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OLIGOGALACTURONIDE SIGNALLING TRASDUCTION

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I. INTRODUCTION

I. 1. Plant immunity

In the course of their development, plants have to face a wide range of potential pathogens, including viruses, bacteria, fungi, oomycetes, nematodes, and insects.(Garcia-Brugger et al., 2006).However, disease is an exception rather than a rule and infections occur in only limited cases. To effectively ward off pathogenic microbes, plants must recognize the intruders and activate a battery of defenses that collectively arrest the pathogen. Unlike vertebrate animals that possess both acquired immunity and innate immunity, plants rely solely on innate immunity. The long history of plant–pathogen associations led to the evolution of multiple surveillance mechanisms in the plant (Zhang et al., 2010)Such protective mechanisms are found in all multicellular organisms and are collectively referred to as innate immunity (Medzhitov and Janeway, Jr., 1997; Akira et al., 2006). Because of their sessile lifestyle, plants cannot run away from invaders and need to defend themselves from threatening organisms by mounting a wide array of defense responses in a timely manner. Due to the absence of an adaptive immune system, plants rely on a so-called “innate immune system”, analogous to that found in animals (Nurnberger et al., 2004; Gomez-Gomez, 2004)

Plant pathogens use diverse life strategies. Pathogenic bacteria proliferate in intercellular spaces (the apoplast) after entering

through gas or water pores (stomata and hydathodes, respectively), or gain access via wounds. Nematodes and aphids feed by inserting a stylet directly into a plant cell. Fungi can directly enter plant epidermal cells, or extend hyphae on top of, between, or through plant cells. Pathogenic and symbiotic fungi and oomycetes can invaginate feeding structures (haustoria), into the host cell plasma membrane. Haustorial plasma membranes, the extracellular matrix, and host plasma membranes form an intimate interface at which the outcome of the interaction is determined (Jones and Dangl, 2006). Therefore, to be pathogenic, most microbes must access the plant interior, either by penetrating the leaf or root surface directly or by entering through wounds or natural openings such as stomata, pores in the underside of the leaf used for gas exchange. Once the plant interior has been breached, microbes are faced with another obstacle: the plant cell wall, a rigid, cellulose-based support surrounding every cell. Penetration of the cell wall exposes the host plasma membrane to the microbe, where they encounter extracellular surface receptors that recognize pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) (Nurnberger and Kemmerling, 2006). Perception of a microorganism at the cell surface initiates PAMP-triggered immunity (PTI) (Dodds and Rathjen, 2010), which usually halts infection before the microbe gains a hold in the plant. Signals similar to PAMPs may arise from the plant itself because of the

damage caused by microbes, which are now described as damage-associated molecular patterns (DAMPs) (Lotze et al., 2007) and can trigger PTI as well.

To evade PTI, adapted pathogens secrete effector molecules into the plant cells that interfere with recognition at the plasma membrane and suppress pattern-triggered responses (Fig.I. 1). Effectors may also enforce metabolic shifts on the host plant which are beneficial for the attacker. Once pathogens acquired the capacity to suppress primary defences, plants developed a more specialized mechanism to detect microbes, referred to as effector-triggered immunity (ETI) (Dodds and Rathjen, 2010).In turn, plants express intracellular resistance (R) proteins that directly or indirectly recognize the effectors or sense their presence through perturbation of endogenous effector targets. The resulting Effector-triggered immunity (ETI) is aqualitatively stronger and faster immune reaction then those triggered by MAMPs (Dodds and Rathjen, 2010). ETI and MTI responses are often overlapping although distinct differences exist. For example, the hypersensitive response (HR), a type of localized programmed cell death, most often follows R-mediated resistance, while callose deposition and cell wall fortification are commonly associated with PRR-triggered resistance.

Not surprisingly, pathogens seem to have adapted effectors to interfere with ETI. These effectors may in turn be sensed by

another set of R proteins, reflecting an evolutionary arms race between the plant and the microbe.

For many years view of the plant immune system was represented as a four phased 'zigzag' model (Fig.I. 2). In phase 1, PAMPs are recognized by PRRs, resulting in PTI that can halt further colonization. In phase 2, successful pathogens deploy effectors that contribute to pathogen virulence.

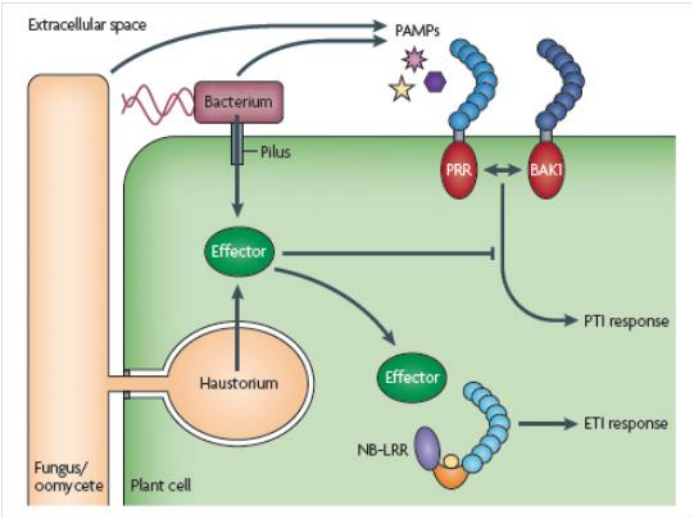

Fig I.1: **The plant immunity**. Recognition of pathogen-associated molecular patterns (such as bacterial flagellin) by cell surface pattern recognition receptors (PRRs) promptly triggers PTI leading to basal immunity. Many PRRs interact with the related protein BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1) to initiate the PTI signalling pathway. Pathogenic bacteria use the type III secretion system to deliver effector proteins that target multiple host proteins to suppress basal immune responses. Plant resistance proteins (such as NB-LRR) recognize effector activity and restore resistance through effector-triggered immune responses (ETI). Adapted from (Dodds and Rathjen, 2010).

Effectors can interfere with PTI. This results in effector-triggered susceptibility (ETS). In phase 3, a given effector is 'specifically recognized' by one of the NB-LRR proteins, resulting in effector-triggered immunity (ETI). Recognition is either indirect, or through direct NB-LRR recognition of an effector. ETI is an accelerated and amplified PTI response, resulting in disease resistance and, usually, a hypersensitive cell death response (HR) at the infection site. In phase 4, natural selection drives pathogens to avoid ETI either by shedding or diversifying the recognized effector gene, or by acquiring additional effectors that suppress ETI. Natural selection results in new R specificities so that ETI can be triggered again.

In recent work (Boller and Felix, 2009) it was proposed a new way to explain plant immunity in which effective innate immunity in plants, as in the case of innate immunity in vertebrates, is mediated through a single overarching principle, the perception of signals of danger. What may be categorized as PAMPs (or MAMPs), DAMPs, and effectors, might appear to the plant as one and the same type of signal that indicates a situation of danger (Fig.I. 3).

Indeed, gene expression data indicate that considerable overlap exists between the defense response induced by MAMPs, effectors, and endogenous elicitors. It remains to be seen, as an important challenge for future research, how signalling through

MAMPs, endogenous DAMPs, and effectors converges into a stereotypical defense response.

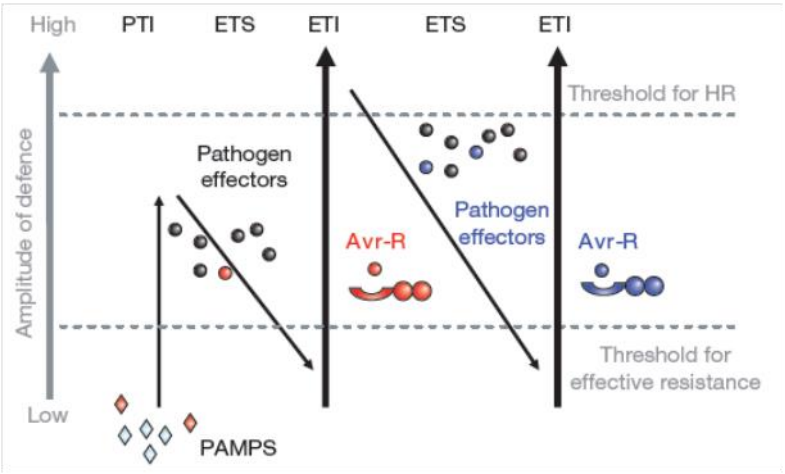

Fig I. 2: **A zigzag model illustrates the quantitative output of the plant immune system.** In this scheme, the ultimate amplitude of disease resistance or susceptibility is proportional to [PTI – ETS1ETI]. In phase 1, plants detect microbial/pathogen-associated molecular patterns (MAMPs/ PAMPs, red diamonds) via PRRs to trigger PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, pathogen isolates are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue)—these can help pathogens to suppress ETI. Selection favours new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI. Adapted from (Jones and Dangl, 2006).

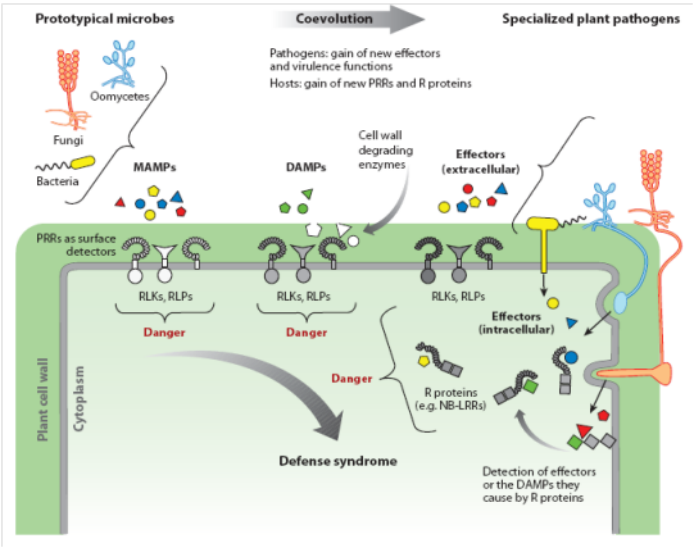

Fig.I. 3: **Microbe-associated molecular patterns (MAMPs), damage-associated molecular patterns (DAMPs), and effectors are perceived as signals of danger.** Extracellular MAMPs of prototypical microbes and DAMPs released by their enzymes are recognized through pattern recognition receptors (PRRs). In the course of coevolution, pathogens gain effectors as virulence factors, and plants evolve new PRRs and resistance (R) proteins to perceive the effectors. When MAMPs, DAMPs, and effectors are recognized by PRRs and R proteins, a stereotypical defense syndrome is induced. RLK, receptor-like kinase; RLP, receptor-like protein; NB-LRR, nucleotide binding-site–leucine-rich repeat. Adapted from (Boller and Felix, 2009).

I. 2. Basal defence

Induction of PTI in response to PAMPs or DAMPs occurs in both host and non-host plant species and is based on basal defense mechanisms. Studies of the effects of PAMPs and DAMPs point to a stereotypical response, indicating that the plant

defensive responses overlap considerably. Indeed, the early physiological and biochemical events and the signaling requirements are similar for each type of interaction. Differences exist in the timing and strength of the responses (Da Cunha et al., 2006).

Very Early Responses (1–5 Minutes):

- **Ion fluxes**

Among the earliest and most easily detectable physiological response to MAMPs and DAMPs in plant cell cultures, occurring ~0.5–2 min, is an alkalinization of the growth medium due to changes of ion fluxes across the plasma membrane (Boller, 1995; Nurnberger et al., 2004). These changes include increased influx of H^+ and Ca^{2+} and a concomitant efflux of K^+; an efflux of anions, in particular of nitrate, has also been observed (Wendehenne et al., 2002). The ion fluxes lead to membrane depolarization. PAMPs and DAMPs are known to stimulate an influx of Ca^{2+} from the apoplast and cause a rapid increase in cytoplasmic Ca^{2+} concentrations, which might serve as second messenger to promote the opening of other membrane channels (Blume et al., 2000; Lecourieux et al., 2002), or to activate calcium-dependent protein kinases (Boudsocq et al., 2010).

- **Oxidative burst**

PAMPs and DAMPs induce, with a lag phase of ~2 min, a rapid and transient ROS production, known us oxidative burst (Chinchilla et al., 2007). Reactive oxygen species (ROS) can act as antibiotic agents directly and may contribute indirectly to defense by causing cell wall crosslinking.

Indeed, the quantities of reactive oxygen species produced can be cytotoxic and thus are expected to be antimicrobial. Also, reactive oxygen species drive the rapid peroxidase-mediated oxidative cross-linking of cell wall lignins, proteins, and carbohydrates, thereby reinforcing the wall against enzymatic maceration by the pathogen (Cote and Hahn, 1994a). In recent years, it has become evident that at low/moderate concentration ROS are an important second messengers in plants controlling processes such as growth, development, response to biotic and abiotic environmental stimuli, stomatal closure and programmed cell death (Bailey-Serres and Mittler, 2006).

It comes the evolution of efficient scavenging mechanisms that make the plant cells able to overcome ROS toxicity and led to the use of several of these ephemeral reactive molecules as signal transducers.

O_2- generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are generally considered to be a major enzymatic source of ROS in the oxidative burst of plant cells

challenged with pathogens or elicitors (Torres and Dangl, 2005; Torres et al., 2006).

In Arabidopsis, several genes encoding proteins with high similarity to the mammalian NADPH oxidase gp91phox subunit have been characterized. Among them, AtrbohD is required for the production of ROS during infection with different bacterial and fungal pathogens, including *B. cinerea* (Torres and Dangl, 2005) (Torres et al., 2006).

Besides NADPH oxidases, other enzymes appear to be important in the elicitor-mediated oxidative burst, including include class III peroxidases, oxalate oxidases, amine oxidases (Allan and Fluhr, 1997),lipoxygenases, quinone reductase (Dumas et al., 1993) which generate either O_2- or H_2O_2.

- **Activation of MAPKs**

An early response to PAMP and DAMP signals is an activation of Mitogen-Activated Protein Kinase (MAPK) cascades (Pedley and Martin, 2005).

Mitogen-activated protein kinases constitute central points of cross-talk in stress signaling in plants including the protection against microbial invasion. It has become evident that mitogen activated protein kinase (MAPK) cascades play some of the most essential roles in plant signal transduction pathways from cell

division to cell death. The MAPK phosphorylation cascade is a highly evolutionarily conserved signaling modules with essential regulatory functions in eukaryotes, including yeasts, worms, flies, frogs, mammals and plants(Tena et al., 2001).

A MAPK cascade consists of a core module of three kinases that act in sequence: a MAPK kinase kinase (MAPKKK) that activates, via phosphorylation, a MAPK kinase (MAPKK), which activates a MAPK (Fig. 4). The A. thaliana genome is characterized by 20 MAPKs that are activated by about 10 MAPK kinases (MAPKK), which themselves are under the regulatory control of approximately 60 MAPKK kinases (Nurnberger et al., 2004).

The high number of genes for MAPK cascade components indicates that plants rely heavily upon MAPK cascades for signal transduction.

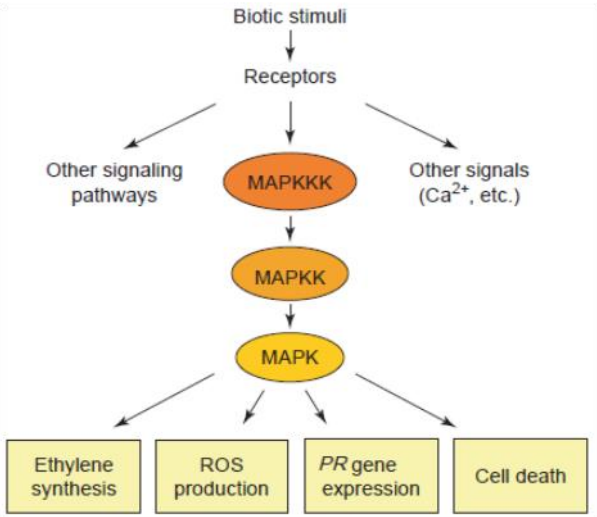

Figure 4. MAPK cascades and the cellular responses they influence following the recognition of microbial pathogens. Adapted from (Pedley and Martin, 2005).

Of the 20 identified MAPKs in Arabidopsis, only three MAPKs such as MPK3, MPK4 and MPK6 are known are known to play a key role in the regulation of signaling mediated by PAMPs (Cheong and Kim, 2010)

In particular, in Arabidopsis, a MAPK cascade, leading to AtMPK3 and AtMPK6 activation, is required for flg22-mediated responses (Asai et al., 2002). In *Arabidopsis* stimulated with flg22, a transient increase in AtMPK6 activity was observed, starting with a lag phase of ∼1–2 min and peaking after 5–10 min

(Nuhse et al., 2000). DAMPs such as AtPep1 similarly induce a MAPK cascade (Huffaker et al., 2006).

- **Phospatase and Changes in protein phosphorylation**

Protein phosphorylation is one of the major mechanisms for controlling many cellular processes in all living organisms. Those, the balance in the phospho-regulation is critical to maintain a normal cell survival state. Protein kinases and phosphatases have a pivotal role in maintaining the phospho-regulation in normal conditions and modulate this balance in adverse conditions (Singh and Pandey, 2012)

In particular the protein phosphatases (PPs), neutralize the action of the protein kinases by dephosphorylation, ensuring fast regulation of signaling. The essential nature of the protein phosphatase function is reflected in its highly conservation throughout evolution (Rodriguez, 1998).

Inactivation of MAPKs can be performed by different PPs. In plants have been characterized several protein phosphatases, that are able to inactivate MAPKs, at least *in vitro*.

Both in animal and in plants, the protein phosphatases, based on the amino acid residue they dephosphorylate, are classified into two major groups, serine/threonine (Ser/Thr) and tyrosine phosphatases . In plants the phosphatases are, also, defined by at least three distinct families. The PPP and PPM families consist of

Ser/Thr phosphatases, and the protein tyrosine phosphatase (PTP) family includes both tyrosine-specific and dual-specificity phosphatases (DSPs) (Luan, 2003).

The PPP family, according to the adopted nomenclature for the human proteins, includes PP1, PP2A and PP2B, whereas PPM family includes the protein phosphatases of type 2C (PP2C) and other Mg2+ dependent phosphatases (Singh and Pandey, 2012).

PP1C and PP2B share a common and highly conserved catalytic domain structure, while PP2C are highly diversified.

Unlike animals that produce only a few isoforms of PP2C, in higher plants the PP2C are the major class of protein phosphatise (Luan, 2003).

Recent microarray analysis, in rice, have demonstrated that the most of the differentially expressed genes, under different abiotic and biotic stress, belonged to PP2C (Fig. 5). This suggests that PP2C genes are involved in multiple cellular processes.

In Arabidopsis two of the most studied protein phosphatases PP2C are ABI1 and ABI2. These have been characterised as the main components of ABA signalling under abiotic stresses and during development. In particular AB1 and AB2 regulate negatively the ABA signalling. Moreover, Arabidopsis PP2C genes have been involved in other pathways to regulate plant growth development and defence response. Kinase associated protein phosphatase (KAPP) is a types of PP2C phosphatases, which negatively regulate CLAVATA1 and FLS2 in Arabidopsis.

Early Responses (5–30 Minutes)

- **Ethylene biosynthesis.**

Among the earliest responses to MAMPs is an increased production of the stress hormone ethylene. An early ethylene burst is observed after plants are attacked by pathogens. The impact of ethylene in studies disease resistance is quite variable; results seem to vary depending on the pathosystem and the conditions employed, and the fact that many pathogens are also able to produce ethylene makes interpretation of the results difficult (Argueso et al., 2007). Generally, an increased activity of l-aminocyclopropane-1-carboxylate (ACC) synthase activity can be detected within 10 min of treatment with MAMPs (Spanu et al., 1994).

- **Receptor endocytosis**

In plants, endocytosis and endosomal trafficking are important mechanisms to both inactivate receptors and down-regulate signaling and for signaling via several plasma membrane kinase receptors(Geldner and Robatzek, 2008).

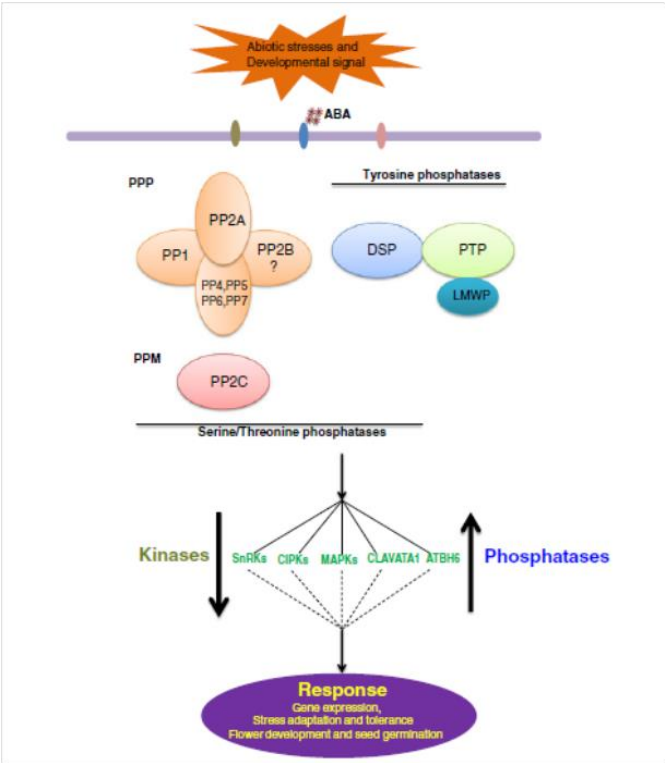

Figure 5. The expression of different phosphatase is differentially regulated in response to stresses and developmental triggers. Protein phosphatases interact with several signaling components such as ser/thr protein kinases i.e. SnRK2s, CBL-interacting protein kinases (CIPKs), mitogen activated protein kinases (MAPKs), receptor like kinases such as CLAVATA1 and transcription factors such as ATBH6 in different signaling pathways and regulate their activity. These components act upon downstream signaling elements to generate a cellular response

It has now become evident that key signaling components are localized exclusively to endosomes and that endocytosis is endocytosis is needed to put in contact them with their activated receptors, for such as steroidal plant hormone receptor Brassinosteroid Insensitive 1 (BRI1), which controls cell expansion and division (Geldner and Robatzek, 2008).

- **Gene activation**

Treatment of *Arabidopsis* plants with flg22 caused the induction of almost 1000 genes within 30 min and the downregulation of approximately 200 genes (Zipfel et al., 2004). The pattern of gene regulation in response to different PAMPs is almost identical, indicating that signaling through various PRR converges at an early step (Zipfel et al., 2006). In fact, fungal chitin and endogenous elicitors such as OG seem to induce a similar set of genes (Ramonell et al., 2002; Ferrari et al., 2007), which suggests a stereotypical gene activation response to all PAMPs and DAMPs. Interestingly, among the induced genes, Receptor-like kinases (RLKs) are overrepresented. *FLS2* and *EFR* are included in the induced genes, indicating that one role of early gene induction is a positive feedback to increase PRR perception capabilities (Zipfel et al., 2004).

Late Responses (Hours–Days).

- **Callose deposition.**

Callose papillae, localized in the cell wall, are effective barriers that are accumulated at the sites of attack during the relatively early stages of pathogen invasion.

Callose is an amorphous, high–molecular weight β-(1,3)-glucan polymer that serves as a matrix in which antimicrobial compounds can be deposited, thereby making targeted delivery of chemical defenses in cellular sites of attack. Callose deposition is typically triggered by conserved pathogen-associated molecular patterns (PAMPs) and by damage molecular patterns (DAMPs) (Gomez-Gomez et al., 1999; Luna et al., 2011). Examples of PAMPs and DAMPs are the 22–amino acid sequence of the conserved N-terminal part of flagellin (flg22) and oligalacturonides (OGs), fragments of homogalacturonan.

Callose accumulation and deposition has been used frequently, to characterize pathogen effectors that interfere with MAMP signaling (Chisholm et al., 2006; Jones and Dangl, 2006).

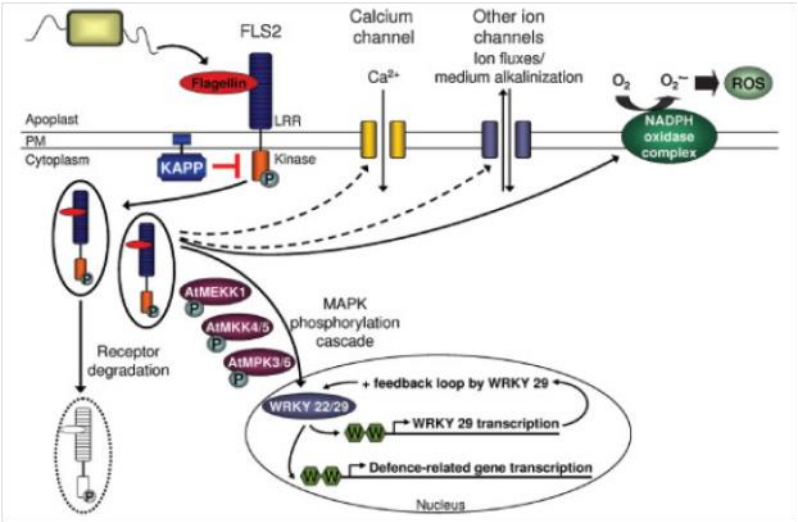

Figure 6: **Plant very early/early responses to PAMPs.** A current model for flagellin signalling in Arabidopsis.

I.3 Signal perception and transduction– a matter of complexity

I.3.1. Innate immunity mediated by PAMP/PRR

PAMPs are highly conserved and ubiquitous molecules widely distributed amongst microbial species (pathogenic or not) where they carry out an essential function, but absent in the potential host species (Nurnberger and Lipka, 2005). A number of PAMPs that fulfill these criteria and elicit a defense response in plants

have been identified from plant pathogens and reviewed in Nurnberger et al. (2004) (Table 1).

Different plant species respond to different PAMPs. For example tobacco responds to cold-shock protein while Arabidopsis does not, and only members of the Brassicaceae have so far been shown to respond to EF-Tu (Felix and Boller, 2003; Kunze et al., 2004). While this represents a diverse set of molecules, within the proteinaceous PAMPs two themes have emerged. These molecules typically contain a short (10–25) amino acid epitope that elicits a stronger defence response than the complete protein.

The crucial perceptive function for PAMPs and DAMPs is assigned to pattern recognition receptors (PRRs), that leading to signal transduction and the activation of a range of basal defence mechanisms including ethylene production, an oxidative burst, callose deposition, induction of defence related gene expression and, in some cases, hypersensitive response (HR)-like cell death (Nurnberger et al., 1994).

The PAMP detection system present in plants corresponds conceptually to that of the innate immune system in animals; both recognize highly conserved microbial molecules and act as an early warning system for the presence of a potential pathogen (Ausubel, 2005).

All known plant PRRs are predominantly located on the plasma membrane, so they are termed membrane-localized receptor-like kinases (RLKs) or receptor-like proteins (RLPs) with modular

functional domains (Fig. 7). RLKs contain an extracellular domain (ECD), a single-pass transmembrane (TM) domain, and an intracellular kinase domain. RLPs contain an ECD and a TM but have only a short cytosolic domain without an obvious signalling domain.

PAMP	Pathogen(s)	Minimal structural motif required for defence activation	Biological response	References
LPS	Gram-negative bacteria (Xanthomonads, Pseudomonads)	lipid A?	oxidative burst, production of antimicrobial enzymes in pepper, tobacco, potentiation of plant defences in response to bacterial infection	(Meyer *et al.*, 2001; Newman *et al.*, 2002; Zeidler *et al.*, 2004)
flagellin	Gram-negative bacteria	flg 22 (amino-terminal fragment of flagellin)	induction of defence responses in tomato, Arabidopsis	(Felix *et al.*, 1999)
elongation factor (EF-Tu)	Gram-negative bacteria	Elf18 (N-acetylated amino-terminal fragment of EF-Tu)	induction of defence responses in Arabidopsis and other Brassicaceae	(Kunze *et al.*, 2004)
harpin (HrpZ)	Gram-negative bacteria (Pseudomonads, *Erwinia*)	undefined	HR-like cell death, induction of defence responses in various plants, systemic acquired resistance to microbial infection	(Wei *et al.*, 1992; He *et al.*, 1993; Lee *et al.*, 2001)
cold shock protein	Gram-negative bacteria, Gram-positive bacteria	RNP-1 motif (amino-terminal fragment of the cold shock protein)	oxidative burst, production of the plant stress hormone ethylene in Solanaceae	(Felix and Boller, 2003)
necrosis-inducing proteins	bacteria (*Bacillus* spp.), fungi (*Fusarium* spp.), oomycetes (*Phytophthora* spp., *Pythium* spp.)	undefined	HR-like cell death, induction of defence responses in many dicot plants	(Bailey, 1995; Fellbrich *et al.*, 2002; Mattinen *et al.*, 2004; Pemberton and Salmond, 2004; Qutob *et al.*, 2002; Veit *et al.*,
transglutaminase	oomycetes (*Phytophthora* spp.)	Pep-13 motif (surface-exposed epitope of the transglutaminase)	induction of defence responses in parsley, potato	(Nürnberger *et al.*, 1994; Brunner *et al.*, 2002)
lipid-transfer proteins (elicitins)	oomycetes (*Phytophthora* spp., *Pythium* spp.)	undefined	HR-like cell death, induction of defence responses in tobacco, systemic acquired resistance to microbial infection	(Osman *et al.*, 2001)
xylanase	fungi (*Trichoderma* spp.)	TKLGE pentapeptide (surface-exposed epitope of the xylanase)	HR-like cell death, ethylene production in tobacco, tomato	(Enkerli *et al.*, 1999; Ron and Avni, 2004)
invertase	yeast	N-mannosylated peptide (fragment of the invertase)	activation of the phenylpropanoid pathway, ethylene production in tomato	(Basse *et al.*, 1993)
β-glucans	fungi (*Pyricularia oryzae*), oomycetes (*Phytophthora* spp.), brown algae	tetraglucosyl glucitol, branched hepta-β-glucoside, linear oligo-β-glucosides	induction of defence responses in legumes, tobacco, rice	(Klarzynski *et al.*, 2000; Fliegmann *et al.*, 2004; Yamaguchi *et al.*, 2000)
sulphated fucans	brown algae	fucan oligosaccharide	induction of defence responses in tobacco, systemic resistance to viral infection	(Klarzynski *et al.*, 2003)
chitin	all fungi	chitin oligosaccharides (degree of polymerization > 3)	induction of defence responses in tomato, Arabidopsis, rice, wheat, barley	(Baureithel *et al.*, 1994; Ito *et al.*, 1997)
ergosterol	all fungi		induction of ion fluxes in tomato	(Granado *et al.*, 1995)
cerebrosides A, C	fungi (*Magnaporthe* spp.)	sphingoid base	phytoalexin production in rice	(Koga *et al.*, 1998)

Table 1: **Selected pathogen-associated molecular patterns (PAMPs) and their plant defence-inducing activities.** Adapted from (Nurnberger et al., 2004).

Notably, in contrast to mammals, no intracellular NB-LRR protein recognizing a PAMP has yet been identified in plants (Monaghan and Zipfel, 2012).

The best-studied plant PRRs are the RLKs FLS2, EFR and XA21. In plants, the first identified and best studied PRR is FLS2, the flagellin receptor(Gomez-Gomez and Boller, 2000). It is characterized by a N-terminal signal peptide, 28 LRRs, a transmembrane domain, and a cytoplasmic kinase. FLS2 orthologs have a highly conserved architecture, suggesting functional importance for the conserved features. Also it is known to perceive a motif of 22 amino acids of the flagellin protein of bacterial flagella (flg22)(Monaghan and Zipfel, 2012).

Flagellin is the main component of the flagellar filament of eubacteria and its perception is the best-characterized PAMP detection system to date in plants. In various plant species, synthetic peptides representing the most highly conserved part of the N terminus of bacterial flagellin, such as the 22-amino-acid peptide flg22, act as potent elicitors at subnanomolar concentrations (Felix et al., 1999). In *Arabidopsis*, flg22 also induces callose formation, accumulation of the defense protein PR1, and strong inhibition of seedling growth (Gomez-Gomez et al., 1999). Growth inhibition was used in a mutant screen, yielding a number of mutants that were insensitive to flg22.

To find which of these LRRs might be involved in flagellin perception, Dunning and coworkers (Dunning et al., 2007) did an

extensive, site-directed, mutational analysis of the x positions in all the LRRs and tested the functionality of the recombinant receptors by expressing them in *fls2* mutants of Arabidopsis, using growth inhibition by flg22 as a bioassay.

In each of the 28 LRRs, they either replaced two of the x positions with alanines or changed one of the x positions with randomizing mutagenesis.

Both approaches identified the x positions of LRRs 9–15 as important for FLS2 function. These x positions in β-strands of LRR 12–14 showed particularly high conservation in FLS2 orthologs of 18 different Brassicaceae.

Upon flagellin perception, FLS2 rapidly associates with another LRR–receptor-like kinase (RLK), BAK1, thereby initiating downstream signaling (Lu and Higgins, 1999). Also, recently, it was demonstrated that after binding of flg22, FLS2 accumulates in mobile intracellular vesicles. This ligand-induced FLS2 endocytosis is followed by receptor degradation possibly via lysosomal and/or proteasomal pathways. Endocytosis and downstream signalling are closely linked but it is not yet known if the actual internalization is required for signal transduction.

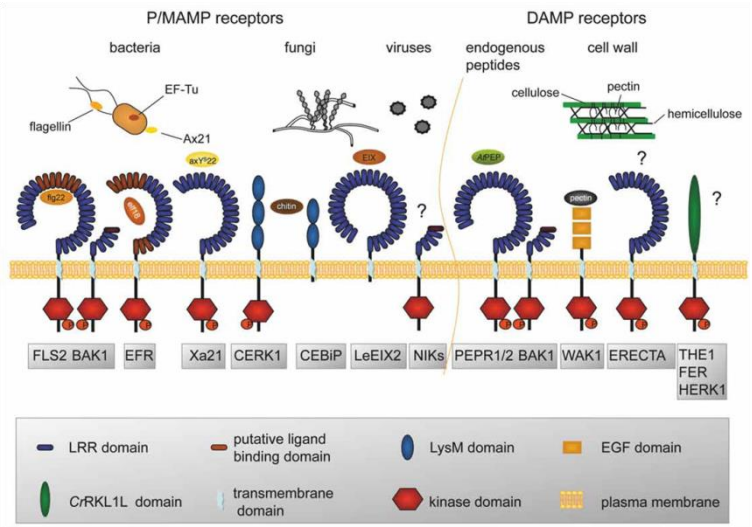

Figure 7. Known pattern recognition receptors from plants. PAMPs derived from different pathogens are perceived by membrane-associated pattern recognition receptors.

PAMPs such as flagellin and EF-Tu are recognized by the LRR-RLKs FLS2 and EFR, respectively. BAK1 was known to be a small co-receptor LRR-RLK that that interacts with several ligand binding receptors such as FLS2, PEPR1/2 and BRI1. The Arabidopsis CERK1 is required for chitin signaling .Chitin binding was shown for the LysM-RLP CEBiP from rice. The fungal PAMP xylanase, is recognizedby the LRR-RLPs *Le*EIX1/2. The LRR-RLK PEPR1 recognizes a plant-encoded DAMP released after wound, *At*PEP1.

Cell wall fragments can bind to WAK1 and activate oligogalacturonide-dependent defense responses. Other RLKs known to be involved in developmentalprocesses as the LRR-RLK ERECTA and the *Cr*RLK1L proteins FERONIA, HERCULES and THESEUS might beinvolved in damage associated defence responses. Perception of the different elicitors via the specific PRRs leads to activation of innate immune responses. PAMP: pathogen-associated molecular pattern; EF-Tu: elongation factor Tu, LRR-RLK: leucine-rich repeat receptor kinase, LysM: lysine motif, RLP: receptor-like protein, *Cr*RLK1L: *Catharanthus roseus* RLK1-like(Postel and Kemmerling, 2009)

Another well known PRR, belonging like FLS2 to subfamily XII, is EFR(Zipfel et al., 2006). The LRR-RK EFR is the PRR for EF-Tu and his structure is highly similar to FLS2, with a 21-LRR extracellular domain, a transmembrane domain, and a cytoplasmic Ser/Thr kinase domain (Nicaise et al., 2009).

The extracellular domain of EFR is highly glycosylated, which seems to be important for ligand binding. Indeed mutation of a single predicted glycosylation site compromises elf18 binding despite correct localization of the mutated protein to the plasma membrane(Schwessinger and Ronald, 2012).

Unlike FLS2, EF-Tu responsiveness was found only in Brassicaceae species, suggesting that EFR is an innovation of this family (Nicaise et al., 2009).

Both FLS2 and EFR signalling pathways rapidly converge at a very early stage of signaling. Indeed the activation of both receptors determine identical calcium-associated plasma membrane anion channel opening as an initial step in the pathogen defence pathway (Jeworutzki et al., 2010).

Apart from the two best studied PRRs, FLS2 and EFR, many advances have been made on the identification of the receptors involved in the perception of fungal chitin or chito-oligosaccharides, the latter responsible for the induction of defence responses in plants. Chitin (a polymer of *N*-acetyl-D-glucosamine) is a major component of fungal cell walls and it is

found in insect exoskeletons and crustacean shells but not in plants. It was shown that the chitin elicitor binding protein (CEBiP) is responsible for binding of chito-oligosaccharides in rice. This protein also belongs to the family of RLPs and is characterized by a extracellular LysM domains, a single TM domain and a cytoplasmic C-terminal tail without a kinase domain Recently, it was demonstrated that CEBiP co-operates together with the rice LysM-receptor kinase *Os*CERK1. Indeed *Os*CERK1 is required for full chitin responsiveness in rice and directly interacts with CEBiP, forming ligand-induced heteromeric complexes in vivo(Shimizu et al., 2010). CERK1 (synonymous to LysM-RLK1) was first identified as the chitin receptor in *Arabidopsis* (Schwessinger and Ronald, 2012).

In *Arabidopsis*, mutations in *AtCERK1* abolish sensitivity to chitin fragments. Moreover it has been suggested that AtCERK1 is involved not only in chitin perception, as the mutant *AtCERK1* is more susceptible to bacterial pathogens, which do not contain chitin(Gimenez-Ibanez et al., 2009).

I. 3. 2 Innate immunity mediated by DAMP/PRR

In addition to the detection of dangerous non-self by means of PAMPs, plants and animals can also sense infectious-self or modified-self. In the danger model first described by Matzinger (1998), danger is defined as harmful conditions which can be sensed by both animals and plants, to start defense mechanisms.

Endogenous molecules which are released after damage by wounding or pathogen attack can function as danger-associated molecular patterns (DAMPs) to induce defence responses as PAMPs (Boller and Felix, 2009; Mazzotta and Kemmerling, 2011).

Many plant pathogens produce lytic enzymes to breach the structural barriers of plant tissues. The products generated by these enzymes may function as endogenous elicitors. Such DAMPs typically appear in the apoplast and, as in the case of PAMPs, can serve as danger signals to induce innate immunity (Matzinger, 2002).

I.3. 2. 1 An example of DAMP/PRR pair: AtPep1 is perceived by PEPR1

*At*Pep1 is a 23-aa endogenous peptide, processed from the 92 elicitor from *Arabidopsis* amino acid precursor protein *At*PROPEP1 that is upregulated after wounding or jasmonate/ethylene application. AtPep1 and its homologues regulate expression of the defence protein PDF1.2 through the JA/Et defence signalling pathway (Huffaker et al., 2006). The identification of the six paralogues of AtproPep1 in Arabidopsis, and of orthologues in widely diverse plant species (Huffaker et al., 2006), suggests that AtPep1 may be a member of a diverse family of peptide signals that have roles as endogenous signals for defence.This elicitor signals the activation of components of

the innateimmune response against pathogens (Yamaguchi et al., 2006).The receptor of *At*PEP1 is PEPR1 and was purified after photolabeling with its radioactive marked ligand, providing the first known DAMP/pattern recognition receptor couple in Arabidopsis (Krol et al., 2010). The receptor contains domains that are typical for LRR receptor-like kinases (LRR-RLKs), including 26 extracellular LRRs, cysteine pairs flanking the LRR region, a transmembrane region and an intracellular protein kinase domain. A related LRR-RLK was recently identified, called PEPR2 as a second receptor for AtPep1 (Krol et al., 2010). These proteins belong to the LRR-RLK XI subfamily to witch belong also HAESA, CLV1, BAM1, BAM2 and BAM3.

I.3. 2. 2 A second DAMP/PRR pair: OGs and WAK1.

I.3. 2. 2. 1 Oligalacturonides a class of DAMP

Oligogalacturonides (OGs) are linear molecules of two to about twenty α-1,4-d-galactopyranoslyuronic acid (GalA) residues. OGs were the first plant oligosaccharins, biologically active carbohydrates that act as signal molecules, to be discovered (Bishop et al., 1981; Hahn, 1981). OGs are released upon fragmentation of homogalacturonan (HG) from the plant primary cell wall (Cote et al., 1998) by wounding or by pathogen-secreted cell wall-degrading enzymes (for example polygalacturonases,

PGs). Indeed, PGs are not elicitors *per se*, but are rather able to release elicitor-active molecules from the host cell wall. When the activity of a fungal PG is modulated by apoplastic PG-inhibiting proteins (PGIPs), long-chain oligogalacturonides are produced (De Lorenzo et al., 2001; De Lorenzo and Ferrari, 2002) (Fig. 8). Accordingly OGs are generated by the host cell during the infection process.

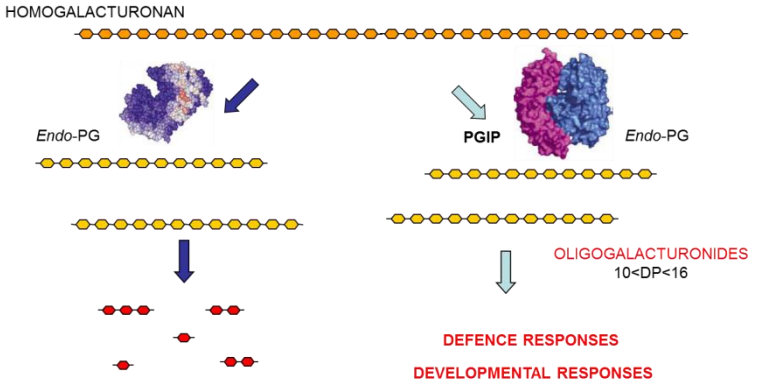

Figure 8. Model for the OG accumulation during pathogen infection.

Pectins are one of the most accessible components of the cell and one of the first targets of digestion by invading pathogens (Pagel and Heitefuss, 1990). OGs are released when PGs and endopectate lyases (PLs) secreted from the pathogen degrade the

homogalacturonan in the cell (Cote et al., 1998). The OGs released are a carbon source for the pathogens, but can also be detected by plants as signals to initiate defense responses.

Recently it has been proposed the existence of a system, called "pectin integrity monitoring system" or PIMS, according to the plant wall integrity may be efficiently watched by monitoring the pectin status (De Lorenzo et al., 2011). In the PIMS, OGs act as indicators of cell wall integrity both in adverse conditions and during normal growth. Also PIMS includes the inhibitors of fungal and insect PGs (PG- inhibiting proteins or PGIPs), which guard the cell wall by limiting HGA degradation.

Chemically pure OGs can act as endogenous elicitors (Galletti et al., 2009). OGs are biological active when their degree of polymerization (DP) is comprised between 10 and 15 (Côté and Hahn, 1994). This size is optimal for the formation of Ca2+-mediated intermolecular cross-links resulting in structures called "eggboxes"(Braccini and Perez, 2001) (Cabrera et al., 2008). Exogenously added OGs inhibit the light-induced opening of stomata in tomato and *Commelina communis L.* leaves (Lee et al., 1999) and elicit a variety of defense responses, including accumulation of phytoalexins (Davis et al., 1986), glucanase and chitinase (Davis and Hahlbrock, 1987; Broekaert and Pneumas, 1988). Stomatal openings provide access to inner leaf tissues required by many plant pathogens (Agrios, 1997), suggesting that the constriction of stomatal apertures is beneficial for plant

defense. Recently the use of model plant, *Arabidopsis* has provided a useful tool to advance our knowledge of the OG biology. Notably, the responses triggered by OGs in *Arabidopsis* largely overlap those activated by MAMPs. These biological responses comprise both plant defense responses, such us induction of marker gene expression, callose and ROS production and accumulation, and plant growth and development (Cote and Hahn, 1994b) (Fig. 9).

One of the first responses observed after the addition of OGs that is clearly involved in plant defense is the production of active oxygen species, including H_2O_2, and O_2- (Low and Merida, 1996). This oxidative burst occurs within a few minutes after the addition of OGs to suspension-cultured soybean (Legendre et al., 1993), tobacco (Rout-Mayer et al., 1997; Binet et al., 1998) and tomato (Stennis et al., 1998) cells. Recently it was shown that, in Arabidopsis, production of H_2O_2 in response to OGs is mediated by AtrbohD (Fig.7) (Galletti et al., 2008).

OGs initiate signaling cascades that activate a plant defense. OGs rapidly activate AtMPK3 and AtMPK6 (Denoux et al., 2008), suggesting that, even though OGs and flg22 are perceived by distinct receptors, the signaling pathways mediated by these elicitors converge very early.

Arabidopsis full-genome expression analysis reveals that OGs influence the expression of ~4000 genes (Ferrari et al., 2007). Some of these, such as *AtWRKY40* (At1g80840), encoding a

transcription factor that acts as a negative regulator of basal defense (Xu et al., 2006), *CYP81F2* (At5g57220), encoding a cytochrome P450 and *RetOx* (At1g26380), encoding a protein with homology to reticuline oxidases, a class of enzymes involved in secondary metabolism and in defense against pathogens (Dittrich and Kutchan, 1991), are rapidly and strongly up-regulated upon exposure to elicitor. Early activation of genes in response to OGs is independent of SA, ET, and JA signaling pathways and of AtRbohD (Galletti et al., 2008).

Exogenous treatment with OGs protects grapevine (*Vitis vinifera*) and Arabidopsis leaves against infection with the necrotrophic fungus *Botrytis cinerea* (Aziz et al., 2004; Ferrari et al., 2007), suggesting that production of this elicitor at the site of infection, where large amounts of PGs are secreted by the fungus, may contribute to activate defenses responses.

In addiction to defense responses, OGs induce responses involved in plant growth and development. Exogenously added OGs influence the growth and development of plant tissues (Cote and Hahn, 1994a). Biologically active OGs inhibit root formation (Bellincampi et al., 1993) and increase stomata formation (Altamura et al., 1998) on tobacco leaf explants incubated in media with specific phytohormone concentrations.

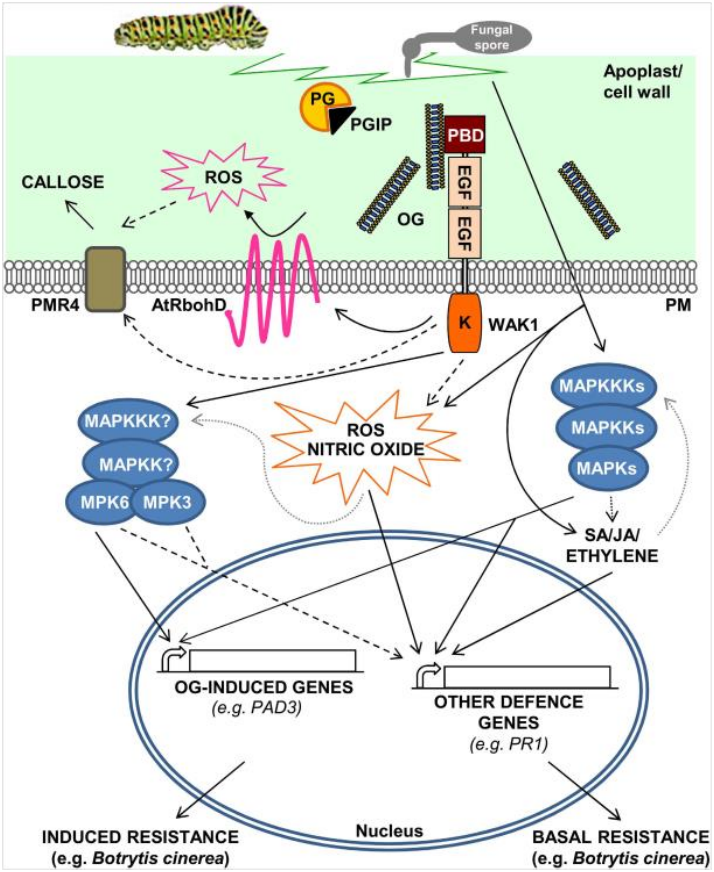

Figure 9. A model of defence responses triggered by OGs in Arabidopsis

OGs, released from the cell wall after degradation of homogalacturonan, are accumulated in the apoplast through the activity of PG/PGIP. OGs are perceived by WAK1 and trigger defense responses such as ROS accumulation through the activation of the NADPH oxidase AtRbohD, nitricoxide production, callose deposition, and MAPK-mediated activation of defense gene expression.

Auxins and in particular indole-3-aceticacid(IAA), are crucial for plant growth and development (Leyser,2002). OGs are able to antagonize the physiological responses induced to auxins ,as described for the first time by Branca et al. (1988). OGs have been subsequently shown to inhibit auxin-induced root formation in tobacco and *Arabidopsis* leaf explants as well as in thin cell-layer explants (Bellincampi et al., 1993; Savatinet al.,2011).

In particular the activity of OGs not only affects long-term responses to auxin such as adventitious root formation, but also early responses such as the up-regulation of *IAA5*, *SAUR16*, and *SAUR-AC1* (Savatin et al., 2011). OGs are also involved in fruit ripening. They have been shown to induce ethylene production in the fruits of tomato (Brecht and Huber, 1988; Campbell and Labavitch, 1991) and citrus (Baldwin and Biggs, 1988).

Pectic fragments that elicit ethylene production have been extracted from tomato fruit at the breaker stage of ripeness. This suggests that OGs, presumably released by PGs, could be involved in initiating the ripening process (Melotto et al., 1994), since exogenous ethylene initiates the ripening process and the production of ethylene is required for ripening (Theologis et al., 1993). The role of OGs in fruit ripening, however, seems to be complex and is not understood.

I.3. 2. 2. 2 Oligalacturonides are important local wound signal

Plants are continuously exposed to agents of wound-causing and immediate tissue damage such as herbivore feeding and adverse weather conditions, endanger plant survival by exposing the plant to water loss and further invasion by pathogens (Leon et al., 2001; Delessert et al., 2004). Plants have developed the ability to sense the mechanical damage and hence they are able to respond by activating either local or systemic or both defences similar to those activated by pathogen infection(Ferrari et al., 2013). The wounding responses (WR) involves a rapid oxidative burst (Bradley et al., 1992), the expression of wounding marker genes (Reymond et al., 2000) and the accumulation of pathogenesis-related proteins (Chang et al., 1995)

Ryan *et al*., have been demonstrated that, in tomato, the peptide signal systemin induced the systemic response to wounding and the PIs accumulation, and suggest that the OGs to be able to induce, as well as, the PIs accumulation. Also, in tomato, has been observed that OGs are released by a PG that is specifically systemin induced after wounding(Ryan and Jagendorf, 1995). Therefore the OGs are supposed to be involved in the wounding.

However, OGs are likely to act only as local signals, because of their oligoan- ionic nature and limited mobility in the tissues (Baydoun and Fry, 1985).

The activation of wound-responses requires, also, the involvement of hormones, such as jasmonate and ethylene.

In particular tomato plants, in response to mechanical injury, produce and accumulate jasmonate that mediate the wound-activated gene expression.

Instead the ethylene is suggested to potentiate to systemin activated-wound signaling, through the octadecaniod pathway. Has been proposed that, in solanacee, the roles of systemin and of oligalacturonide are closely linked to the activity of these hormones in activating wound defences. Moreover several wound responsive genes are up-regulated by OGs independently of JA. Probably, in solanacee, there are two different wound- signaling pathways , one dependent of JA and systemin, for the systemic response, and one dependent to OGs, functioning only locally(Leon et al., 2001).

In *Arabidopsis*, like in tomato ,both JA and ethylene are required for a stronger and more rapid expression of several wound-responsive genes (Moffat et al., 2012), and local and systemic responses to wounding are different. However, there are important differences between the wound responses of tomato and *Arabidopsis*(Ferrari et al., 2013). For example, differently to solanacee plants, in *Arabidopsis* ethylene act as a regulator in the cross- talk between JA-dependent and independent pathway, determining the activation of local or systemic wound responses. Moreover the genes codifying for systemin are absent in

Arabidopsis. It must be also noted that both wounding (Cheong et al., 2002) and OGs (Branca et al., 1988; Bellincampi et al., 1996; Ferrari et al., 2008; Savatin et al., 2011) repress auxin responses, supporting the hypothesis that OGs mediate at least some responses induced by mechanical damage(Ferrari et al., 2013).

I.3. 2. 2. 3 OGs are perceived by the Wall-Associated Kinase1 (WAK1) in Arabidopsis

Although its eliciting activity is well documented, the perception system for OGs has been elusive. Interestingly, the extracellular domain of an Arabidopsis wall-associated RLK named WAK1 has high affinity to OGs, particularly to the elicitor-active egg-box form of OG (Cabrera et al., 2008). This finding opened the prospect that WAK1 or its homologs might be part of the perception system for OGs. Indeed a recent work reveals through a domain swap approach a role of the WAK1 protein as a receptor of oligogalacturonides (Brutus et al., 2010). Authors firstly, through a test-of-concept study, demonstrated the possibility of obtaining functional plant chimeric receptors and devise an appropriate design for their construction. Specifically, it was analyzed the amenability of the Arabidopsis EFR, a LRR receptor kinase for recognition of the microbeassociated molecular pattern (MAMP) EF-Tu and its derived peptide elf18 as a recipient

protein structure. EFR was chosen because it is functional when expressed in Nicotiana species (Zipfel et al., 2004), unlike the Arabidopsis FLS2, receptor for flagellin and its derived peptide flg22 (Robatzek et al., 2007). Next, they obtained chimeras between EFR and Arabidopsis WAK1 and demonstrated that WAK1 is capable to sense OGs in vivo and trigger a defense response that mirrors that normally activated by OGs (Brutus et al., 2010).

I.3. 3 PRRs do not signal alone

I.3. 3. 1 The receptor-like kinase SERK3/BAK1 is a central regulator of PRR-mediated signalling

The activation of RLK , mediated by ligand binding to the extracellular

domain, leads to conformational changes and to a sequential auto- or trans-phosphorylation of specific residues in the cytoplasmic domain serving as docking sites for downstream signaling partners, and/or direct phosphorylation of signaling substrates (Schwessinger and Ronald, 2012). The phosphorylation is performed by kinases, that can be divided into RD and not-RD kinases depending to the presence of a conserved arginine (R) immediately preceding the invariant aspartate in subdomain VI required for catalytic activity(Dardick and Ronald, 2006).

Notably a RD-type regulator, a LRR-RLK named BRI1-ASSOCIATED KINASE 1 (BAK1), is required as signaling partner of several RLK, both RD and not-RD. BAK1 belongs to the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family which contains five LRR-RLKs belonging to subgroup II (Hecht et al., 2001), and is also named SERK3. It is characterized by a small extracellular domain with 4 and a half LRR repeats, a SPP motif, the serine and proline rich domain that is characteristic of the SERK protein family, a single trans-membrane domain, a cytoplasmic kinase domain and a short C-terminal tail (Chinchilla et al., 2009). BAK1 was initially identified as a positive regulator of the brassinosteroid signaling, forming *in vivo* a complex with the receptor BRI1.

It is known that BAK1 also forms complexes with FLS2, EFR, AtPEPR1 and AtPEPR2 (Kemmerling et al., 2011)(Fig. 10), but is not involved in ligand binding. Therefore BAK1 does not act as a co-receptor but rather as a signal transducer most probably relying on its kinase activity.

Current knowledge suggested a model of activation of the PRR, and in particular of FLS2, mediated by BAK1. In this model in a first step FLS2 perceive flagellin independently of BAK1. The ligand binding to the LRR domain of the receptor may coincide with conformational changes in the ectodomain of FLS2.

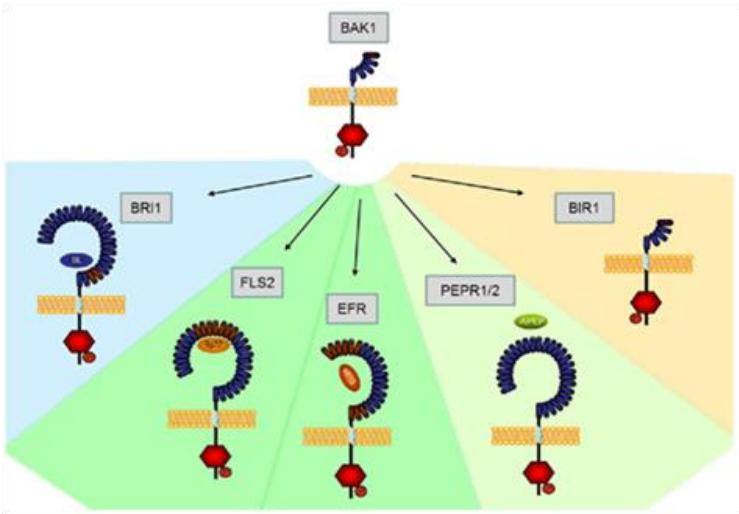

Figure 10. Schematic overview of *Arabidopsis* LRR-RLKs interacting with BAK1. While currently BRI1 is the only known BAK1-interacting LRR-RLK involved in developmental processes (blue area), several other RLKs are involved in plant innate immunity (PTI, green area; ETI orange area) (Kemmerling et al., 2011).

These modifications might then allow association of FLS2 with BAK1, probably trough some residues in the ectodomain of BAK1. The interaction between their ectodomain may lead to the interaction of kinase domains and consequently to an event of trans-phosphorylation (Fig. 11)(Chinchilla et al., 2009)

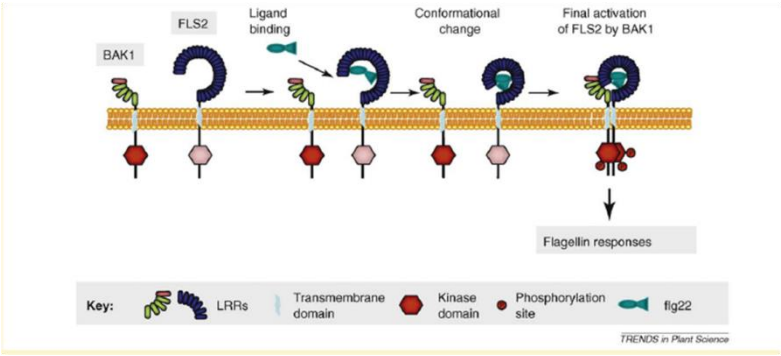

Figure 11. Schematic model of activaction of FLS2 mediated by BAK1. Adapted from (Chinchilla 2009).

The biochemical function of BAK1 and the precise mechanism underlying activation of the flagellin receptor remain unclear.

Some data suggest that BAK1 is a target of bacterial effectors. Two functionally related effectors, AvrPto and AvrPtoB from *Pseudomonas syringae* directly target BAK1, and interfere with the formation of FLS2/BAK1 and BRI1/BAK1 complexes(Shan et al., 2008). Indeed it is well known that these effectors suppress the convergent defense signaling stimulated by flg22 and some other PAMPs and that plants overexpressing AvrPto mimic BR-insensitive phenotype. Additionally it is possible that BAK1 is involved in the regulation of signaling pathways of others PRRs.

I. 3. 3. 2 KAPP and GRP-3 may regulate signalling mediated by OGs

A combination of *in vitro* and *in vivo* studies by two-hybrid, co-immunoprecipitation and gel filtration chromatography experiments showed that WAK1 interacts and form a complex with GRP-3, a glycine rich extracellular protein and with KAPP, a kinase associated protein phosphatase (Park et al., 2001);(Anderson et al., 2001).

The glycine-rich protein superfamily corresponds to a large and complex group of proteins that share the presence of a high content and repetitive sequences of glycine residues based on (Gly-X)n motifs that are usually found in β-plated sheets with antiparallel strands or form flexible coiled structures and are thought to be involved in protein-protein interactions. The GRPs isolated, based on their primary structure and functional domains, are divided into five classes: structural proteins in the cell wall, which contain signal peptide followed by a glycine-rich region with GGGX repeats; class II, characterized by GRPs that contain a glycine rich-region followed by a cysteine-rich region at their C-terminus; class III that show a lower glycine content; class IV GRPs that are also known as RNA-binding proteins, or RNA-GRPs. These GRPs may contain several motifs which include the RNA-recognition motif, the cold-shock domain and

zinc fingers (Mousavi and Hotta, 2005).Indeed, the Class IV GRPS are subdivided into IVa (which contain one RRM motif besides the glycine-rich domain), IVb (one RRM and a CCHC zincfinger), IVc (a cold-shock domain and two or more zinc-fingers) and IVd (two RRMs). Finally, class V that comprised GRPs with a high glycine content but with mixed patterns of repeats(Mangeon et al., 2010) (Fig. 12).

GRP genes encoding proteins, initially isolated from plants, have been reported in a wide variety of organisms from cyanobacteria to animals (Sachetto-Martins et al., 2000). Despite the extensive number of reports describing the occurrence of these genes in different species, very little is known about their biological role in plants (Fusaro et al., 2001);(Mangeon et al., 2009); (Bocca et al., 2005). GRPs may have very diverse localisation and functions.

The only common feature among all different GRPs is the presence of glycine repeat domains, which, in mammalian keratins, are highly fexible and may play a role in the protein-protein interaction.

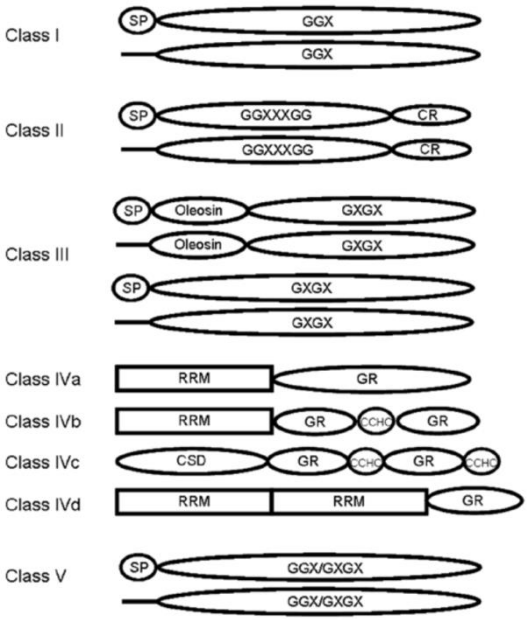

Figure 12. Schematic representation of plant glycine-rich proteins classification. SP, signal peptide; CR, cysteine-rich domain; Oleosin,Oleosin-conserved domain; RRM, RNA-recognition motif; GR, Glycine rich domain; CCHC, zinc-finger; CSD, Cold-shock domain. Glycine-rich repeats are indicated as GGX, GGXXXGG, GXGX and GGX/GXGX, where G represents glycine and X any amino acid. Adapted to (Mangeon et al., 2010).

GRPs are developmentally regulated and are also induced by physical, chemical and biological factors, such as auxin, abscisic acid (ABA), osmotic and water stress, circadian rhythm, cold, light and pathogens (Sachetto-Martins et al., 2000).

These data together with the broad expression pattern diversity of GRPs suggest that these proteins are involved in important

cellular processes. They have been involved in RNA chaperone, binding and splicing activity, flowering, pollen recognition (Mayfield et al., 2001). They have been involved in callose deposition and inhibition of the long distance movement of Turnip vein clearing tobamovirus (TVCV) in tobacco plants (Ueki and Citovsky, 2005) plant cold acclimation (Kwak et al., 2005) and antimicrobial activity (Sachetto-Martins et al., 2000).

Among the 30 genes expressed in Arabidopsis (Sachetto-Martins et al., 2000), five GRPs are secreted; they have significant amino acid identity to each other outside of the glycine-rich domain and are clustered in tandem one locus on chromosome 2. Among these secreted proteins there is GRP-3. GRP-3 is induced by ethylene, salicylic acid, ABA treatments and abiotic stress, such as drying and water (De Oliveira et al., 1990). Moreover, *GRP-3* mRNA is mainly expressed in stems and leaves as opposed to the faint signals detected in roots, flowers and siliques (De Oliveira et al., 1990).

The protein sequence is composed of a putative signal peptide sequence, followed by a glycine-rich region and a cysteine rich C-terminus (De Oliveira et al., 1990).This structure classifies GRP-3 as a Class II GRP (Fusaro et al., 2001); (Bocca et al., 2005); (Mangeon et al., 2010). A previous work has shown that GRP-3 specifically interacts with WAK1 in its C-terminal region and can binds not only WAK1, but even WAK3 and WAK5 (Park et al., 2001). The expression of WAK1 and GRP-3 was up-regulated by

exogenously added GRP-3 protein, suggesting that they are regulated by a positive feedback loop (Park et al., 2001). Moreover, *WAK1* and *GRP-3* genes are expressed in the same tissues and induced by SA treatment (Park et al., 2001). By gel filtration analysis and co-immunoprecipitation, it has been demonstrated that WAK1 and GRP-3 are associated in the multimeric complex with the kinase-associated protein phosphatase (KAPP) (Park et al., 2001). The interaction between the kinase domain of WAK1 with KAPP occur only in presence of GRP3 (Park et al., 2001). Notably, KAPP binds not only WAK1 but even WAK2 (Anderson et al., 2001). KAPP belongs to the PPM (protein phosphatases family), that comprises the Mg^{2+}-dependent protein phosphatases that include PP2C and pyruvate dehydrogenase phosphatase (Cohen, 1997). Sequence analyses showed that KAPP contains three different functional domains: an N-terminal type I membrane anchor, a kinase-interacting FHA domain and a carboxy-terminal type 2C protein phosphatase catalytic domain.

The FHA domain is a phosphoprotein-binding domain known as the forkhead associated domain. This domain has been identified in many signalling proteins, including protein kinases, protein phosphatases, adenylate cyclases, proteases, kinesins, zinc-finger proteins and glycoproteins. Recent data suggest that all these proteins may regulate many different signaling pathways through their interaction with phosphorylated protein targets. KAPP uses

its KI-FHA to bind epitopes of RLKs activated by phosphorylation of serine or threonine residues. In particular KAPP interacts with RLKs from Arabidopsis that include HAESA (formerly RLK5,implicated in abscission dynamics in Arabidopsis) (Stone et al., 1994) CLAVATA1 (CLV1, implicated in shoot meristem development) (Williams et al., 1997) RLK4, TMK1 (Braun et al., 1997), WAK1 (Park et al., 2001) FLS2 (Gomez-Gomez et al., 2001) BAK1 (Li et al., 2002), and SERK1 (Shah et al., 2002). Moreover, it has been demonstrated that the Kinase interaction domain of KAPP binds RLKs *in vitro* in a phosphorylation-dependent manner and does not bind kinase-inactive mutants of RLKs (Williams, 1997; Stone et al., 1998).

Park and collaborators have demonstrated that WAK1, GRP-3, and KAPP are associated into a 500-kDa complex *in vivo* that may represent the activated signalosome. Furthermore in this work they shown that the receptor WAK1 appears in a 100-kDa and in a 78-kDa protein, and suggest that only the slowly migrating 100-kDa protein appears to be the functional WAK1, because this protein but not of78 kDa is associated with GRP-3 and KAPP to form the 500 kDa complex. It is possible that either GRP-3 invokes the modification the 78 kDa protein, resulting in the appearance of 100-kDa Wak1, or GRP-3 prevents the 100-kDa WAK1 from being cleaved a 78-kDa protein. The detailed molecular mechanism underlying the communication between

Wak1 and GRP-3 remains to be elucidated in detail(Park et al., 2001).

In this work I've analyzed the involvement of GRP-3 and KAPP in the OGs signaling.

II.METHODS

II.1 Plant Material

Arabidopsis (*Arabidopsis thaliana*) Columbia-0 (Col-0) wild-type seeds were purchased from Lehle Seeds. *grp3* (SALK_084685.46.60, Col-0), *kapp* (SAIL_1255-D05, Col-0) T-DNA insertional mutants (in the Col-0 background) were purchased from European Arabidopsis Stock Centre (uNASC). Homozygous mutants were isolated by PCR-based genotyping using gene specific PCR primers listed in table1.

The construct 35S::KAPP-YFP was kindly provided by Prof. Elliot M. Meyerowitz (California Institute of Technology, Pasadena).

OGs with an average DP of 10 to 16, as assessed by matrix-assisted laser desorption/ionization time-of-flight MS, were kindly prepared by Gianni Salvi and Daniela Pontiggia (Università di Roma "La Sapienza") as previously described (Bellincampi et al., 2000). The flg22 peptide were synthesized by Prof. Maria Eugenia Schininà ("Sapienza", Università di Roma).

II.2 Construction of tagged vectors

The entire open reading frame (ORF) of *GRP-3* was amplified by polymerase chain reaction from GRP-3 cDNA obtained from Riken (pda02854), using the primers listed in Table 1. The cDNA was cloned into pB7m34GW vector downstream of the

Cauliflower mosaic virus 35S promoter and fused at the C-terminal to CFP (Cyan Fluorescent Protein) and RFP using the Gateway Cloning System (Invitogen). In particular pEN-GRP3 entry clones was generated in the pDONR221/Zeo vector (Life Technologies). Subsequently multisite recombination was performed by using the pEN-R2-F-L3, pEN-R2-C-L3 and pEN-R2-3XHA-L3 vectors, which contain the 35S promoter, the CFP coding sequence and the RFP coding sequence tag respectively, and pB7m34GW as destination binary vector that confers the phosphinothricin resistance. All Gateway compatible vectors were previously described (Karimi et al., 2002) and obtained from Plant System Biology (Ghent University; http://gateway.psb.ugent.be/). I primers used in the the Gateway Cloning System were listed in Table 1.

The construct was confirmed by sequencing (Primm) and used to transform *Agrobacterium tumefaciens* GV3101) (KonczandSchell, 1986) for Arabidopsis transient and stable expression.

Transient expression in seedlings was performed as previously described (Li et al., 2009). All constructs were transformed into Arabidopsis ecotype Col-0 using the floral dipping method. Transformants were selected on MS agar medium containing 50 µg/ml phosphinothricin. . Resistant seedlings were transferred to soil and are currently under characterization.

II. 3 Growth Conditions and Plant Treatments

Arabidopsis plants were grown on a soil Compo Agricoltura at 22°C and 70% relative humidity under a 12-h light/12-h dark cycle (approximately 120 μmol m^{-2} s^{-1}).

For seedling assays, seeds were surface sterilized and germinated in multiwell plates (approximately 10 seeds/well) containing 0.5X Murashige and Skoog (MS; (Murashige and Skoog, 1962) medium (1 ml/well; Sigma-Aldrich) supplemented with 0.5% sucrose. After 9 days, the medium was replaced with fresh one and, after 24 h, OGs and flg22 (50 μg/ml and 100 nM, respectively) were added to the medium and seedlings were incubated for 1h and 3 hours at room temperature.

Seedlings were grown at 22°C and 70% relative humidity under a 16 h/8 h light/dark cycle (approximately 120 μmol/m^2/s).

For elicitor treatments in adult plants, a solution containing 70 μg mL^{-1} OGs or 100nM flg22 or OG 3/6 as control, was uniformly sprayed on 4-week-old plants until run off

II.4 Pathogen infections

All pathogen infections were conducted on rosette leaves of 4-week-old plants.

B. cinerea growth and inoculation were performed as previously described (Ferrari et al., 2007; Galletti et al., 2008).

Pectobacterium carotovorum subsp. *carotovorum* (strain DSMZ 30169) was obtained by DSMZ GmbH (Braunschweig, Germany). Bacteria were cultivated in Luria-Bertani (LB) liquid medium (Duchefa Biochemie, Haarlem, The Netherlands) for 16-18 h at 28°C, 340 rpm. Bacteria were then collected by centrifugation (8000 x g for 10 min) and suspended in a 50 mM potassium-phosphate buffer (pH 7.0) at a final OD_{600} = 0.05, corresponding to a concentration of 5×10^7 colony forming units ml^{-1}. Arabidopsis leaves were detached and placed in Petri dishes containing 0.8% plant agar with the petiole embedded in the medium. Two scratches were made on the epidermis of the adaxial surface of each leaf, at the sides of the mid rib, using a sterile needle. A droplet of 5 µl of the bacterial suspension was placed on each scratch. Plates were wrapped with transparent plastic film and incubated at the same conditions as the leaves inoculated with *B. cinerea*. The area of water-soaked lesions was determined 16 hours after inoculation. Infection was performed by inoculating about three leaves per plant (at least four plants per genotype).

II.5 Gene Expression Analysis

Gene expression analysis were performed as previously described (Galletti et al., 2011) with slight modifications. Seedlings or leaf tissues were frozen in liquid nitrogen, homogenized with a MM301 Ball Mill (Retsch) and total RNA was extracted from at least 3 independent

replicates, each composed by 20 seedlings or at least 3 adult leaves form different plants, with Isol-RNA Lysis Reagent (5 Prime) according to the manufacturer's protocol. RNA (2 µg) was treated with RQ1 DNase (Promega) and first-strand cDNA was synthesized using ImProm-II reverse transcriptase (Promega) according to the manufacturer's instructions. Quantitative RT-PCR analysis was performed by using a CFX96 Real-Time System (Bio-Rad). cDNA (corresponding to 50 ng of total RNA) was amplified in a 20 µl reaction mix containing 1X GoTaq Real-Time PCR System (Promega) and 0.5 µM of each primer. Data analysis was done using LinRegPCR software. Expression levels of each gene, relative to *UBQ5*, were determined using a modification of the Pfaffl method (Pfaffl, 2001) as previously described (Ferrari et al., 2006) and expressed in arbitrary units. Primer sequences are shown in Table1.

II.6 Bioassays

Callose deposition was detected on leaves from 4-week-old plants sprayed with elicitors or wounded with forceps. After 24 h, for each treatment, about eight leaves, from at least five independent plants, were cleared and dehydrated with 100% boiled ethanol. Leaves were fixed in an acetic acid: ethanol (1:3) solution for 2 h, sequentially incubated for 15 min in 75% ethanol, for 15 min in 50% ethanol, and for 15 min in 150 mM phosphate buffer, pH 8.0, and then stained for 16 h at 4°C in 150 mM phosphate buffer, pH 8.0, containing 0.01% (w/v) aniline blue. After staining, leaves were mounted in 50% glycerol and examined by

epifluorescence microscope (Nikon, Eclipse e200) equipped with 10x or 4x magnification objective. Filter cube used was UV filter (Ex 330/380 EM 400; and the excitation was detected using a cooled charge-coupled device CCD camera (Nikon DS-Fi1C) Acquisition software is Nis Elements AR (Nikon). Callose quantification was performed by using ImageJ software. Callose deposition was replicated at least five independent times.

The H_2O_2 concentration in the incubation medium of treated seedlings (about 100–120 mg (10 seedlings) in 1 Ml of medium) was measured by the FOX1 method (Jiang et al., 1990), based on the peroxide-mediated oxidation of Fe2+, followed by the reaction of Fe3+ with xylenol orange dye (o-cresolsulfonephthalein 3#,3##-bis[methylimino] diacetic acid, sodium salt; Sigma). This method is extremely sensitive and used to measure low levels of water-soluble H_2O_2 present in the aqueous phase. To determine H2O2 concentration, 500 µl of the incubation medium were added to 500 µl of assay reagent (500 Mm ammonium ferrous sulfate, 50 Mm H_2SO_4, 200 Mm xylenol orange, and 200 Mm sorbitol). Absorbance of the $Fe3^+$-xylenol orange complex (A560) was detected
after 45 min of incubation. The specificity for H_2O_2 was tested by eliminating H_2O_2 in the reaction mixture with catalase. Standard curves of H_2O_2 were obtained for each independent experiment.

Data were normalized and expressed as micro molar H_2O_2/g fresh weight of seedlings.

II.7 Spinning Disk Microscopy Analyses

For confocal microscopy analyses, seedlings were grown for 6 days in Petri dishes containing MS medium agar plates supplemented with 1% sucrose. An inverted spinning-disk confocal microscope (CarvX, CrEST) was used for localization analyses. Imaging was performed using CFI Planfluo 40x (1,4 NA) oil immersion objective (NIKON) through 70 µm pinhole disk set at 6000 rpm. CFP and YFP were excited using 458 nm and 520 nm laser light, respectively; while RFP was excited using 558 nm laser light. Detection was performed using a cooled charge-coupled device CCD camera (CoolSNAP HQ2, Photometrics) and omega band-pass filters XF100-2 (for GFP and DCF-DA) and XF101-2 (for PI). The CCD camera, Z-motor and Confocal head were controlled by Metamorph software (Molecular Devices).

II.8 Statistical Analysis.

Experiments were run in duplicate or triplicate and repeated in a minimum of three independent trials. Data are represented as means ± standard error (s.e.m.). Unpaired *t*-test with equal

variance was used to calculate two-tailed P value to estimate statistical significance of differences between two treatment groups in the whole study. Statistical significant *P* values are indicated in the figures.

Prediction of GRP-3 cellular localization was performed using the Arabidopsis Cell Efp (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi) and SUBA (http://suba.plantenergy.uwa. Edu.au) browsers.

Table 1 ***List of primers used***

GENE	Forward Primer	Reverse
KAPP (At5g19280)	TCAGTGGTTTGT CCTTGGATC	ATCATGATGCTTT TCTCGTGG
GRP-3(At2g05520)	ACATCATTAGCC AACGCTTTG	TTTCCTCCATTGT CACCGTAG
Ret-ox(At1g26380)	CGAACCCTAAC AACAAAAAC	GACGACACGTAA GAAAGTCC
UBQ5 (At3G62250)	GTTAAGCTCGCT GTTCTTCAGT	TCAAGCTTCAACT CCTTCTTTC
WRKY40(At1g807540)	TGCACTTACCCT CCTTCG	GACAGTAGAAGC CGGTTGGT
FRK1 (At2g19190)	TGCACTTACCCT CCTTCG	GACAGTAGAAGC CGGTTGGT
RAP2 (At1g78080)	TTATTACCCGGA TTCAACGTT	CCGTAAGCGAAA CAAGATCC
WR3	GACCTGCCCAC ACAAGATCA	TGGAGGCAATAT CTAGGGACGC

KAPP GATEWAY	GGGGACAAGTT TGTACAAAAAG CAGGCTCCATG GCGATGATAGG GATGAAC	GGGGACCACTTT GTACAAGAAAGC GGGTACAGGGAA GTATCGAAATCTA A
GRP-3 GATEWAY(CFP)	GGGGACAAGTT TGTACAAAAAA GCAGGCTCCAT GGCTCCAAG GCT TTGGTT	GGGGACCACTTT GTACAAGAAAGC TGGGTAGTGACG GGCTGAGTCTGA
GRP-3 GATEWAY(RFP)	GGGGACAAGTT TGTACAAAAAA GCAGGCTCCAT GGCTTCCAAGG CTTTGGTT	GGGGACCACTTT GTACAAGAAAGC TGGGTATTAGGC GCCGGTGGA
GRP-3 pSATN-6 HindIII/BamHI	ATGCAAAGCTT ATGGCTTCCAA GGCTTTGG	TGCATCCTAGGGT GACCGGGCTGAG TCTGA
WAK2(At1g21270)	TTGCTTATACGC AGCTAGTCAA	CTGGATCTAACTA GCCGAACAC
FLS2 (At5g46330)	AAACAGAGCTT TGAACCAGAGA	AGTGAGATCAAG AACCTGGAGA
EFR (At5g20480)	GGGTAATCTTA GGGCTGATT	CTGGACGAGT TATTTCCAAG
WAK1 (At1g21250)	ACAGCACTTGTC TCGATTCT	TCTTTACGCTTGC AGCTCAT

AIM OF THE WORK

Fragments of HGA, named oligogalacturonides (OGs), function as danger signals and induce the expression of defense genes and proteins, protecting plants against fungal diseases: their accumulation at the apoplastic level determines the activation of defense responses (Brutus et al., 2010). Like hyaluronan fragments, OGs are regarded as damage-associated molecular patterns (DAMPs). The OGs, are formed by hydrolysis of homogalacturonan, main component of pectin, by hydrolytic enzymes, such as endopolygalacturonase (PG), secreted by pathogenic organisms during the infection process. Members of the Wall-Associated Kinase (WAK) family are candidate receptors of OGs, due to their ability to bind in vitro these oligosaccharides.

In the lab where I performed this work, it had previously demonstrated that the Arabidopsis Wall-Associated Kinase 1 (WAK1) is a receptor of OGs. On the other hand, WAK1 has been described to form a complex with an apoplastic glycine-rich protein (GRP-3) and a cytoplasmatic kinase-associated protein phosphatase (KAPP). Using Arabidopsis *grp-3* and *kapp* null insertional mutants, I show in this thesis that the two proteins act in the perception/transduction of the OG signal and in the regulation of the wound response.

III.RESULTS

III.1 GRP-3 and KAPP regulate the response to OGs and flg22

Two elements may be important in the perception/signal transduction cascade mediated by OGs: the PP2C phosphatase named KAPP and the glycine rich protein GRP-3. *In vitro* and *in vivo* analyses have shown that WAK1 interacts with KAPP through the kinase domain and with GRP-3 through the extracellular domain (Park et al., 2001).

III.2 *grp-3* and *kapp* seedlings have a prolonged defence genes expression after OGs or flg22 treatment

To elucidate whether KAPP and GRP-3 mediate OG signaling, I used homozygous Arabidopsis Col-0 mutants lines carring a T-DNA insertion in the *KAPP* and *GRP-3* genes (Fig. 1A and B). Both mutant lines carred a single insertion, as shown by segregation analysis of the antibiotic resistance behaviour that showed, for both mutants, a 3:1 segregation of resistant *versus* susceptible. *Kapp* and *grp-3* mutant seedlings showed no expression of full length transcripts of the corresponding mutated genes and therefore represent null mutants (Fig 1 C).

Response to elicitors was first examined in *kapp* and *grp-3* seedlings by monitoring the expression of genes that are known markers of the response to OGs and MAMPs (Denoux et al.,

2008; Galletti et al., 2011). These are *RetOx* (At1g26380), encoding a protein with homology to reticuline oxidases, *WRKY40* (At1g807540), encoding a transcription factor that acts as a negative regulator of basal defence responses (Chen et al., 2010) and *FRK1*, encoding a flg22-induced receptor-like kinase (de Torres et al., 2003). In seedlings treated with OGs or flg22, expression of *RetOx* is known to peak at 30 min and to decrease at 3 h, whereas that of *FRK1* and *WRKY40* peaks at 30 min and decreases nearly to basal levels at 1 h.

Ten-day-old *kapp* and *grp-3* seedlings were treated with OGs (50 µg/ml), flg22 (10 nM) or water for 1 h and 3 h, and expression of the marker genes was evaluated by quantitative RT-PCR (qRT-PCR). In both *kapp* and *grp-3* seedlings, levels of marker gene transcripts, upon water treatment, were comparable to those of the wild type (Fig. 2). After elicitation with either OGs or flg22, both mutants showed an accumulation of *RetOx* transcripts comparable to that of wild type at 1 h hour after treatment, and higher than that the wild type at 3 h. *FRK1* and *WRKY40* transcripts, instead accumulated at higher levels at both 1 h and 3 h in the two mutants in response to OGs and flg22 (Figure 2). These results indicate that elicitor induced up-regulation of gene expression is more prolonged in the *kapp* and *grp-3* mutants, and suggest a negative role of KAPP and GRP-3 on the duration of the elicitor-triggered responses.

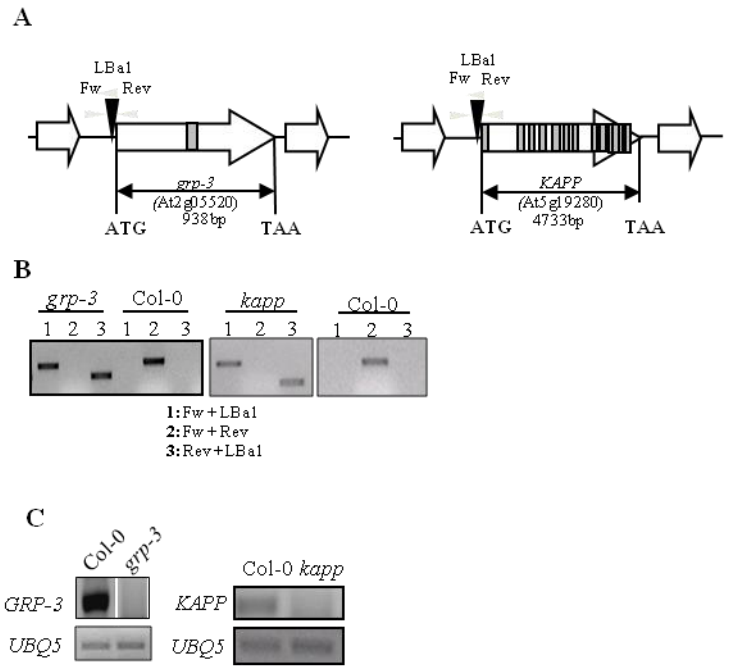

Figure 1. The lines *grp-3* and *kapp* knock out are null mutants. A) In both *grp-3* (SALK_084685.46.60, Col-0) and the *kapp* mutant (SAIL_1255-D05, Col-0) the T-DNA insertion site is within the 5'UTR of the genes (black arrowhead). Neighbour genes are shown as block arrows with arrowheads indicating the 3' terminus. Introns are indicated as grey boxes. The gray arrowheads indicate the forward and left-border primers used for diagnostic PCR (shown in B). B) PCR-based genotyping using gene specific PCR primers, performed to select homozygous mutants. C) Analysis of *GRP-3* and *KAPP* transcripts was performed by RT-PCR (40 cycles using 400 ng of cDNA as a template) in 10-day-old wild type (Col-0), *grp-3* and *kapp* seedlings. *UBQ5* was analysed as a control to show that equal amounts of cDNA were used. Mutants are homozygous for the insertions, which functionally disrupt the expression of the *GRP-3* and *KAPP* gene. A single insertion is present in each mutant, as shown by segregation analysis of the antibiotic resistance gene (3:1, resistant:susceptible)

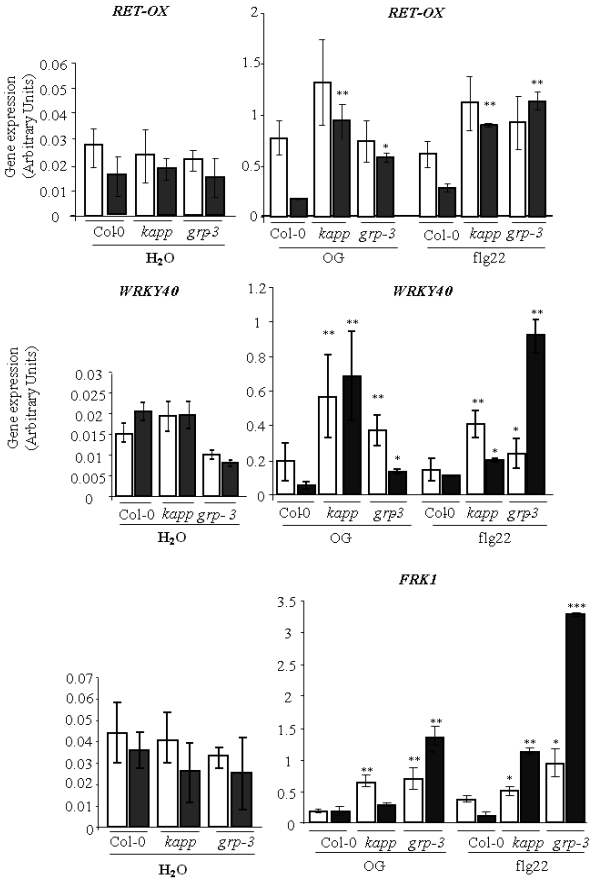

Figure 2. Arabidopsis *kapp* and *grp-3* mutant seedlings show a prolonged expression of defense marker genes in response to elicitor treatment. Ten-day-old seedlings were treated with OGs (50 µg/ml) or flg22 (10 nM) or water and elicitor-induced accumulation of *RetOx*, *WRKY40* and *FRK1* transcripts was analysed after 1 h (white bar) and 3 h (gray bar). Analyses were performed by qRT-PCR and transcript levels are shown as the mean of at least three independent experiments (±SE; n=20 in each experiment) normalized to *UBQ5* expression. Asterisks indicate statistically significant differences between elicitor treatment of mutant seedlings and Col0, according to Student's *t* test (*, P < 0.05; **, P < 0.001;***, P<0,0005).

III.3 GRP-3 and KAPP are involved in ROS production

In recent years, it has become apparent that ROS play an important signaling role in plants processes such as growth, development, response to biotic and abiotic environmental stimuli, and programmed cell death. The evolution of highly efficient scavenging mechanisms most likely enables plant cells to overcome ROS toxicity and led to the use of several of these reactive molecules as signal transducers. Moreover, the ROS-mediated signaling is controlled by a delicate balance between production and scavenging. These reactive molecules are generated at a number of cellular sites, including mitochondria, chloroplasts, peroxisomes, and at the extracellular side of the plasma membrane (Bailey-Serres and Mittler, 2006). However, although the highly compartmentalized nature of ROS is fairly well defined, little is known about the initiation of ROS signaling, the sensing and response mechanisms, and how the delicate balance between production and scavenging is controlled.

To understand whether KAPP and GRP-3 participate in the regulation of elicitor-induced ROS production, accumulation of H_2O_2 was measured after treatment with OGs (100 µg/ml) or flg22 (100nM), in *kapp* and *grp-3* leaves and seedlings. In seedlings, H_2O_2 accumulated in the growth medium was determined using a xylenol orange-based assay. This method is extremely sensitive and is normally used to measure low levels of

water-soluble H_2O_2. For both knock out lines, the amount of H_2O_2 produced, after treatment with either flg22 or OGs, was significantly higher than that of Col-0 seedlings (Fig. 5).

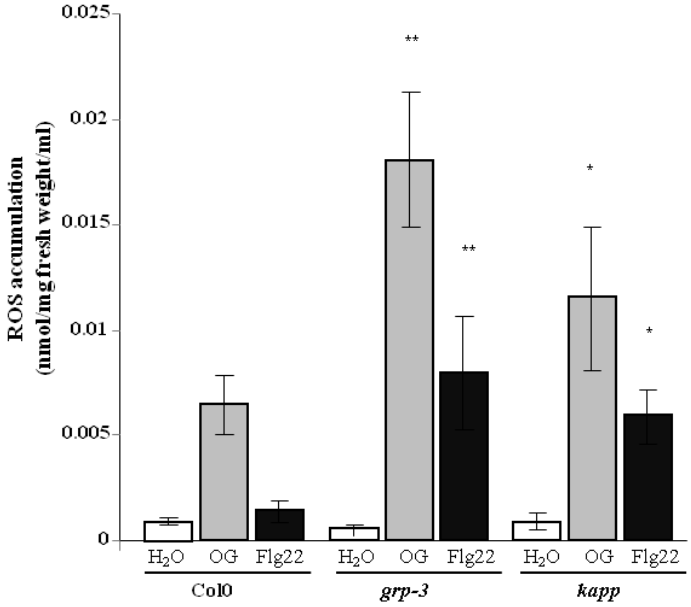

Figure 5. *kapp and grp-3* seedlings show enhanced oxidative burst in response to OGs and flg22. Accumulation of extracellular H_2O_2 in response to water (white bars), OGs (gray bars) or flg22 (black bars) in *kapp* and *grp3* mutant seedlings, measured by using a xylenol orange-based assay. Results are means ((± SE) of four independent experiments, each comprising 4 replicates of 10 seedlings. Asterisks indicate statistically significant difference between control and mutant seedlings, according to student T-test (* p<0,0005; ** p<0,000005)

III.4 grp-3 and kapp adult plants show enhanced sensitivity to sprayed DAMPs and PAMPs

Deposition of callose, a β-1 ,3-glucan synthesized between the cell wall and the plasma membrane, is among the most studied defence responses activated by both MAMPs and DAMPs (Galletti et al., 2008; Clay et al., 2009; Luna et al., 2011). Callose accumulation is considered a marker of the response PTI (PAMP-triggered immunity) (Nicaise et al., 2009). I investigated whether the callose deposition response was also affected by the lack of KAPP and GRP-3 function. Leaves of four-week-old *kapp*, *grp-3* and Col-0 plants were sprayed with OGs (70 µg/ml), flg22 (100 nM), water or short OGs (OG3-6) which are biologically inactive, and callose was visualized after 24 h by anyline blue staining. *Grp-3* mutants showed an enhanced sensitivity to water spraying (Fig. 3). Wild type leaves showed a moderate response to both OGs and flg22, consisting of few dots or isolated patches of callose deposits (Fig. 3). In *kapp* and *grp3* leaves, callose deposition in response to OGs and flg22 was significantly higher than that observed in response to water treatment; notably it was also significantly higher than in elicitor-treated Col-0 leaves. Treatment with short inactive OGs (OG3/6) resulted in a weak but significant response only in *grp-3* plants (Figure 3). These data support the hypothesis that GRP-3 and KAPP are general negative regulators of the response to elicitors.

(A)

	Score	Callose deposition description
	1	No deposition
	2	Few dots or limited areas of dots
	3	Diffused dots and/or 2 patches of dots
	4	Lots and extended dots and numerous patches

(B)

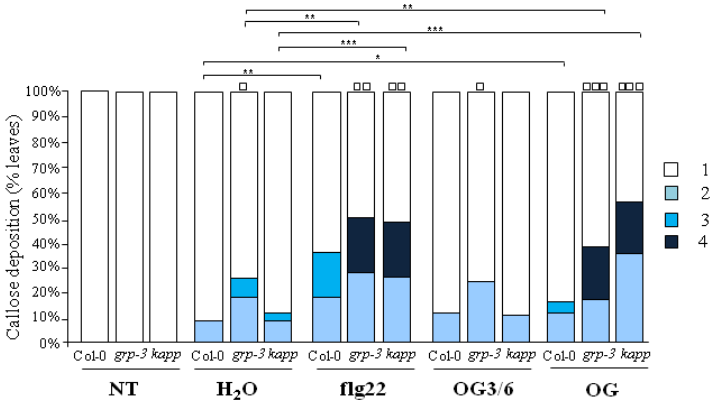

Figure 3. Arabidopsis *kapp and grp-3* mutants show enhanced callose deposition in response to sprayed elicitors. Leaves were sprayed with H_2O, flg22 (100 nM),) short and biologically inactive OGs (OG 3/6, 70 µg/ml) and OG (70 µg/ml). Callose deposits observed 24 h after treatment is expressed as a score that varies between 1 (no deposition), 2 (few dots or limited areas of dots), 3 (diffused dots and/or 2 patches of dots) and 4 (lots and extended dots and numerous patches). Representative drawings of callose deposition for each score is shown in panel A. In panel B, the histograms show the percentage of leaves with a specific callose deposition score. Values are the mean of five independent experiments (n=12 in each experiments). White squares directly above bars indicate statistically significant difference between Col-0 plants and transgenic plants. Asterisks above connection lines indicate statistically significant difference between water and elicitors treatment in each genetic background. Statistical analysis was performed according to Fisher's exact test (* $p< 0,05$; ** $p<0,005$; *** $p<0,0001$).

III.5 GRP-3 and KAPP regulate local response to wounding

OGs have been proposed as important signals in the wound response (Ryan and Jagendorf, 1995; Leon et al., 2001). Since they are negatively charged and have a limited mobility, the activity of these oligosaccharides as a wound signal is thought to be restricted to the areas that are close to the damaged or wounded tissue (Baydoun and Fry, 1985).

The observation that *grp-3* and *kapp* mutants display an increased response to OGs prompted to investigate whether these mutants show alterations in the wound response. To this aim, I first analyzed callose deposition in leaves in response to mechanical damage inflicted using forceps. Unlike wild type plants that show callose deposition only at the edge of the wounded tissue *kapp* and *grp-3* plants showed callose deposition (Fig. 6) also in a region surrounding the wound site (i.e. the proximal region), up to a distance of 0.5 ± 0.1 mm from the wounded site, indicating an enhanced response in a very localized aria proximal to the wound site in the mutant plants.

To corroborate this conclusion, I investigated whether to the increased callose deposition in the proximal region corresponded an increased expression of wound-response marker genes. The expression of genes that are known to be expressed after wounding was therefore analysed; in particular the genes examined were *RAP2* (At1g78080), encoding a AP2 domain-

containing protein RAP24 transcription factor (Delessert et al., 2004), and WR3 (At5g50200), encoding a high-affinity nitrate transporter known to be induced in a manner independent of JA synthesis and perception (Titarenko et al., 1997; Rojo et al., 1998). All these gene are induced early and locally upon wounding.

Four week-old leaves (2 leaves from at least 3 different plants) were wounded in the central part of the lamina by forceps. After 30 and 60 min, tissues corresponding to the region strictly proximal to the wound site were collected for the analysis of the expression of wound response marker genes by quantitative RT-PCR (qRT-PCR). Unwounded leaves were used as a control. Both knock out mutants showed increased expression of the wound response marker genes in the proximal region, at both time points analysed (Fig. 7 and 8). Basal levels of gene transcripts were slightly lower in *grp-3* leaves. Instead, *kapp* unwounded leaves showed basal expression level of all genes similar or slightly higher than those of the wild type.

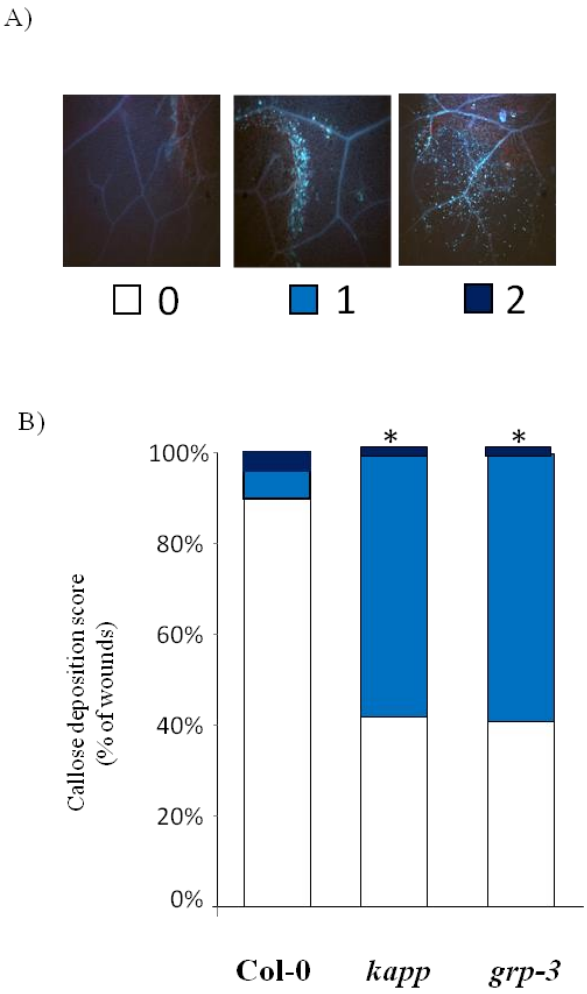

Figure 6. *kapp* and *grp-3* plants show enhanced local response to wounding. Callose deposition is expressed as scores that vary between 0 (no deposition), 1 (few dots) and 2 (numerous dots). A) Representative callose deposition for each score is shown; all images are at the same scale. B) the histograms show the percentage of leaves with a specific callose deposition score. Values are means (± was SE) of four independent experiments (n = 10 in each experiment). Asterisks indicate statistically significant difference between control and transgenic plants, according to Fisher's exact test (* p<0,001)

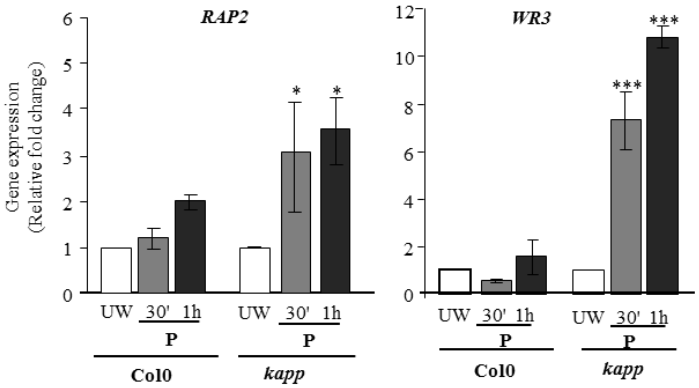

Figure 7. Arabidopsis *kapp* mutant plants show a higher expression of wounding marker genes in the area proximal to the wound site. Four-week-old leaves were wounded using forceps. The accumulation of *RAP2* or *WR3* transcripts in unwounded leaves (UW) and in the proximal zone (P) was analyzed by Real-Time PCR, 30 (gray bars) and 60 min (black bars) after wounding. *UBQ5* transcripts were used as a reference. The gene expression is expressed as fold change relative to unwounded sample. Analyses were performed by qRT-PCR and transcript levels are shown as the mean of at least three independent experiments (±SE; n=4 in each experiment. Asterisks indicate statistically significant differences between wounded mutants leaves and Col0, according to Student's *t* test (*, $P < 0.005$; **, $P < 0.0005$, ***,$P<0.00005$).

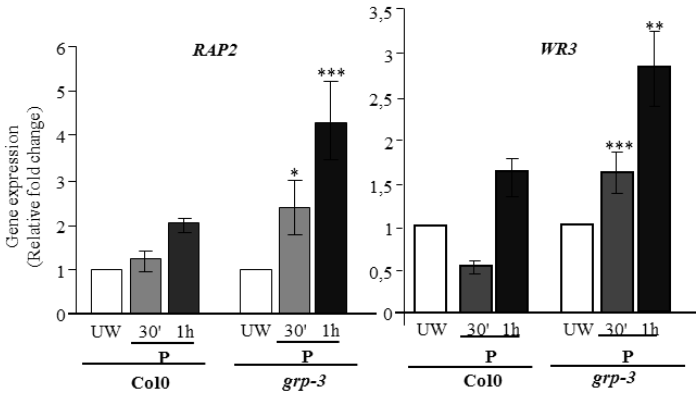

Figure 8. Arabidopsis *grp-3* mutant plants show a higher expression of wounding marker genes in the area proximal to the wound site. Four-week-old leaves were wounded using forceps. The accumulation of *RAP2* or *WR3* transcripts in unwounded leaves (UW) and in the proximal zone (P) was analyzed by Real-Time PCR, 30 (gray bars) and 60 min (black bars) after wounding. *UBQ5* transcripts were used as a reference. The gene expression is expressed as fold change relative to unwounded sample. Values are means ± SE of three independent experiments (n=3). Asterisks indicate statistically significant differences between wounded mutants leaves and Col0, according to Student's *t* test (*, P < 0.005; **, P < 0.0005, ***,P<0.00005).

III.6 KAPP and GRP3 are required for basal pathogen defence

It was previously reported that *WAK1* overexpression confers enhanced resistance to the fungus *B. cinerea* (Brutus et al., 2010). To better understand the involvement of KAPP and GPR-3 in defence responses, pathogen resistance was analysed in *kapp* and *grp-3* mutants. WT, *kapp* and *grp-3* plants were inoculated with *B. cinerea* spores or with *Pectobacterium carotovorum*. Lesion

development in both mutants plants was reduced by about 50% and 20%, with *B. cinerea* spores or with *Pectobacterium carotovorum*, respectively (Fig. 9), indicating that KAPP and GPR3 are necessary for basal resistance to these pathogens.

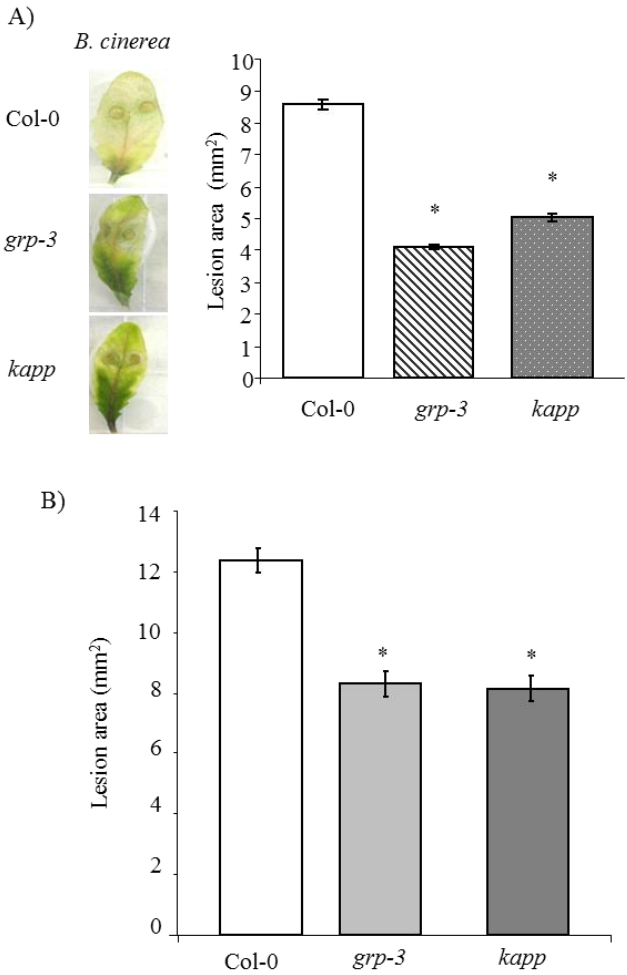

Figure 9. KAPP and GRP3 are required for basal resistance against *B. cinerea* and *Pectobacterium carotovorum*. A) Leaves from 4-week-old wild-type Col-0 and KO mutant were inoculated with *B.cinerea* (5 x 10^5 conidiospores mL^{-1}) and after 48h lesion area was analyzed . B) Infection with *P. carotovorum* (5 X 10^7 CFU/ml) . After 16 hours lesion areas were analyzed. Values are means ± SE of at least 16 lesions. Asterisks indicate statistically significant differences against control (Col-0), according to Fisher's exact test (*p < 0.005; **p <0.0005).

III.7 grp-3 and kapp plants show altered basal expression of genes codifying for DAMP or PAMP recognition receptors

Overexpression of the receptors involved in plant immunity greatly enhances plant defense against pathogens. Recently, it has been demonstrated that the expression of EFR, a PRR from the cruciferous plant *Arabidopsis thaliana*, confers responsiveness to bacterial elongation factor Tu in *Nicotiana benthamiana* and *Solanum lycopersicum*, making them more resistant to a range of phytopathogenic bacteria from different genera (Lacombe et al., 2010). Moreover, Brutus et al.,(2010) showed that the overexpression of the OGs receptor, WAK1, confers resistance to *B. cinerea.*

Whether the increased resistance to pathogens observed in *kapp* and *grp-3* mutants is related to an altered basal expression of genes encoding receptors involved in immunity was investigated. The genes examined were *EFR* (At5g20480), *FLS2* (At5g46330) and *WAK1*; in parallel, also the expression of WAK2 (At1g21270) was examined. Leaves from four-week-old rosettes were collected and expression of the genes was evaluated by quantitative RT-PCR (qRT-PCR). The analyses showed a higher expression of all the genes examined in *grp-3* plants compared to the control, while only expression of EFR and FLS2 was altered in *kapp* mutant plants(Fig. 10).

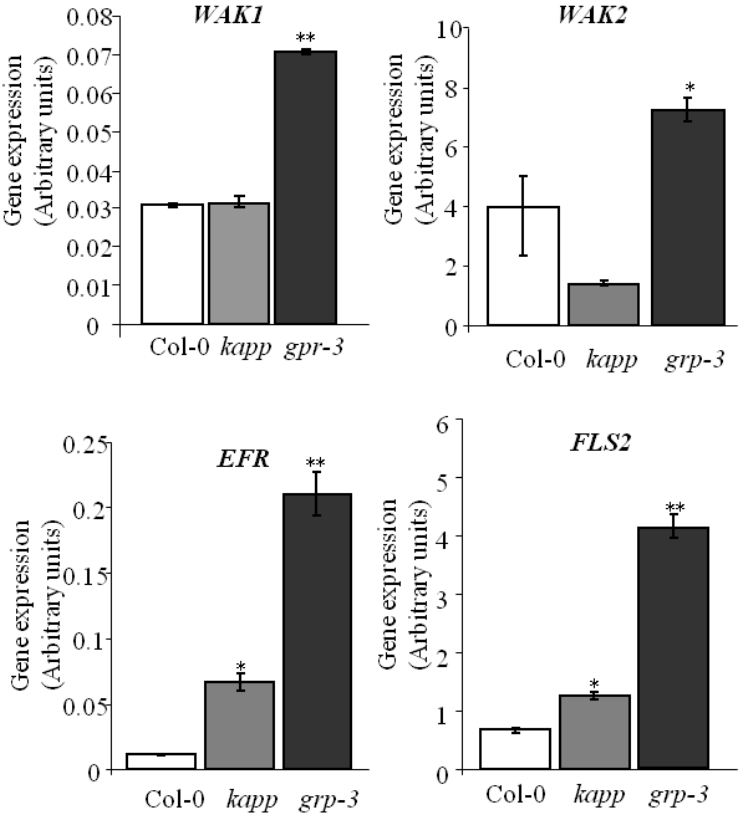

Figure 10. **Arabidopsis *kapp* and *grp-3* mutant show an enhanced basal level expression of DAMP and PAMP receptors**. Basal levels of of *EFR, FLS2 WAK1 or WAK2* transcripts in adult leaves was analyzed by Real-Time PCR, using *UBQ5* for normalization. Values are means (± was SE) of three independent experiments (n=3). Asterisks indicate statistically significant differences between wounded mutants leaves and Col0, according to Student's *t* test (*, $P < 0.05$; **, $P < 0.005$).

III.8 GRP-3 is putatively localized in the apoplast

A requisite for the physical and functional interaction between WAK1, GRP-3 and KAPP is that the proteins co-localize in the cell. The plasma membrane localization of WAK1 in leaves of transgenic Arabidopsis plants (Brutus et al., 2010) and of KAPP in cowpea mesophyll protoplasts (Shah et al., 2002) has been previously described, using variants that had been fused to the GFP and YFP, respectively. Instead, localization of GRP-3 has never been assessed. The *GRP-3*-encoded product exhibits a putative N-signal peptide (von Heijne, 1988) and no other membrane spanning domains or canonical organelle retention signals, suggesting a cell wall localization.

I investigated the localization of both GRP-3 and KAPP by both transiently and stably expressing fluorescent forms of the proteins in Arabidopsis plants. Confocal microscopy analyses confirmed the localization of fluorescent KAPP-YFP on plasma membrane (Fig. 11A). GRP-3-CFP fluorescence was instead localized in the cortical region of the cell in a reticulate pattern, typical of the ER (Figure 11B) (Batoko et al., 2000; Simpson et al., 2009; Rinne et al., 2011). This results is apparently in contrast with the notion that glycine-rich protein are structural component of the plant cell wall (Ringli et al., 2001). Moreover for several GRPs, like for GRP-3, extracellular localization is predicted due to the presence of a putative N-terminal peptide for export of the proteins

(Nielsen et al., 1997). The discrepancy can however be explained by a very low fluorescence of CFP at low pH (5.8) conditions, which are typical of the cell wall (Scott et al., 1999).

In fact GFP and color mutant derivatives such as CFP and YFP have a pK of 8.1 and are unstable at low pH (Haseloff et al., 1997); these features hamper their detection. To overcome this problem, GRP-3 was fused to RFP (Red Fluorescent Protein), expressed both transiently and stably in Arabidopsis WT plants, and its localization was analyzed by spinning disk microscopy analyses. GRP-3 fluorescence was observed at the cell periphery, likely in correspondence of the cell wall (Figure 11C). Taken together, these results support the hypothesis that GRP-3 and KAPP may form a complex with WAK1 at the plasma membrane level.

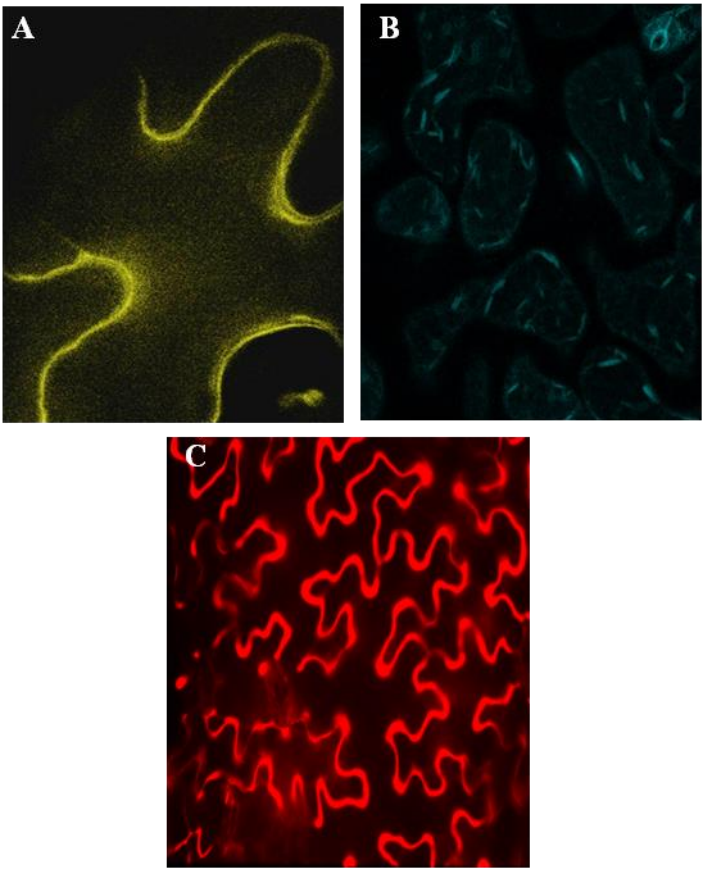

Figure 11. Localization of KAPP and GRP-3 in epidermal Arabidopsis seedlings cells by spinning disk microscopy analyses. KAPP-YFP and GRP-3-CFP protein fusions were stably expressed in transgenic Arabidopsis plants. A) KAPP-YFP exhibits a plasma membrane localization, in agreement with literature data. B) GRP-3-CFP labels, with a reticulate pattern, the cell periphery (cortical region). C) GRP-3-RFP labels the cell periphery, likely the apoplast.

IV.DISCUSSION

Plants have evolved adaptive mechanisms that allow them to survive in an ever changing environment. Since plants are sessile, they must be able to sense their natural environment and undergo changes in their physiology and development in response to those environmental cues whether they are adverse or beneficiate (Osakabe et al., 2012). The first line of microbial recognition leading to active defence responses relies on the perception of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) (Zipfel, 2009). Although the number of identified bacterial PAMPs recognized by plants is increasing constantly, very few plant PRRs have been discovered. Most of PRR characterized, correspond to transmembrane proteins with a ligand-binding ectodomain. The perception step is followed by activation of complex downstream signaling networks that trigger transient changes in defence gene expression. Indeed, PRRs interact, in a highly dynamic manner, with other components, that act as signaling adapters or amplifiers to achieve full functionality. These components include:

- co-regulators, such as BAK1, that several reports suggest as a signal "amplifier" rather than an integral component of downstream signaling pathways (Nicaise et al., 2009). Formation of receptor complexes linking extracellular perception to intracellular signal

transduction is a common theme in plant and animal signaling.

- protein kinases (PK); protein phosphorylation occurs in diverse cellular processes as a means of controlling protein activity. Signalling via the MAPK network relies on directional and sequential phosphorylation events between three elements, MAPK kinase kinases, MAPK kinases, and MAPKs (Nicaise et al., 2009). MAPKs are involved in various processes in eukaryote cells, including plant defence. Within the first 5 min after perception step, nearly 20 phosphoproteins showed an increase in phosphorylation status, as visualized by two-dimensional electrophoresis analysis of in vivo-labeled proteins (Lecourieux-Ouaked et al. 2000).
- protein phosphatases (PP); phosphorylation/dephosphorylation events are efficient regulatory mechanisms for signaling pathways involving kinases. Based on the amino acid residue they dephosphorylate, protein phosphatases have been classified into two major categories namely serine/threonine phosphatases and tyrosine phosphatases.
- Second messengers; the signal propagation is amplified through a complex network with many branches, each

being controlled by a combination of second messengers including free calcium, AOS, NO^-, cytosolic pH and membrane potential changes, cGMP, cADPR, SA, JA, ethylene, and probably metabolites of primary metabolism (Garcia-Brugger et al., 2006).

The contribution and regulation of each of these compounds in plant defence are still poorly defined (Alexandre Robert-Seilaniantz).

In the signaling mediated by OG two factors seem to be involved: the phosphatase KAPP and a glycine-rich protein GRP-3.

Indeed, by a combination of *in vitro* and *in vivo* experiments (yeast two-hybrid, immunoprecipitation, in vitro binding assay and gel filtration chromatography), it has been shown that WAK1 interacts, through its extracellular domain, with the glycine-rich protein GRP-3, and that the WAK1/GRP-3 complex allows interaction with the cytosolic kinase associated protein phosphatase (KAPP) (Park et al., 2001; Anderson et al., 2001).

The kinase-associated protein phosphatase (KAPP) is a member of the protein phosphatase 2C (PP2C) family. KAPP binds the kinase domain of FLS2 in yeast two-hybrid experiments (Gomez-Gomez et al., 2001), and transgenic Arabidopsis plants overexpressing KAPP are affected in flg22 binding and induced responses. Therefore, KAPP is a negative regulator of FLS2 (Gomez-Gomez et al., 2001). The fact that KAPP interacts with

many plant RKs through their phosphorylated kinase domains (Chevalier et al., 2009) suggests that it is a general regulator of RKs. In contrast the role of glycine rich protein is widely unknown.

Interestingly several data suggest that the GRP characterized by a C-terminal Cys-rich region, like GRP-3, are involved in stress responses.

In this study, I demonstrate that GRP3 and KAPP are regulators of OG responsiveness, in particular this protein have an important role as negative regulators of defence. Moreover I show that these protein, through the perception of OG, regulate the wounding responses.

This conclusion is based on the observation that *kapp* and *grp3* insertional mutant plants show increased OG responsiveness, both at the seedling stage and at the rosette stage.

It is well known that the treatment with OGs active the expression of several marker genes. In *kapp* and *grp3* seedlings the OG treatment not only trigger the expression of defence genes, such as *WRKY40, RET-OX* or *FRK1*, but also induce a prolonged expression of these genes.

Also, the lack of *kapp* and *grp3* expression significantly increased the H_2O_2 accumulation in seedlings treated with OGs.

The role of KAPP and GRP3 as negative regulator in the oligalacturonides perception is supported to analysis of callose deposition. The accumulation of callose, a plant b-1,3-glucan

polymer synthesized between the cell wall and the plasma membrane, is a classical marker of PTI responses after treatment with PAMPs or not infectious pathogens (Bestwick et al., 1995; Brown et al., 1998;Gomez-Gomez et al., 1999).

In particular adult plants *kapp* and *grp3* show an increased callose deposition in response to sprayed OGs in treatment conditions in which the wild type plants show a weak response.

Notably the same responses was observed, in each experiment performed, after flg22 treatment. This result is in agreement with the observation that KAPP overexpressing plants mimick the *fls2* mutant phenotype (Gomez-Gomez et al., 2001), whereas is unexpected for *grp3* mutant. It is possible that, like KAPP, GRP-3 is involved to negatively regulate the signalling mediated to both PAMPs and DAMPs.

In addition the involvement of these mutants in the signalling mediated by OG is highlighted by basal resistance of these mutants to different microbial and fungal pathogens. *Botrytis cinerea* and *Pectobacterium carotovorum* are known release, during the infection, a large amount of pectinolytic enzymes.

Furthermore, KO mutants for KAPP and GRP3 also have increased expression of genes codifying for DAMPs or PAMPs recognition receptor, such as FLS2 and EFR.

Taken together, our results support a possibility that KAPP and GRP3 protein have an important role in the regulation of many

physiological processes, activated by both OGs and PAMP (flagellin).

OGs are thought to be released from plant cell walls upon partial degradation of HGA by microbial PGs during infections (Cervone etal., 1989) or by the action of endogenous PGs induced by mechanical damage (Orozco-Cardenas and Ryan, 1999). The signalling activity of OGs is a clear indication that plants have evolved mechanisms to monitor HGA degradation for the early detection of tissue damage (Ferrari et al., 2013). Moreover since 90s OGs have been proposed as important local signals in the wound response.

So, because the *grp-3* and *kapp* mutants display an enhanced response to OGs, I investigated if these protein are involved in the wound response.

Interestingly adult mutant leaves, mechanical wounded, show a strong callose deposition, not only in the wounded site, but even in proximal zone, where Col-0 not show callose accumulation.

This data suggest a higher responsiveness of these plants to mechanical damage.

The importance of KAPP and GRP3 in wound-triggered response is also confirmed by analysis of expression of two wounding marker genes, such as *RAP2* and *WR3*, in the wound proximal region.

Indeed, mechanical damage caused an increased expression of these genes, in the wound proximal region of *kapp* and *grp-3* mutants, compared to the wild type.

Finally, confocal microscope analysis, using fluorescent version of GRP-3 and KAPP proteins, suggest that these proteins are localized in the apoplast and in plasma membrane, respectively. This localization supports the hypothesis that these two proteins can form a complex with WAK1 for the signal transduction mediated by OG.

All in all, since KAPP and GRP3 bind WAK1, an OG receptor, and because they show an enhanced OG and wound responsiveness, we can correlate the role of OG as local signal molecule accumulated during cell wall degradation due to the wound process.

Additional insights in the role of KAPP and GRP3 in DAMPs and PAMPs signalling and wounding response would be obtained after the characterization of the transgenic lines overexpressing this protein.

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