the cytosol and they are formed through the endosomal sorting complex required for transport (ESCRT) machinery (HENNE; BUCHKOVICH; EMR, 2011). Posttranslational modifications have been shown to regulate sorting of endocytic cargos. Ubiquitination is considered a targeting signal that directs membrane proteins for degradation (SCHEURING et al., 2012) and phosphorylation has also been reported as an important cue to regulate endocytosis; for instance, plasma membrane and constitutive endocytosis of PIN auxin transporters are misregulated when their phosphorylation state is altered (MICHNIEWICZ et al., 2007; ZHANG, JING et al., 2010).

1.4.4 Receptor-Mediated Endocytosis

Receptor-mediated endocytosis (RME) is the uptake of soluble ligands from the extracellular space, after binding to specific plasma membrane-localized receptors (DI RUBBO; RUSSINOVA, 2012). The first indications of RME in plants were obtained from the study of a ^{125}I-labeled elicitor that was able to bind the cell surface and to internalize in a temperature- and energy-dependent process into a soybean cell suspension, but neither the identity of the receptor nor the nature of the elicitor molecule were known (HORN; HEINSTEIN; LOW, 1989). Subsequently, studies of the plasma membrane receptors have demonstrated the involvement of RME in different physiological processes, including plant development and plant immunity, mainly through research on the BR receptor BRI1 (IRANI et al., 2012) and flg22 receptor FLS2 (BECK et al., 2012; ROBATZEK; CHINCHILLA; BOLLER, 2006).

The internalization of receptors can either be constitutive or ligand induced. BRI1 and FLS2 can constitutively recycle in the absence of their respective ligands, BR and flg22 (BECK et al., 2012; GELDNER et al., 2007). Moreover, after treatments with the ligands, both receptors undergo ligand-induced endocytosis (BECK et al., 2012; IRANI et al., 2012). However, differences in the temporal dynamics of the ligand-induced endocytosis of BRI1 (~2 min after ligand application) and FLS2 (~30 min after ligand application) are evidenced (BECK et al., 2012; CHOI et al., 2013; IRANI et al., 2012), suggesting that the RME pathways are regulated by distinct mechanisms in plant cells, probably related to the ligand nature.

Usually, after the ligand-binding of the receptors, various signaling events are triggered, leading to specific cell responses (GELDNER; ROBATZEK, 2008; HAN; SUN; CHAI, 2014).Therefore, RME functions as a spatial and temporal modulator of the signaling outputs by (i) attenuating the signaling strength or duration by moving the activated receptor to lytic compartments or (ii) re-localizing active receptor to signaling endosomes from where signaling is sustained or initiated *de novo* (DI RUBBO; RUSSINOVA, 2012; GELDNER; ROBATZEK, 2008; SORKIN; VON ZASTROW, 2009). Both functions have been well-documented in animals, but the interplay between RME and signaling in plants is still a matter of debate. Recently, in *Arabidopsis* has been found that blocking endocytosis of active BRI1-ligand complexes at the plasma membrane enhanced BR signaling, whereas retaining BRI1-ligand complexes at the TGN/EE did not affect signaling (IRANI et al., 2012). Thus, indicating that the majority of BR signaling is initiated from the plasma membrane pool of BRI1 and that its endocytosis is not essential for the downstream signaling activity. Different from BRI1 seems to be the case of *Le*Eix2 receptor in tobacco, as ligand-induced signaling triggered by EIX is

impaired when endocytosis of *Le*Eix2 is blocked (BAR; AVNI, 2009; 2014). Similar observations have been reported for the FLS2 endocytosis that, when blocked with wortmannin (an inhibitor of phosphoinositide 3-kinase and phosphoinositide 4-kinase) or by mutations in its phosphorylation sites, leads to defective pathogen responses due to impaired flg22 signaling (ROBATZEK; CHINCHILLA; BOLLER, 2006; SALOMON; ROBATZEK, 2006). Furthermore, recent work suggests that FLS2 trafficking might be associated differentially with the immunity responses triggered by flg22 (SMITH et al., 2014a, 2014b). These findings imply that appropriate RME trafficking may be required for specific signaling responses that could also take place in endosomal compartments; however, this remains to be clarified.

As the plant genomes encode hundreds of plasma membrane receptors (SHIU; BLEECKER, 2003; SHIU et al., 2004), it is tempting to argue that RME is a mechanism that is amply used to modulate physiological responses. However, future studies are needed to clarify the modulation of endocytic pathways, the machinery involved, and the biological role of endocytosis during signal transduction cascades. So far, RME has been mainly studied through live imaging of genetically engineered fluorescent protein-tagged receptors (GELDNER; ROBATZEK, 2006), but RME can also be visualized with fluorescently labeled ligands (DI RUBBO; RUSSINOVA, 2012). This approach would allow faster assays, because different plant genotypes could be assessed when treated with labeled ligands, avoiding plant transformation or crossing steps that are needed when fluorescent protein-tagged receptors are used. Nevertheless, labeling molecules is a challenging task, because addition of an extra molecule can easily abolish or modify the biological activity. Recently, a BR analog labeled with a small fluorophore, Alexa Fluor 647, allowed the specific tracking of the endocytosis of the BRI1-ligand complexes in *Arabidopsis* meristem root tip cells, hence showing the potential of this approach (IRANI et al., 2012). Therefore, for further understanding of plant endocytosis, the identification of *bona fide* endocytosed ligand cargos that can be labeled with molecular probes without compromising their biological activity is required.

1.5 Aims of this thesis

The main focus of my Ph.D. research has been to understand the subcellular dynamics of plant signaling peptides and their interplay with signaling that have not been reported yet. To this end, I chose as a model the well-characterized plant immunity-related peptide *At*Pep1, because information about its signaling responses and components are available, including the identification of its plasma membrane-localized receptors. Moreover, *At*Pep1 is able to trigger quick, but transient immunity responses, thus providing an excellent scenario to study trafficking and signaling in general. Therefore, the aims of this thesis were the following:

- Develop a fluorescent tool to study the subcellular dynamics of *At*Pep1
- Investigate whether *At*Pep1 and its receptor undergo internalization
- Elucidate the trafficking pathway, mechanism and machinery of the *At*Pep1 internalization
- Examine the role of endocytosis during the *At*Pep1 signaling
- Clarify the role of BAK1, the coreceptor of *At*Pep1 receptors, in the internalization and signaling of *At*Pep1

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5 CONCLUSION AND FUTURE PERSPECTIVES

In the past years, a growing number of signaling peptides have been discovered that play an active role in cell-to-cell communication networks in plants and knowledge has been acquired about their responses and signaling components (ALBERT, 2013; GHORBANI et al., 2014). Nonetheless, the subcellular fate and dynamics of these molecules remain to be elucidated as well as their association with signaling responses. In this thesis, we characterized a fluorescent probe that allowed us to examine the subcellular dynamics of the plant endogenous elicitor peptide *At*Pep1 and its receptors *in vivo*, hence, providing new information to the understanding of endocytic trafficking and its interplay with signaling during plant immunity responses. This work also opens research avenues in the field and proposes novel technical approaches to carry out future investigations.

5.1 Fluorescently labeled ligands as a tool to study ligand-receptor pairs

Plant genomes are predicted to encode hundreds of plasma membrane-localized receptor kinases (RKs) that potentially could recognize ligands by their extracellular domains, thus triggering signaling events that lead to specific cell responses (GELDNER; ROBATZEK, 2008; SHIU et al., 2004). Ligands are self- or not self-produced molecules, they can have different biological structures, among which peptides, steroids and oligosaccharides are found (ALBERT, 2013; GELDNER; ROBATZEK, 2008; HOTHORN et al., 2011). Within the endogenous ligands, numerous genes (approximately 1000) are found in *Arabidopsis thaliana* that encode small proteins giving rise to secreted peptides that would bind RKs (LEASE; WALKER, 2006). Nonetheless, in spite of the large ligand-receptor pairings that are possible, only a few ligand-receptor combinations have been identified and experimentally demonstrated. Characterization of these regulatory pairs is essential for the advancement of our understanding of communication networks in plants, but this task is challenging because the genes encoding RKs and peptides are often redundant and their low expression is restricted to a few cells and/or particular developmental stages or stress conditions (BUTENKO et al., 2014).

In this thesis, we showed that the peptide *At*Pep1 can be fluorescently tagged without loss of its biological activity and that at low concentrations the labeled *At*Pep1complexed with its receptor underwent internalization. This observation was similar to that reported previously for the fluorescent brassinosteroid (BR) AFCS and its receptor BRASSINOSTEROID INSENSITIVE1 (BRI1) (IRANI et al., 2012), thus showing that ligands with different natures, such peptides and steroids, can be labeled with fluorescent dyes and their subcellular dynamics can be assessed *in vivo.* Moreover, our results, showing that endocytosis of the *At*Pep1-induced

PEPRs was triggered by ligands, together with previous reports on ligand-induced endocytosis of RKs such as FLAGELLIN-SENSITIVE2 (FLS2) and BRI1 (IRANI et al., 2012; ROBATZEK; CHINCHILLA; BOLLER, 2006), confirmed that this process is a hallmark of RKs. Thus, we propose the use of labeled ligands combined with the ectopic expression of receptor genes in suitable plant cells for matching ligand-pair candidates by assessing endocytosis as read-out of their interaction. This strategy would avoid the use of radiolabeled ligands to identify receptor pairs that is technically inconvenient because of the manipulation of the radioactive material, high costs and short half-life of the probes (BUTENKO et al., 2014; YAMAGUCHI; PEARCE; RYAN, 2006).

In addition, redundancy, specificity and low expression of the potential receptors can be overcome by ectopic expression of fluorescently tagged candidates in plant cell systems, employed as models for cell biology studies. These systems include the transient *Agrobacterium*-mediated gene expression system in leaves of *Nicotiana benthamina* (WYDRO; KOZUBEK; LEHMANN, 2006), and that explored in this thesis, namely expression of GFP-fused PEPRs in the root epidermal meristem cells in *Arabidopsis*, by means of transgenic lines of the *At*Pep1 receptors under the *RPS5A* promoter (WEIJERS et al., 2001). However, this approach should be consider as an initial indicator of ligand-receptor interaction, as molecules could be co-internalized without necessarily interacting, therefore the ligand-receptor interaction must be further supported through additional approaches.

5.2 Release of *At*Peps, the missing piece of the puzzle

In this thesis, we elucidated the internalization pathway of the peptide *At*Pep1, which is probably similar to the other members of the family, because they all trigger a comparable set of downstream signaling responses (BARTELS et al., 2013; YAMAGUCHI et al., 2010). However, the internalization of *At*Peps would happen after the release of active peptides from their precursor proteins (PROPEPs) into the extracellular space, which has not been shown yet.

We found that PROPEP1-GFP was attached to the tonoplast, in agreement with previous reports (BARTELS et al., 2013) and additionally; we observed that probably it is accumulated into the vacuole. Thus, it is possible that the vacuole is an organelle where PROPEP1 is stored before the active *At*Pep1 is released. Mechanism, localization and timing of release of active *At*Peps are largely unknown, but still of great interest in the field (BARTELS; BOLLER, 2015). In an attempt to clarify these issues, we analyzed the subcellular localization of PROPEP1-GFP when treated with a cocktail composed by the MAMPs flg22 and *At*Pep1 that generates a biotic stress. As we did not observe any apparent changes, we hypothesized that the PROPEP1 would

be delivered only when the cell integrity is compromised and, subsequently, that the protease action would cleave the active *At*Pep1, which, in turn, would bind the receptors located at the plasma membrane of neighboring cells, thus triggering defense responses. This assumption fits into the proposed damage model for activation of the *At*Peps-PEPRs (BARTELS; BOLLER, 2015). However this could be not the case for all PROPEPs, because PROPEP3-YFP accumulated in the cytosol (BARTELS et al., 2013). The accumulation of PROPEPs at two different subcellular compartments might hint at differences between release of the active *At*Peps and their contributions to the cellular immunity. Furthermore, because our transgenic plants expressing *35S:PROPEP1-GFP* did not up-regulate the A*t*Pep1-responsive genes, as seen for the lines expressing *35S:PROPEP1* (HUFFAKER; RYAN, 2007), we cannot disregard that the vacuolar localization and/or the non-secretion of PROPEP1-GFP upon biotic stresses are due to a disfunctionality of this chimeric protein.

Therefore, based on our data and published results, we propose that future experiments with the aim at solving the secretion mechanism of *At*Peps should consider the generation of transgenic plants expressing PROPEPs with a fluorescent tag located between the C-terminus of the active peptide-containing PROPEPs and the N-terminus with the precursor protein, thus allowing an eventual C-terminus cleavage given origin to possibly active GFP-*At*Peps. These plants should be exposed to different stresses, such as MAMPs and damage treatments, and subsequently the subcellular localization of the chimeric proteins should be assessed *in vivo*. Damage treatment could be carry out through laser ablation associated with confocal microscopy. This minimally invasive approach would allow mimicking more accurately the injury caused by invasive organisms.

Approaches that don not include the use of fused proteins can also be proposed, such as immunolocalization and cell fractionation. Immunolocalization would permit to identify the localization of PROPEPs or *At*Peps *in situ* at subcellular resolution avoiding the risk of inducing side effects by a fusion protein, such as misexpression, mistargeting and altered stability; however, its use depends on specific antibodies that would recognize the protein of interest (PACIOREK et al., 2005). Currently, it has not been reported any antibody that would recognize PROPEPs or *At*Peps, which limits the use of this technique to study their subcellular dynamics. Cell fractionation that allows the separation of organelles according to their density, combined with mass spectrometry could also help to identify the subcellular localization of PROPEs and/or *At*Peps under different physiological conditions.

5.3 *At*Pep1/PEPR internalization reveals different endocytic pathways

In plants, endocytic trafficking requires sequential steps through the *trans*-Golgi network/early endosome (TGN/EE) that is the first place where endocytosed material is delivered. Moreover, the TGN/EE functions as a sorting station from where the cargo is further sorted back to the plasma membrane or into multivesicular bodies (MVBs)/late endosomes that fuse with the vacuole to release their intraluminal vesicles for degradation (VIOTTI et al., 2010).

Here, we found that the *At*Pep1-PEPR complexes are transported from the plasma membrane to the vacuole following a common endocytic trafficking route. However, distinct modes of receptor-mediated endocytosis (RME) might coexist in plant cells, because the spatio-temporal cellular dynamics of *At*Pep1-PEPRs showed unique features in comparison with the described subcellular dynamics of AFCS-BRI1 and partially overlapped with the FLS2 behavior upon flg22 treatment. These features can be listed as: (i) inactive PEPRs from the plasma membrane did not accumulate in large bodies in the presence of the fungal toxin brefeldin A (BFA) that inhibits protein secretion and vesicle recycling of endocytosed proteins to the plasma membrane (GELDNER et al., 2003), as seen for FLS2 and BRI1 that undergo constitutive endocytic recycling under steady-state conditions (BECK et al., 2012; GELDNER et al., 2007; IRANI et al., 2012); (ii) the temporal dynamics of the internalization of *At*Pep1-PEPR complexes differed from those of BRI1-AFCS that internalized more quickly (2 min after ligand application) (IRANI et al., 2012), but matched more those of the FLS2 receptor, in which endosomes were visualized in leaves 30 min after flg22 application (ROBATZEK; CHINCHILLA; BOLLER, 2006); (iii) the molecular probe of *At*Pep1 (TAMRA-*At*Pep1) presented low colocalization with the TGN/EE marker VHA-a1, as seen for flg22-activated FLS2 (CHOI et al., 2013), in contrast to the AFCS-BRI1 complex that highly colabeled with this TGN/EE marker (IRANI et al., 2012); and finally, (iv) the endocytic trafficking of *At*Pep1-PEPR complexes was not blocked by ConcA, which inhibits the V-ATPase activity in the TGN/EE, leading to an increase in the pH of this organelle and blocking the vacuolar transport of endocytic cargos, as observed for AFCS-BRI1 (DETTMER et al., 2006; IRANI et al., 2012; SHEN et al., 2013). For flg22-activated FLS2, trafficking was not largely impaired in the presence of ConcA and colocalization with endosomal compartments occurred (BECK et al., 2012), suggesting that its transport is not blocked as for *At*Pep1-PEPR complexes.

The apparent existence of distinct RME pathways raises interesting questions about their regulation. As *At*Pep1 and the bacterial peptide flg22 are elicitor peptides that, albeit with a different origin, activate similar swift and transient plant immunity responses under biotic

stresses (BARTELS; BOLLER, 2015), whereas AFCS is an analog of the ubiquitous plant hormone BR mainly associated to plant growth and development (IRANI et al., 2012), it is tempting to argue that RME is modulated by the nature of the ligand.

This ligand modulation could be mediated by pH changes, because an early hallmark of *At*Pep1 and flg22 activities is the induction of changes in the ion fluxes, including H^+ and Ca^{2+} influxes, leading to membrane depolarization and extracellular alkalinization (KROL et al., 2010; MITHÖFER; EBEL; FELLE, 2005; YAMAGUCHI; PEARCE; RYAN, 2006). Interestingly, the opposite effect is triggered by BR that induces hyperpolarization of the plasma membrane and slight medium acidification (ZHANG et al., 2005). Furthermore, regulation and pH homeostasis within intracellular compartments are well known to be essential for the viability of all eukaryotic cells, in which each endomembrane compartment presents particular luminal pH-dependent environments that assure their optimal operation and fulfillment of their specific functions (CASEY; GRINSTEIN; ORLOWSKI, 2010; SCHUMACHER, 2014; SHEN et al., 2013). In animals, the pH participation during the endomembrane trafficking is evidenced by its role that modulates luminal processes, such as proteolytic processing and receptor-ligand interaction. Moreover, pH has been shown to affect recruitment of the trafficking machinery components to the cytosolic membrane face and cargo sorting (HUANG; CHANG, 2011; MARANDA et al., 2001; MARSHANSKY; FUTAI, 2008).

Based on the results of this thesis and previous literature reports, we hypothesize that a refined model for RME exists that relies on a pH-dependent cargo sorting mechanism that operates at the plasma membrane, as illustrated by the subcellular dynamics of *At*Pep1-PEPRs and AFCS-BRI1 (Figure 1). Thus, we propose a scenario in which after the *At*Pep1 binding of PEPRs a rapid change is triggered in the ion fluxes, leading to membrane depolarization and extracellular alkalinization and dictating the cues for recruitment of the endocytic machinery. Moreover, the alkaline environment would maintain the interaction between *At*Pep1 and PEPRs that had been shown to depend on the pH (TANG et al., 2015). Later, between 15-20 min after the high alkalinization activity has been found (HUFFAKER; PEARCE; RYAN, 2006), the transport of *At*Pep1-PEPR complexes from the plasma membrane to the intracellular compartments begins. Then, the *At*Pep1-PEPR complexes are transported via a VHA-a1-negative SYP42/SYP61/ARA7-positive TGN/EE population or subdomains that rapidly mature, first into ARA7/ARA6 compartments and then into ARA7/ARA6/VAMP727-positive compartments, which fuse with the vacuole where the *At*Pep1-PEPR complexes are released for final dissociation and degradation. Moreover, the compartments through which the *At*Pep1-

PEPR complexes are transported might have an alkaline pH, thus ensuring that peptides and receptors reach the lytic vacuoles as a complex.

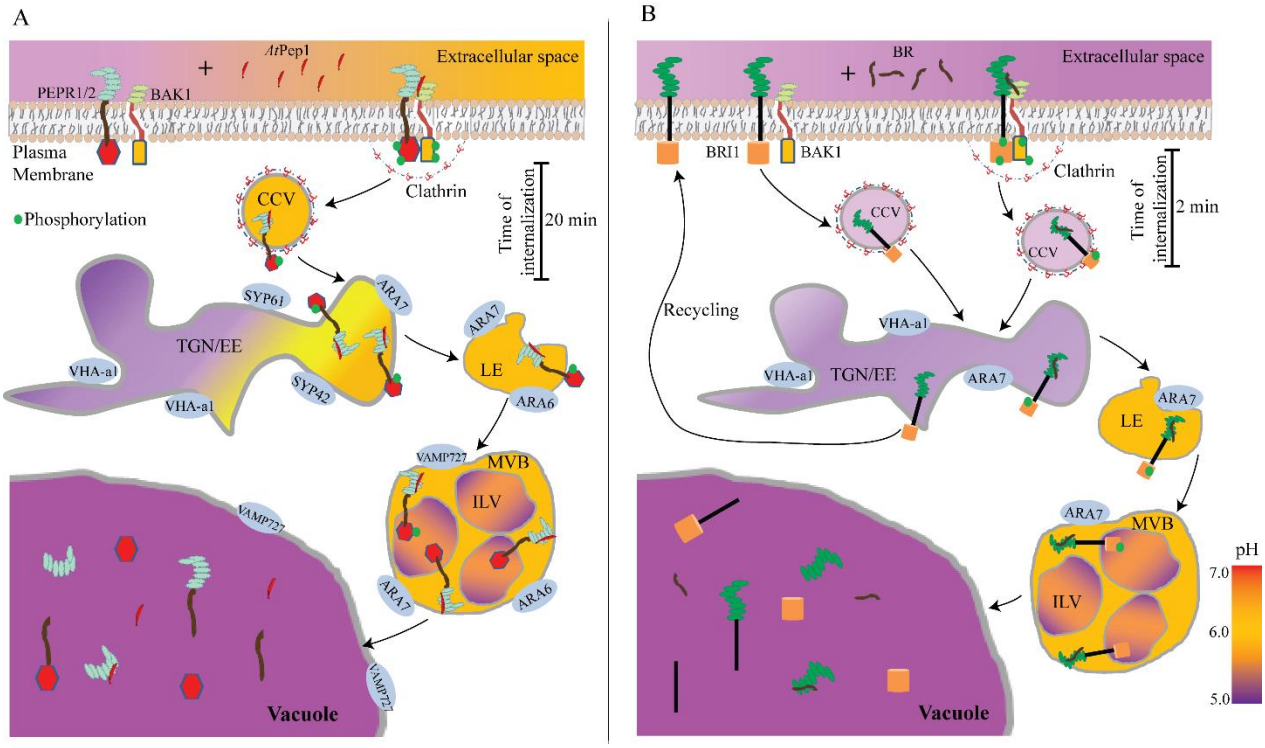

Figure 1 – Schematic overview of the endocytic pathways of PEPRs and BRI1. (A) After *At*Pep1 binding, PEPRs associates with BAK1, triggering phosphorylation of the kinase domains and extracellular alkalinization. After 20 min *At*Pep1-PEPRs undergoes endocytosis mediated by clathrin and are sorted in specific SYP42/SYP61/ARA7-positive subdomains of the *trans*-Golgi network/early endosomes (TGN/EE). Here, the complex rapidly maturate into ARA7/ARA6-positive late endosomes (LE) and is directed to the vacuole via ARA7/ARA6/VAMP727-positive multivesicular bodies (MVB). (**B**) Independent of ligand BRI1 undergoes constitutive endocytosis. In this case BRI1 is targeted to TGN/EE, from where it is recycled back to the plasma membrane. *Upon brassinosteroid (BR) binding, BRI1* form complex with BAK1, triggering phosphorylation events and slight extracellular acidification. Quickly, activated BIR1 undergoes endocytosis mediated by clathrin and is sorted to TGN/EE. Subsequently, BR-BRI1 mature to late endosomes and is targeted to the vacuole via MVB. Endocytosis of BR-BRI1 requires a functional TGN/EE localized V-ATPase VHA-a1, but not *At*Pep1-PEPRs. For simplicity only the PEPRs, BRI1 and BAK1 are shown in the schematic representation, without referring to other regulators of PEPRs and BRI1. CCV, clathrin coated vesicle; ILV, *intraluminal vesicles*

However, to verify our hypothetic model, we need to prove that the VHA-a1-negative TGN/EE population or subdomains indeed exist or appear under *At*Pep1 elicitation. To this end, it would be interesting to generate plant genotypes expressing TGN markers (VHA-a1 and SYP42 or SYP61) in combination with PEPRs and the endosomal marker ARA7 fused to

fluorescent proteins with different spectrums, and to evaluate their localization upon *At*Pep1 stimulation over time. Another point that has to be assessed is the pH behavior in the different endocytic compartments during the ligand-induced internalization of PEPRs and BRI1, which would provide information to understand how different cargos influence the pH of subcellular compartments and the relationship with RME. An interesting strategy would be to fuse PEPRs and BRI1 to pH-fluorescent protein sensors, such as PEpHluorin and PrpHluorin, that permit *in vivo* measurements (SHEN et al., 2013) and to assess the pH of the different endomembrane compartments through which ligand-activated PEPRs and BRI1 are transported.

5.4 The *At*Pep1-PEPR system, a new suitable model to study clathrin-mediated endocytosis in plants and its implication in immunity

In this thesis, we provide solid proof that the endocytosis of *At*Pep1-PEPRs is mediated by clathrin proteins, supporting its role as the primary endocytic route in plants (BAISA; MAYERS; BEDNAREK, 2013). Previously, endocytosis of BRI1, a membrane-localized RK structurally similar to *At*Pep1 receptors had also been found to depend on clathrin (IRANI et al., 2012) indicating that clathrin-mediated endocytosis (CME) can be a conserved mechanism for RK internalization. We propose that at least two different ligand-induced endocytic pathways exist; as exemplified by the spatio-temporal dynamics of *At*Pep1-PEPRs and AFCS-BRI1, part of their endocytic machinery is probably pretty well conserved, including clathrin proteins, but some specificity-dictating components, which remain to be identified, might also occur. This specificity could be determined by adaptor proteins that recruit clathrin to perform the coat assembly of the specific cargos, because clathrin cannot bind directly to the membrane (LEE; HWANG, 2014; SCHMID; MCMAHON, 2007).

The role of adaptor proteins for cargo selection has been characterized extensively in animal and yeast cells (SCHMID; MCMAHON, 2007; TRAUB, 2009). Although plant CME is far away from having well-defined CME network as described in animals, in the past decade, significant progress has been made in understanding and identifying the CME components in plants. For instance, the role of the adaptor protein complex 2 (AP2) that represents the core complex during the cargo recognition/selection of the CME in animals has also been reported to mediate CME in plants (DI RUBBO et al., 2013); more recently, the TPLATE adaptor complex (TPC) that consists of eight core subunits has been found to accumulate at the plasma membrane, preceding the recruitment of future components for formation of clathrin coat vesicles (GADEYNE et al., 2014). However, to get a deeper insight into the CME in plants, it is still necessary to identify the functional CME constituents as well as to elucidate how they

participate in the endocytosis of cargos with different physiological roles and subcellular dynamics. A starting point could be the identification of the interactome of proteins that undergo CME, providing refined information about the missing CME components. To this end, PEPRs appear excellent candidates due to the *At*Pep1 inducibility, temporal dynamics and transient behavior of their endocytosis. Hence, it would be possible to sequentially assess the endocytic machinery associated with PEPRs through the different steps of CME. These endocytic features of PEPRs also turn these receptors into good candidates to study *in vivo* the recruitment dynamics of the clathrin assembly machinery at the plasma membrane by means of high-end microscopy techniques, such as total internal reaction fluorescence (TIRF)/variable angle epifluorescence microscope (VAEM) and Spinning Disc Microscopy, which allow the evaluation of the behavior of candidate proteins at the plasma membrane.

During this thesis, we also detected that CME impairment compromised the *At*Pep1 responses, thus providing evidence that pattern-triggered immunity (PTI) can be regulated by CME, a physiological role not reported so far. Although the defects in the *At*Pep1 responses could not be attributed to a specific inhibition of the endocytosis of *At*Pep1-PEPRs because the CME is affected in a general manner, these defects could also reflect impairment of the endosomal signaling that would not operate when endocytosis is blocked. Therefore, tools have to be developed that allow specific blocking of the *At*Pep1-PEPR endocytosis. Remarkably, we identified putative endocytic motifs at the cytoplasmic domain of the *At*Pep1 receptors (Figure 2) that are carried by proteins interacting with the AP2 complex and clathrin (GADEYNE et al., 2014; TRAUB, 2009). It would be worthwhile to generate specific mutations into these endocytic motifs and to evaluate whether through this approach the *At*Pep1-PEPR trafficking could be blocked specifically. This tool could be used to address issues about endosomal signaling in plants.

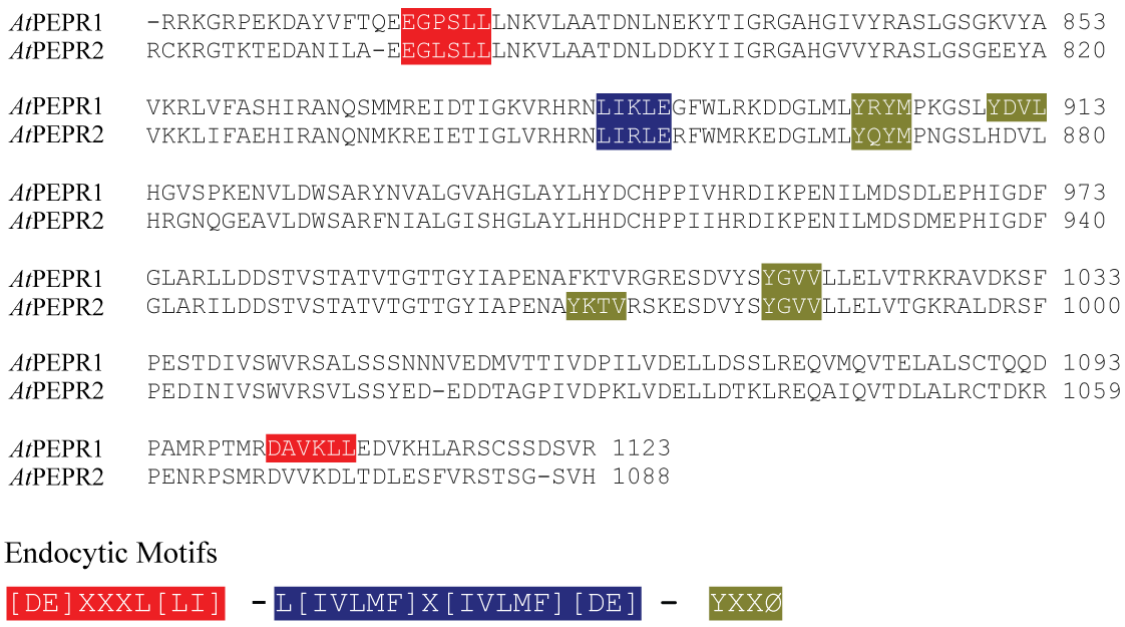

Figure 2 – Alignment of the cytoplasmic domain of *At*Pep1 receptors displaying putative protein endocytic motifs. [DE]XXXL[LI]and YXXØ function as cargo sorting signals; L[IVLMF]X[IVLMF][DE] is a clathrin binding motif. Endocytosis motifs were manually searched using the ScanProsite tool from the bioinformatics research portal expasy (http://prosite.expasy.org/scanprosite/)

5.5 BAK1 modulates endocytosis and signaling of the plant elicitor peptide *At*Pep1

Through the use of the hypoactive kinase *bak1-5*, we showed that endocytosis of *At*Pep1 is modulated by BAK1, which interacts with *At*Pep1 receptors in a ligand-dependent manner. As *bak1-5* presents altered phosphorylation patterns (SCHWESSINGER et al., 2011; WANG et al., 2014), it is possible to argue that phosphorylation events occurring after the PEPR1-BAK1 heterodimerization upon *At*Pep1 treatments dictate the bases for recruitment of the endocytic machinery. Moreover, we also confirmed that the *At*Pep1 signaling is largely impaired in *bak1-5*, thus raising the question whether there is interplay between endocytosis and signaling or whether they are processes regulated independently by phosphorylation. Examination of different phosphorylating *bak1* mutants would help to clarify this issue. Another mutant worthwhile to assess is *bak1-4*/proBAK1:BAK1* that expresses a BAK1-5 kinase inactive in the null *bak1-4* mutant and does not strongly inhibit immunity responses, as seen for *bak1-5* and *bak1-4*/proBAK1::BAK1-5 (SCHWESSINGER et al., 2011); therefore, if endocytosis and signaling would regulate each other, a lower impact in the *At*Pep1 internalization is expected in this genotype.

Interestingly, we also found that overexpression of BAK1 leads to a dominant-negative effect in *At*Pep1 endocytosis and signaling, suggesting that BAK1 could be required to attenuate and balance the system after *At*Pep1 elicitation; however, this effect is not well understood and

further experiments are needed. It would be relevant to generate transgenic plants with different levels of BAK1 expression and to examine whether there is a dose-response effect in the endocytosis and signaling, as expected if this effect indeed represents a biological process.

An open question that remains to be elucidated is whether BAK1 undergoes internalization together with the *At*Pep1-PEPR complexes. To address this question is a challenging task because the C-Terminal fusion proteins antagonize the BAK1 activity linked to immunity responses (NTOUKAKIS et al., 2011); hence, various versions of biologically active fluorescent BAK1 tags have to be engineered. A good strategy would be to introduce the fluorescent protein into the juxtamembrane domain of BAK1 without compromising the activity of the kinase domain localized at the C-terminus. As a specific antibody of BAK1 is available, another approach that could be explored to solve this question is the antibody-based immunological detection, thus avoiding the risks of side effects by the fusion of a protein to BAK1.

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Curriculum vitae

Professional profile

Professional experience in genetics, plant physiology, and molecular biology. Proficiency at analysis of gene expression analysis, plant genetic transformation (sugarcane and *Arabidopsis*), confocal microscopy, in-vitro tissue culture, data acquisition and processing. With ability to work with a great diversity of personalities and people from divergent backgrounds and skill sets.

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Meetings attended

2015

- 26th International Conference on Arabidopsis Research (ICAR), Oral Presentation
  Paris, France
- VIB seminar 2015, Oral presentation
  Blankenberge, Belgium

2014

- 17th European Network of Plant Endomembrane Research (ENPER) meeting, Oral Presentation
  Lecce, Italy

2010

- XII Brazilian congresso of Plant physyology, Poster Presentation
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Teaching experience

- October - December 2014: Supervision of Masters 1 thesis in Biochemistry and Biotechnology, Ghent University
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