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PART I – GENERAL INTRODUCTION

I. 1. PLANT IMMUNITY

During evolution, plants, due to its sessile lifestyle, have evolved a sophisticated immune system of the so called "innate" type to combat microbial attach. On the other hand, in order to be pathogenic, microbes must reach the plant interior either by penetrating the leaf or root surface directly or by entering through wounds or natural openings such as stomata. Once inside, they have cross another obstacle, the plant cell wall, a rigid, cellulose-based support surrounding every cell, before encountering the host plasma membrane. Here, they have to face a sentinel system that comprises the so called Pattern Recognition Receptors or PRRs (Jones and Dangl, 2006; Boller and Felix, 2009). The detection of specific Pathogen-Associated Molecular Pattern (PAMPs), also indicated as microbe-associated molecular patterns (MAMPs), by PRRs represents the first of the two layers into which plant immunity is distinguished. This type of resistance is referred to as PTI, for PAMP-Triggered Immunity (Chisholm et al., 2006). PAMPs belong to the class of the so-called general elicitors, which also include damage-associated molecular patterns (DAMPs). DAMPs, originally indicated as endogenous elicitors, arise from the plant itself due to damage caused by microbes or mechanical injury (Darvill and Albersheim, 1984; Boller and Felix, 2009).

To counteract PTI, microbes have evolved effectors, against which plants have in turn evolved a second layer of plant surveillance, known as Effector-Triggered-Immunity (ETI). This is a very robust response typically mediated by Resistance (R) proteins, which are intracellular proteins of the nucleotide-binding site-LRR (NB-LRR) class that mediate, directly or indirectly, recognition of pathogen effectors, originally indicated as Avirulence (Avr) proteins (Jones and Dangl, 2006). Activation of R protein-mediated resistance also suppresses microbial growth, but only when the invader is already inside after a limited proliferation (Figure 1). ETI is often characterized by a local programmed cell death termed Hypersensitive Response (HR) at the infection site: here, a limited number of plant cells, those that take contact with the pathogen, die quickly, determining a necrotic lesion where the pathogen is confined and blocked (Godiard et al., 1994).

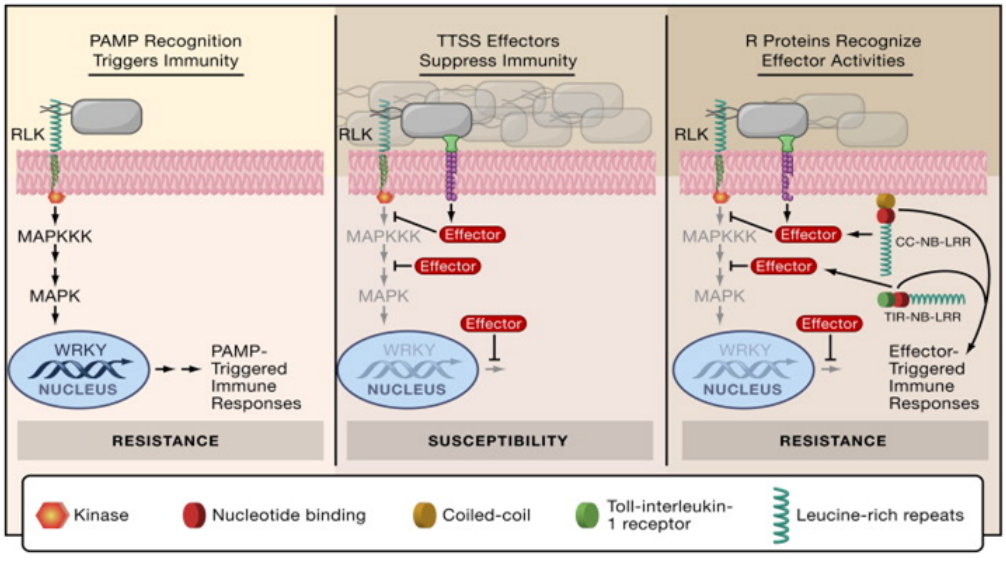

Figure 1. Model for the Evolution of Bacterial Resistance in Plants
Left to right, recognition of pathogen-associated molecular patterns (such as bacterial flagellin) by extracellular receptor-like kinases (RLKs) promptly triggers basal immunity, which requires signaling through MAP kinase cascades and transcriptional reprogramming mediated by plant WRKY transcription factors. Pathogenic bacteria use the type III secretion system to deliver effector proteins that target multiple host proteins to suppress basal immune responses, allowing significant accumulation of bacteria in the plant apoplast. Plant resistance proteins (represented by CC-NB-LRR and TIR-NB-LRR; see text) recognize effector activity and restore resistance through effector-triggered immune responses. Limited accumulation of bacteria occurs prior to effective initiation of effector-triggered immune responses. Adapted from Chisholm et al. (2006).

On the other hand, Boller and Felix (2009) postulate only one form of plant innate immunity, where both PTI and ETI coexist and DAMPs are included. These authors envision that effective innate immunity in plants, as in vertebrates, is mediated through a single overarching principle, the perception of signals of danger (Matzinger, 2002; Lotze et al., 2007; Rubartelli and Lotze, 2007). PAMPs, DAMPs, and effectors might appear to the plant as one and the same type of signal that indicates a situation of danger (Figure 2). Indeed, gene expression data indicate that considerable overlap exists between the defense response induced by MAMPs, DAMP and effectors (Tao et al., 2003; Navarro et al., 2004; Thilmony et al., 2006; Wise et al., 2007).

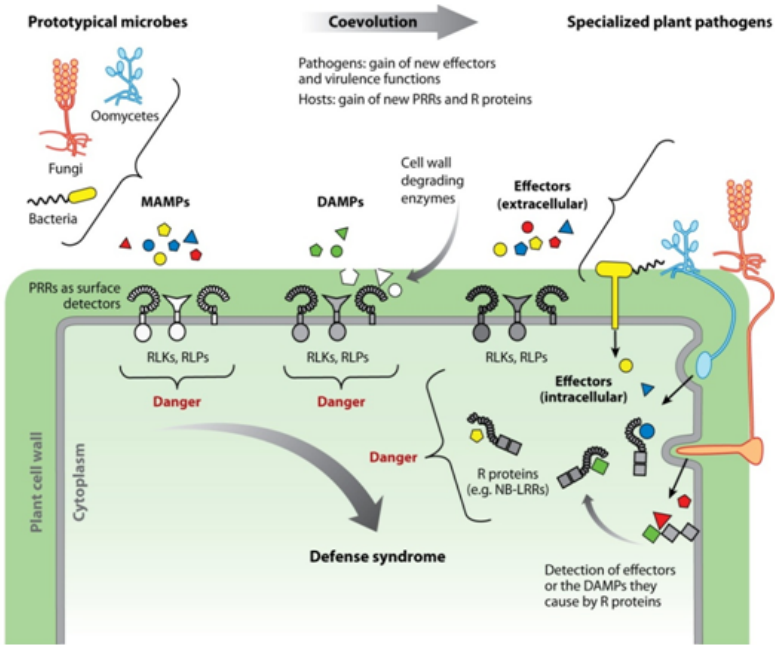

Figure 2. Microbe-associated molecular patterns (MAMPs), damage-associated molecular patterns (DAMPs), and effectors are perceived as signals of danger. Extracellular MAMPs of prototypical microbes and DAMPs released by their enzymes are recognized through pattern recognition receptors (PRRs). In the 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 et al. (2009).

The recognition of specific or non-specific elicitors activates PRRs or R proteins and is followed by a complex spectrum of reaction including molecular, morphological and physiological changes (Altenbach and Robatzek, 2007) that constitute the immune response (see following section).

I. 2. THE PLANT DEFENSE RESPONSE

I. 2. 1. Basal defense

Induction of basal defense mechanisms occurs in response to PAMPs in both host and non-host plant species through a complex signal transduction pathway that includes a rapid depolarization of the plasma membrane potential, a rapid oxidative burst, and the activation of intracellular kinase cascades, generally followed by protein phosphorylation and changes in gene expression (Schwessinger and Zipfel, 2008).

Very Early Responses (1-5 Minutes)

Among the earliest and most easily recordable physiological responses to PAMPs and DAMPs in plant cell cultures, starting after a lag phase of ~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) which resulted in cytoplasmic acidification increasing 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. The rapid increase in cytoplasmic $Ca2^+$ concentrations 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). In this regard, it is interesting that production of secondary metabolites is enhanced in response to changes in cytoplasmic pH (Roos et al., 1998).

Another very early response to PAMPs and DAMPs, with a lag phase of ~2 min, is the oxidative burst (Chinchilla et al., 2007). Reactive oxygen species (ROS) may act as secondary stress signals to induce various defense responses (Apel and Hirt, 2004). The quantities of ROS produced can be cytotoxic and thus are expected to be antimicrobial and are thought to have direct (through cytotoxicity) and indirect (through signaling) roles in the plant cell death required for the HR. In addition, ROS 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, 1994).

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).

AtrbohD is NADPH oxidase 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).

Other enzymes appear to be important in the elicitor-mediated oxidative burst, including apoplastic oxidases, such as oxalate oxidase (Dumas et al., 1993), amine oxidase (Allan and Fluhr, 1997), and pH-dependent apoplastic peroxidases (Frahry and Schopfer, 1998; Bolwell et al., 1995), which generate either O_2^- or H_2O_2.

Activation of Mitogen-Activated Protein Kinase (MAPK) cascades is another early response to PAMP and DAMP (Pedley et al. 2005). The MAPK phosphorylation cascade is a highly conserved signal transduction mechanism that plays a key role in regulating many aspects of growth and development in eukaryotes. 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 Once activated, MAPKs phosphorylate a number of different target proteins including transcription factors, other targets protein kinases, phospholipases, and cytoskeletal proteins, all of which effect changes in gene expression and/or physiological responses appropriate to the stimulus in question (Widmann et al., 1999).

Early Responses (5–30 Minutes)

Among the early responses there is an increased production of the stress hormone ethylene, receptor endocytosis as described for certain receptors in animals, and a substantial transcriptional reprogramming involving activities of WRKY transcription factors. Activation of transcription of genes related to the pathogenesis such as lytic enzymes (chitinase, glucanase, protease), proteins and metabolites with antimicrobial activities (defensins and phytoalexins, respectively) is also induced (Kombrink and Somssich, 1995).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). 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).

A secretory machinery becomes also engaged in the execution of immune responses. Vesicle-associated and SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) protein-mediated exocytosis pathways are involved to drive secretion of antimicrobial cocktails comprising proteins, small molecules, and cell wall building blocks into the apoplastic space. These pathways have important functions also in plant development and might have been recruited for immune responses. The driving forces of such focal accumulation is the rapid changes of the actin and tubulin network. Rearrangements of cytoskeleton produce a kind of physical barrier by locally increasing the density of cellular components (Frey and Robatzek, 2009). Bacteria and fungi have evolved molecules that intercept the secretion machinery by blocking vesicle formation from intracellular membranes (Kwon et al., 2008).

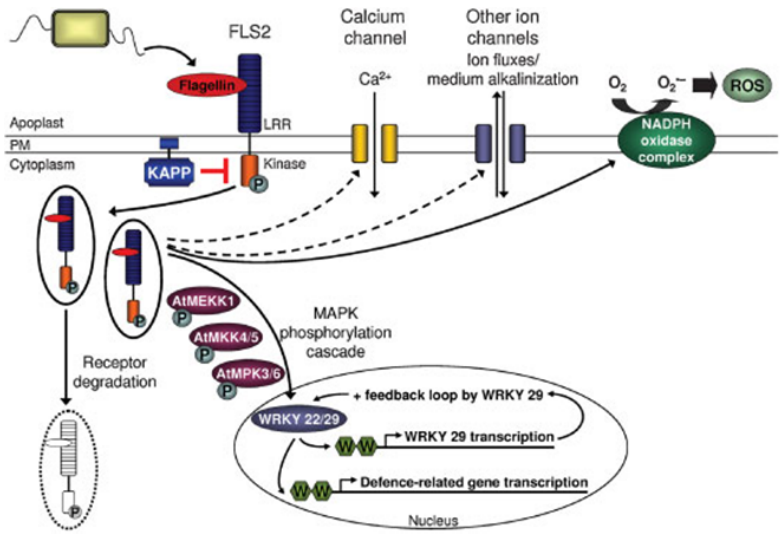

Figure 4. Plant very early/early responses to PAMPs. A current model for flagellin signalling in Arabidopsis.

Late Responses (Hours–Days)

Later changes include a series of immune responses, including callose deposition. Arabidopsis leaves treated with flg22 and fixed and stained with aniline blue about 16 h later display strong accumulation of fluorescent spots thought to represent callose deposits (Gómez-Gómez et al., 1999). Although the biological foundation of this response is not clear, it has been used frequently, particularly to characterize pathogen effectors that interfere with MAMP signaling (Chisholm et al., 2006; Jones and Dangl, 2006; Abramovitch et al., 2006).

Another late response is certainly stomatal closure. Stomata provide a major entry point for many plant pathogens and *A. thaliana* stomata have been shown to close within 1 h in response to PAMPs as part of PTI (Melotto et al., 2006).

Moreover, newly synthesized cell wall material, into the paramural space between the cell wall and the plasma membrane, serves as a physical barrier at infection sites (Aist, 1976).

In Arabidopsis, a robust bioassay for MAMPs such as flg22 and elf18 (see paragraph I.2.2) is seedling growth inhibition. This response may reflect a physiological switch from a growth to a defense program, and it may be connected to the induction the downregulation of auxin-responsive genes (Bellincampi et al., 1993; Navarro et al., 2006; Savatin et al., 2011).

Another characteristic of PAMP and DAMP elicitors is to activate plant responses involved in defense against pathogens. For example, the pretreatment of Arabidopsis leaves with the MAMP flg22 was reported to enhance

resistance to *Pseudomonas syringae* (Zipfel et al., 2004) and *B. cinerea* (Ferrari et al., 2007). Treatment with exogenous OGs, a class of DAMPs (see paragraphs I.2.3 and I.2.4) enhances resistance against *B. cinerea* in grape (Aziz et al., 2004) and Arabidopsis leaves (Ferrari et al., 2007). However, defense mechanisms that underlie resistance against pathogens are very complex and not fully understood. For a more detailed description see below (paragraph I.3).

I. 2. 2. PAMPs and their receptors

PAMPs are molecular signatures typical of whole classes of microbes, and their recognition plays a key role in innate immunity. A complete description of the responses triggered by MAMPs is beyond the scope of this introduction and the reader can refer to several comprehensive reviews (Boller and Felix, 2009; Zipfel, 2009; Zhang and Zhou, 2010; Segonzac and Zipfel, 2011; Tena et al., 2011). Recognition of PAMPs by PRRs is the prerequisite and the first step to trigger defence reactions effective against the invading microbes. Characterized PRRs belong to the superfamily of surface receptor-like kinases (RLKs) (Boller and Felix, 2009) that generally have an extracellular ligand-binding domain, a membrane spanning region, a juxtamembrane (JM) domain, and a serine/threonine kinase domain. The N-terminal extracellular domain of PRRs defines ligand specificity. The most studied of them include those with leucine-rich repeat (LRR) domains (LRR-RLKs), LysM domains (LYK) and the *Catharanthus roseus* RLK1-like (CrRLK1L) domain. These proteins recognize distinct ligands of microbial origin or ligands derived from intracellular protein/carbohydrate signals. From a simplistic viewpoint, kinases serve as switches that are turned on or off via conformational changes induced by ligand binding. The plant RLKs conserve an aspartate residue in the kinases catalytic loop required for catalytic activity. The activation loop becomes phosphorylated and structurally reoriented to enable substrate access and/or to enhance phosphotransfer efficiency (Adams, 2003). In Ser/Thr kinases the catalytic aspartate (D) is mostly preceded by an arginine (R). This kind of kinases are termed RD kinases, and RD motif facilitates phosphotransfer (Johnson et al., 1996). However most RLKs are non-RD kinases, lacking an arginine preceding the catalytic aspartate (Krupa et al., 2004). RLKs, in general, require additional proteins to modulate their function (Johnson et al., 1996; Dardick and Ronald, 2006). An important example is BRI-associated kinase1 (BAK1), which interacts with many *Arabidopsis* RLKs and is required for their activity (see paragraph IV.1).
The best characterized plant PRRs are LRR-RLKs and include FLS2 (flagellin sensing 2), which recognize a conserved 22 amino-acid peptide (flg22) of the bacterial flagellin, and EFR, which binds a 18 amino-acid epitope (elf18) of the bacterial elongation factor EF-Tu (Boller and Felix, 2009; Lacombe et al., 2010) (Figure 3). A distinct subfamily of RLKs is characterized by LysM motifs and include CERK1 (chitin elicitor receptor kinase 1) and CEBiP (chitin elicitor-binding protein). The proteins act for immune signaling triggered by fungal chitin, a long-chain polymer of an N-acetylglucosamine and the main component of cell walls of higher fungi. LysM motifs are required for glycan binding in the N-terminal ectodomain (Wan et al., 2008; Nakagawa et al., 2011).

FLS2/flg22. FLS2 perceives the conserved peptide of flagellin flg22 present in a broad class of bacterial plant pathogens including *Pseudomonas syringae* pv. *tomato* (Pto) DC3000 (Gomez-Gomez and Boller, 2000). FLS2 consists of an extracellular LRR domain with 28 repeats, a transmembrane domain and a cytoplasmic kinase domain (Boller and Felix, 2009). LRR 9-15 of FLS2 are necessary for flg22 binding (Dunning et al., 2007). The catalytic loop of FLS2 contains the sequence CD instead of the RD. It is well-established that FLS2 forms heterodimers with BAK1 (Chinchilla et al., 2007; Schulze et al., 2010) in the presence of bound flg22. BAK1 is a common component in many RLK signaling complexes and was first identified for its requirement in brassinosteroid signaling via the receptor BRI1

(Li and Nam, 2002). Immediately after FLS2-BAK1 activation, Arabidopsis BIK1 (a receptor-like cytoplasmic Kinase, RLCK) plays a pivotal role in MAMP signaling.

BIK1 interacts with FLS2 and BAK1, and flg22 triggers FLS2- and BAK1-dependent BIK1 phosphorylation (Zhang et al., 2010; Lu et al., 2010). Recently, ligand mediated receptor endocytosis has been identified as an additional FLS2 regulatory mechanism (Robatzek et al., 2006). After binding of flg22, FLS2 accumulates in mobile intracellular vesicles. This ligand-induced FLS2 endocytosis is followed by receptor degradation possibly via endosomal and/or proteasomal pathways (Goehre et al., 2008; Lu et al., 2011; Beck et al., 2012; Choi et al., 2013). Endocytosis and downstream signaling are closely linked but it is not yet known if the actual internalization is required for signal transduction (Robatzek et al., 2006; Beck et al., 2012; Choi et al., 2013). It was suggested that flg22-induced degradation of endogenous FLS2 may serve to desensitize cells to the same stimulus, likely to prevent continuous signal output upon repetitive flg22 stimulation (Smith et al., 2014). Degradation of ligand-bound FLS2 is likely required for receptor turnover from the cell surface. Subsequent replenishment of newly synthesized FLS2 to the site of stimulus perception resulted in resensitization, probably to prepare cells for a new round of flg22 perception and signaling (Smith et al., 2014).

Responsiveness to flg22 is shared by members of all major groups of higher plants, indicating that the PRR for this epitope of bacterial flagellin is evolutionarily ancient. Indeed, orthologs of FLS2 with a high degree of conservation are present in genomes of all higher plants analyzed so far. Only the moss *Physcomitrella patens* contains many LRR-RKs in its genome but does not carry an FLS2 ortholog and also shows no response to flg22 (Boller and Felix, 2009).

EFR/EF-Tu. The elongation factor receptor EFR is another well studied receptor which can perceive the N-terminal acetylated peptide elf18 and elf26 of the bacterial elongation factor Tu (EF-Tu). Transient expression of the EFR gene in *Nicotiana benthamiana*, a plant lacking an endogenous EF-Tu perception system, conferred elf18/elf26 responsiveness to this plant species, directly demonstrating that EFR is the PRR for EF-Tu ((Zipfel et al., 2006) and, furthermore, that downstream elements of PRR activation are conserved between Arabidopsis and Nicotiana.

The extracellular LRR domain of EFR (21 repeats) is highly glycosylated, and this seems to be important for ligand binding as mutation of a single predicted glycosylation site compromises elf18 binding (Haweker et al., 2010). Most likely LRR 1-6 and 19-21 of EFR are necessary for elf18 binding and receptor activation (Albert et al., 2010).

EFR and BAK1 have also been shown to interact in a ligand-dependent manner (Roux et al., 2011). Indeed, many of the signaling components downstream of EFR and FLS2 are shared and activation of EFR leads to activation of similar defence responses as those triggered by flg22 (Zipfel et al., 2006). In fact it has been shown that BIK1 is phosphorylated upon elf18 and flg22 treatment (Lu et al., 2010). Given the many parallels between FLS2 and EFR, it is possible that trans-phosphorylation of the EFR/BAK1 complex also occurs, although direct proof is still lacking.

In contrast to FLS2, N-glycosylation is critical for EFR function and EFR is subject to ER quality control that requires several chaperones involved in ER-QC for full activity (Haweker et al., 2010). Responsiveness to elf18/elf26 was found in various Brassicaceae species but not in members of other plant families tested, indicating that perception of EF-Tu as a PAMP is an innovation in the Brassicaceae (Kunze et al., 2004).

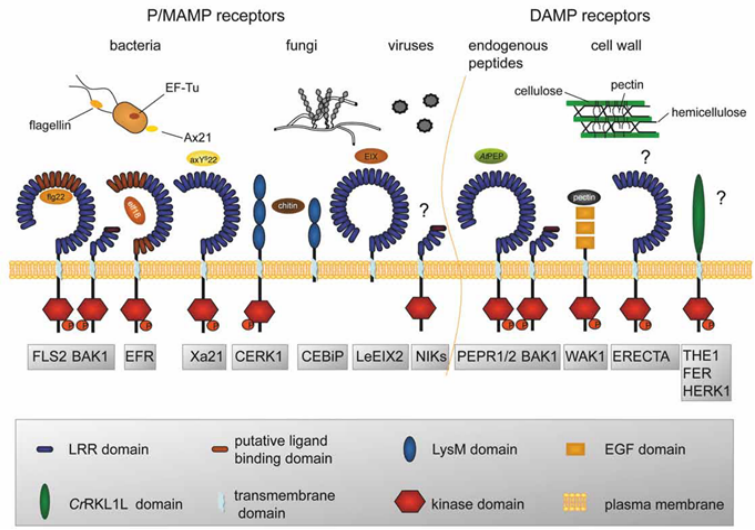

Figure 3. Membrane-associated pattern recognition receptors can perceive microbial patterns (P/MAMP) from different microbes such as bacteria, fungi, oomycetes or viruses. They can also perceive damage associated molecular patterns (DAMP), released after wounding or pathogen attack. DAMPs as e.g. the endogenous peptides AtPEPs are perceived by the redundant LRR-receptors PEPR1 and 2. Cell wall fragments can bind to WAK1 and activate oligogalacturonide-dependent defense responses. Other RLKs known to be involved in developmental processes as the LRR-RLK ERECTA and the CrRLK1L proteins FERONIA, HERCULES and THESEUS might be involved in damage associated defence responses. Adapted from (Mazzotta and Kemmerling, 2011).

Other PRR/PAMP pairs. CERK1/LysM-RLK1 is necessary for fungal chitin perception in Arabidopsis (Miya et al., 2007; Wan et al., 2008) and acts cooperatively with CEBiP in rice (Shimizu et al., 2010), while tomato EIX2 can perceive the fungal ethylene inducing xylanase (EIX) of the ascomycete *Trichoderma viride* (Bar and Avni, 2009). NIK1 to 3 were shown to be involved in virus resistance in tomato and Arabidopsis (Fontes et al., 2004). They belong to the same LRR family II as BAK1 (Santos et al., 2010), a small LRR-RLK with four and a half LRR-repeats that interacts with several ligand binding receptors such as FLS2 (Chinchilla et al., 2007; Heese et al., 2007), EFR (Schwessinger et al., 2011), PEPR1/2 (Postel et al., 2010; Schulze et al., 2010) (see paragraph V.1.3) and BRI1 (Li and Nam, 2002; Nam and Li, 2002). In Arabidopsis the peptidoglycan (PGN) perception system comprises three LysM domain proteins. LYM1 and LYM3 are plasma membrane proteins that lack cytoplasmic signaling domains and that physically bind PGNs. It is hypothesized that LYM1 and LYM3 proteins form a heteromeric PGN-binding module that in conjunction with the transmembrane receptor kinase CERK1 builds a receptor complex that is required for ligand binding and initiation of an intracellular signaling cascade (Willmann et al., 2011).

I. 2. 3. Damage-associated molecular patterns (DAMPS)

In addition to sensing invading microbes by means of PAMPs (infectious non-self), plants and animals can also sense infectious-self or modified-self via damage-associated molecular patterns (DAMPs). 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). Oligosaccharide fragments released from plant cell wall pectin or cuticle owing to pathogens attack, wounding or the action of endogenous degrading enzymes are well known DAMPs.

I. 2. 4. Oligogalacturonides

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) (figure 4). OGs cannot be considered true PAMPs, since they are not derived from the pathogen. However, they are considered the classic examples of DAMPs that are generated by the host cell during the infection process.

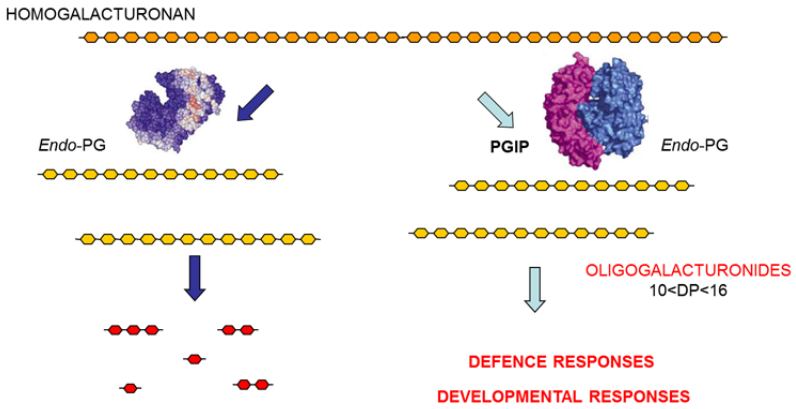

Figure 4. Model for the OG accumulation during pathogen infection.

Chemically pure OGs can act as endogenous elicitors (Galletti et al., 2009). Biological responses to OGs occur in at least five of the six subclasses of dicotyledonous plants Magnoliidae, Hamamelidae, Asteridae, Rosidae, Dilleniidae (Reymond et al., 1996; Cote and Hahn, 1994) in a monocot (Moerschbacher et al., 1999) and a gymnosperm (Asiegbu et al., 1994). A number of different biological responses to OGs have been reported, and the particular response observed depends on the plant species, the bioassay, and the chemical structure of the OG used (Cote et al., 1998). A spectrum of modified and unmodified OGs of various lengths are active in different systems (reviewed by (Cote and Hahn, 1994).

The biological responses of plants to OGs can be divided into two broad categories: plant defense and plant growth and development (Cote and Hahn, 1994).

I. 2. 5. Oligogalacturonide-induced responses involved in plant defense

Pathogens enter plant tissues in at least three ways: digesting cell walls, entering through wounds, and invading through natural openings such as stomata. Pectins are 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. 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., 1986a), 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. 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 (Galletti et al., 2008).

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 *At*RbohD (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. A variety of plant defense responses against microbial pathogens are regulated by the signaling molecules SA, JA and ET. Resistance to Botrytis cinerea induced in Arabidopsis by OGs is independent of SA, ET or JA signaling, but requires *PHYTOALEXIN DEFICIENT3* (*PAD3*) (Ferrari et al., 2007), a gene involved in the metabolism of Trp-derived secondary compounds (Zhou et al., 1999).

I. 2. 6. Oligogalacturonide-induced responses involved in plant growth and development

Exogenously added OGs influence the growth and development of plant tissues (Cote and Hahn, 1994). OGs inhibit auxin-induced pea stem elongation (Branca et al., 1988) and are also active in the tobacco thin-cell layer (TCL) (Tran Thanh Van et al., 1985; Mohnen et al., 1990), and the tobacco leaf explant bioassays (Bellincampi et al., 1993). When biologically active OGs are added to media containing specific phytohormone concentrations, TCLs that would normally form few or no organs form flowers, while TCLs that normally form roots form significantly fewer roots (Eberhard et al., 1989). Biologically active OGs inhibit root formation (Bellincampi et al., 1993) and increase stomata formation (Altamura et al., 1998b) on tobacco leaf explants incubated in media with specific phytohormone concentrations.

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. Tomato fruits expressing antisense PGs mRNA exhibited a 99% reduction in PGs activity and a substantial reduction in pectin depolymerization, but were unaffected in their ethylene production and overall ripening (Smith et al., 1990). Moreover,

the increase in ethylene production during ripening is detected prior to the increase in PGs production, and elevated ethylene levels can induce the accumulation of PGs mRNA (Sitrit and Bennett, 1998). The addition of trigalacturonide to ripening tomato fruit tissue inhibits the increase in PGs mRNA and enzyme activity, as well as fruit ripening (Ben-Arie et al., 1995). These data indicate that although OGs are involved in tomato fruit ripening, their role in the process is not clear.

In every case reported to date where OGs regulate the growth and development of plant tissues, with the exception of fruit ripening, their effect is the opposite of the effect of added auxin (Branca et al., 1988; Eberhard et al., 1989) (Altamura et al., 1998b). Accordingly, OGs inhibit the auxin-induced expression of the plant oncogene *rolB* (Bellincampi et al., 1996), and the auxin induced division of phloem parenchyma cells (Altamura et al., 1998a) in tobacco leaf disk explants. OGs also induce the tobacco leaf explants to produce extracellular H_2O_2 (Bellincampi et al., 2000). However, the H_2O_2 does not appear to have a role in the OG induced signal transduction pathway leading to the inhibition of *rolB* expression (Bellincampi et al., 2000). Recently it was shown that OGs antagonize responses to auxin also in Arabidopsis (Savatin et al., 2011). The mechanism by which OGs act in opposition to the action of auxin is presently unknown.

I. 2. 7. The structural requirements for the biological activity of oligogalacturonides

Many studies attributing biological responses to OGs have used impure mixtures of OG oligomers that often contain sugars other than galacturonic acid (Cote and Hahn, 1994). The results of such studies must be considered carefully, since molecules other than OGs may be responsible for the observed biological activities. Furthermore, studies using mixtures of different sizes of OGA oligomers reveal little about the OG structure that is required for biological activity. Homogeneous, size-fractionated OGs are relatively easy to prepare, and have been used in some studies (Spiro et al., 1993). Most biological responses have been attributed to OGs with a degree of polymerization (DP) from 10 to 16, with the most active sizes being around DP 12 (Spiro et al., 1993). These responses include: the induction of phytoalexins in soybean (Davis et al., 1986b) the induction of casbene synthetase in castor bean (Jin and West, 1984), the induction of PAL in suspension-cultured carrot cells (Messiaen and Van Cutsem, 1994), the induction of *rolB* expression in tobacco leaf explants (Bellincampi et al., 1993), the induction of Ca^{2+} influx in suspension-cultured tobacco cells (Mathieu et al., 1991), and the regulation of organogenesis in tobacco TCL and leaf explants (Marfà et al., 1991; Bellincampi et al., 1993). The same size range of OGs (i.e. DP 10-16) having a GalA or a Δ-4,5 unsaturated GalA residue at the non-reducing terminus induce the production of phytoalexins in soybean cotyledons, indicating that 4,5-unsaturation of the non-reducing terminal GalA does not greatly influence the biological activity of the OGAs (Hahn et al., 1981) (Davis et al., 1986b). In contrast, modifications of the reducing terminus including biotinylation, tyramination, C1 reduction, and C1 oxidation decrease the biological activity of OGs 2–6 fold in the tobacco TCL bioassay and 2–32 fold in suspension-cultured tobacco cell extracellular alkalinization bioassays (Spiro et al., 1998). Chemical esterification of the C6 carboxylates of OGs greatly diminishes their ability to elicit casbene synthetase in castor bean, whereas subsequent de-esterification restores their biological activity (Jin and West, 1984). This shows that, at least in the case of induction of casbene synthetase, free carboxylates are necessary for activity. The minimum size of OGs required for most of the biological activities reported, and the minimum size requirement for the formation of a Ca^{2+}-dependent conformation often called the ‘‘egg-box’’ conformation, coincide at a DP of approximately 10 (Kohn, 1975; Messiaen and Van Cutsem, 1994; Cote and Hahn, 1994). Millimolar Ca^{2+} is required for biological activity of OGs in carrot and tobacco cell suspensions (Messiaen and Van Cutsem, 1994) which suggests

that a Ca^{2+}-dependent conformation formed by OGs with DPs 5-10 is required for biological activity in certain bioassays (Cote and Hahn, 1994). The polyamines spermidine and spermine are believed to selectively prevent OGs from adopting the Ca^{2+}-dependent conformation (Messiaen and Van Cutsem, 1999). Physiological concentrations of these polyamines inhibit the biological activity of OGs in carrot cell suspensions, suggesting that they modulate the biological activity of OGs by preventing the Ca^{2+}-dependent formation of an active conformation (Messiaen and Van Cutsem, 1999). There are several reports showing that OGs other than those with DPs from 10 to 17 are biologically active (Cote and Hahn, 1994). For example, OGs from DP 2 to 30 elicit the expression of proteinase inhibitors (PIs) in tomato seedlings, with the disaccharide being the most active (Farmer et al., 1990; Moloshok et al., 1992). In this system, di- and tri-GalA with Δ-4,5 unsaturated GalA at the non-reducing terminus are active, whereas C1 reduced oligomers are not. OGs with a DP from two to six induce ethylene biosynthesis in tomato plants, with the pentasaccharide being the most active (Simpson et al., 1998). Trigalacturonide is active in inhibiting the production of PGs in ripening tomato fruit tissue. Di- and tri- GalA elicit the accumulation of HGRPs in the cell walls of bean seedlings (Boudart et al., 1995). Di- and tri- GalA also suppress the induction of PAL and the hypersensitive response to a fungal pathogen in wheat leaves (Moerschbacher et al., 1999). Nonreducing-end Δ-4,5 unsaturated di-GalA is active in inhibiting tissue maceration in potato tuber tissue infected with the bacterial soft rot pathogen Erwinia carotovora (Weber et al., 1996).

I. 2. 8. OGs are perceived by the Wall-Associated Kinase 1 (WAK1) in Arabidopsis

Although its eliciting activity is well documented, the perception system for OGs has been elusive. Another group of RLKs is encoded by the wall-associated kinases (WAK) gene family, which comprises five tightly clustered members (*WAK1*-*WAK5*). These receptors contain epidermal growth factor (EGF)-like motifs in the extracellular domain. Interestingly, it was shown that the extracellular domain of WAK1 and WAK2 have high affinity to pectin and OGAs, particularly to the elicitor-active egg-box form of OGA, *in vitro* (Decreux et al., 2006; Cabrera et al., 2008; Kohorn et al., 2009). 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 microbe associated 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 a chimeric receptor comprising the WAK ectodomain fused with the EFR trans membrane (TM) and intracellular kinase domains is able to perceive OGs and induce typical EFR-mediated responses, such as ethylene production and defense gene expression (Brutus et al., 2010), providing the first evidence that WAK1 is capable to sense OGs *in vivo* and trigger a defense response that mirrors that normally activated by OGs. Similarly, a chimeric receptor comprising the TM and cytoplasmic kinase domains of WAK1 fused with the ectodomain of EFR was able to perceive elf18, triggering an oxidative burst in the *efr* Arabidopsis mutant that lacks EFR (Brutus et al., 2010).

I. 2. 9. Transduction of the OG signal

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.

I. 2. 10. Another class of DAMPs: AtPEP

Another class of DAMPs is represented by plant-derived peptide elicitors, which in Arabidopsis are called *At*Pep (Huffaker et al., 2006; Bartels et al., 2013) and in Solanaceae systemins (Pearce et al., 1991). These small peptides are conserved among different plant species and are induced in response to various biotic and abiotic stress conditions, including pathogens and herbivore attacks and wounding, to prolong or amplify immune responses. For a detailed description of *At*Pep biology see section V.1.

I. 3. ARABIDOPSIS DEFENSE AGAINST NECROTROPHIC PATHOGENS

Pathogens can be classified as biotrophic, when require living host cells to establish infection and complete their life cycle, necrotrophic, when kill their host to feed on dead cells and necrotic tissue or hemibiotrophic, when initially behave as biotrophic and as their life cycle progresses they proceed to kill their host (Mengiste, 2012). *Botrytis cinerea* is a broad host-range necrotroph regarded the second most important fungal plant pathogen (Dean et al., 2012). Fungal cell wall components, such as chitin, chitosan and glucans are some of the fungal PAMPs that are involved in the interaction between *B. cinerea* and *Arabidopsis thaliana*.

I. 3. 1. The intriguing roles of the plant cell wall and cuticle in Arabidopsis defense

Defense against necrotrophic pathogens include constitutive and induced physical and chemical barriers. The plant cell wall and the cuticle inhibit the initiation and spread of infection while also serving as sources of elicitors that trigger induced defenses. In particular, the extent of pectin methyl-esterification positively correlates with resistance and plants overexpressing pectin methyl-esterase inhibitors (PMEIs) are less susceptible to the fungus (Lionetti et al., 2007). Mutants in Arabidopsis *REDUCED WALL ACETYLATION2* had decreased levels of acetylated cell wall polymers, resulting in increased tolerance to Botrytis (Manabe et al., 2011). Moreover, mutants of the CesA family of cellulose synthase subunits showed constitutively active JA and ET signaling pathways (Ellis and Turner, 2001), while *myb46* mutant displayed down-regulation of CesA genes, up-regulation of JA/ET-responsive genes (*PDF1.2* and *PR3*) and enhanced resistance to Botrytis (Ramirez et al., 2011a; Ramirez et al., 2011b). Similar to cell wall mutants, Arabidopsis mutants and transgenic plants altered in components of the cuticle were found to be completely resistant to *B. cinerea* (Kurdyukov et al., 2006; Chassot et al., 2007; Bessire et al., 2007). Moreover, transgenic Arabidopsis plants constitutively expressing a fungal cutinase or lipase, each with cutin hydrolytic activity, exhibited enhanced resistance to *B. cinerea* infection (Chassot et al., 2007). This would seem in contrast with the general notion that the cuticle protects plants against abiotic stresses and serves as a barrier to fungal infection. However, the altered cuticle composition was hypothesized to facilitate faster perception of fungal elicitors. This, coupled with an increased cuticle permeability that allows easier diffusion of defense signals to the infection site, faster oxidative burst, and loss of virulence in the pathogen was hypothesized to promote resistance (Kurdyukov et al., 2006; Chassot et al., 2007; Bessire

et al., 2007; Voisin et al., 2009; Mang et al., 2009; L'Haridon et al., 2011). It was also suggested that mutant plants compensate the functional disorder of the cuticle by reinforcing their defenses thereby enhancing resistance independent of the changes in actual cuticle composition (Voisin et al., 2009).

I. 3. 2. PAMP- and DAMP-triggered immunity against Botrytis cinerea

Several findings indicated a growing role of PAMP- and DAMP-triggered immunity (PTI) in Arabidopsis responses to Botrytis. Like flg22 and elf18, recognition of B. cinerea PAMPs also activates components of MAPK cascade including MPK3 and MPK6 (Ren et al., 2008; Pitzschke et al., 2009). Furthermore, overexpression of constitutive active elements that are involved in FLS2 signaling, such as the triple kinase MEKK1, and the double kinase MKK4, as well as pre-treatment of plants with flg22 results in increased resistance to Botrytis (Asai et al., 2002; Ferrari et al., 2007). On the contrary, the loss of function of elements involved in PAMPs signaling, including BAK1, BIK1, and MPK3 results in increased susceptibility to Botrytis (Veronese et al., 2006; Kemmerling et al., 2007; Ren et al., 2008; Galletti et al., 2011; Laluk et al., 2011; Zhang et al., 2013). Immunity triggered by flg22 and oligogalcturonides is similar although flg22 elicit a stronger and sustained effect on gene expression relative to OGs (Denoux et al., 2008). Like flg22, pre-treatment of Arabidopsis plants with OGs prior to B. cinerea inoculation increased resistance to the fungus in a PAD3-dependent manner, independently of SA- and JA/ET-mediated signaling (Ferrari et al., 2007). Polygalacturonases (PGs) are other fundamental components of *B. cinerea* virulence. PGs are sensed by at least two different mechanisms; one (independent of its enzymatic activity) through the recognition of distinctive motifs of the protein activating defense responses in the host (Poinssot et al., 2003). Additionally, PGs act on the host cell wall to degrade pectin, the primary carbon source for the pathogen, releasing OGs. Plant PG-inhibiting proteins (PGIPs) counteract the hydrolytic activity of fungal PGs and favor the accumulation of OGs of a certain length (10 to 15 degrees of polymerization) that showed elicitor activity (Cervone et al., 1989; De Lorenzo et al., 2001; De Lorenzo and Ferrari, 2002) and activated immunity against *B. cinerea* (Ferrari et al., 2007). The Arabidopsis Wall-Associated kinase 1 (WAK1) functions as a receptor of OGs and transgenic plants overexpressing WAK1 are more resistant to Botrytis (Brutus et al., 2010), while intracellular mitogen-activated protein kinase 6 (MPK6) is required for OG-triggered resistance against *B. cinerea* (Galletti et al., 2011).

I. 3. 3. Botrytis induced kinase 1 (BIK1) promotes PTI to necrotrophs

The role of BIK1 in PTI to fungal necrotrophs was recently described (Laluk et al., 2011). BIK1 interacts with and is phosphorylated by FLS2 and BAK1 in response to flg22 (Zhang et al., 2010; Lu et al., 2010). *BIK1* was identified as an early-induced gene during infection of *Arabidopsis* by *B. cinerea* (Veronese et al., 2006). It encodes a typical receptor-like cytoplasmic kinase (RLCK), a subclass of RLKs that lack the extracellular domain (Shiu and Bleecker, 2001). BIK1 is required for responses to ET, including 1-aminocyclopropane-1-carboxylic acid (ACC)-induced triple response and defense gene expression (Veronese et al., 2006; Laluk et al., 2011). Moreover, during *B. cinerea* infection the expression of JA/ET-responsive genes, such as the plant defensin *PDF1.2* and the transcription factors *ETHYLENE RESPONSE FACTORs* (*ERF104* and *ORA59*), is affected in the *bik1* mutant resulting in enhanced susceptibility to Botrytis (Veronese et al., 2006; Laluk et al., 2011). Also flg22-PTI to Botrytis is compromised in *bik1* mutant (Laluk et al., 2011), probably because BIK1 is part of the flg22-induced FLS2 perception complex. Induced *BIK1* expression is strictly dependent on EIN3, a TF that controls ET signaling, and chromatin immunoprecipitation

experiments confirmed that EIN3 associates with the BIK1 promoter (Laluk et al., 2011). BIK1 kinase activity increases in response to the ET-precursor ACC but is blocked when ET perception is inhibited by chemical treatment, suggesting that BIK1 activation is dependent on ET signaling. EIN2, a central regulator of ET signaling, contributes to flg22-PTI to *B. cinerea* (Laluk et al., 2011). However, *ein2* mutant is impaired in all flg22-triggered responses, correlating with decreased *FLS2* transcription and protein accumulation. Like *BIK1*, *FLS2* expression is under control of EIN3 and EIN3-like TFs, which depend on EIN2 activity for their accumulation (Boutrot et al., 2010). BIK1 also interacts with CERK1 (Zhang et al., 2010), suggesting it may play a similar role in pattern-triggered immunity induced by chitin. Furthermore, BIK1 is required for maintenance of normal SA levels in infected plants (Veronese et al., 2006). The contribution of BIK1 to immune responses is at least partially linked to its role in the regulation of SA levels. Intriguingly, removal of SA from *bik1* through genetic crosses to nahG (expressing a salicylate hydroxylase) or *sid2* (a salicylic acid deficient mutant) restored WT susceptibility to *B. cinerea* while had no impact on flg22-PTI to *B. cinerea*, although the response to ET, in terms of ACC-induced hypocotyl growth inhibition and expression of ET-regulated genes, was still compromised in these double mutants (Laluk et al., 2011), suggesting that ET acts upstream of SA and that SA is not required for flg22-PTI to *B. cinerea*. Coherently, EIN3 and EIN3-like proteins suppress SA synthesis through regulation of *SID2* expression (Chen et al., 2009).

I. 3. 4. MAPKs mediate ethylene and camalexin biosynthesis

MAPKs regulation of ET and camalexin biosynthesis has been linked to immune responses to necrotrophs (Ren et al., 2008; Han et al., 2010). It was demonstrated that MPK3 and MPK6 not only control the stability of ACS2 and ACS6 by directly phosphorylation, but also activate their gene expression through WRKY33 phosphorylation, which binds on the W-boxes in the promoter of *ACS2* and *ACS6* and activate their expression (Han et al., 2010) (Li et al., 2012). Consistently, MPK3 and MPK6 are both required for Botrytis-induced ethylene accumulation (Han et al., 2010). Moreover, both MPK3 and MPK6 are required for camalexin biosynthesis triggered by Botrytis (Ren et al., 2008), through WRKY33 phosphorylation, which bind on the promoter of PAD3, a key gene for camalexin biosynthesis (Mao et al., 2011). Interestingly, *wrky33* mutant, which is more susceptible to Botrytis (Zheng et al., 2006), is affected in camalexin but not in ethylene accumulation triggered by Botrytis (Mao et al., 2011; Li et al., 2012).

I. 3. 5. Plant hormones modulate immunity against necrotrophs

Signal transduction by plant hormones is another key component of basal immunity. SA has been traditionally associated with defense against biotrophic and hemibiotrophic pathogens, whereas JA and ET signaling appear to be more important against necrotrophic pathogens (Thomma et al., 1998; Glazebrook, 2005). This remains broadly true, although SA does appear to have a role in local immunity against *B. cinerea*, since treatment with SA before *B. cinerea* inoculation resulted in a significant reduction in the size of lesions; conversely, plants expressing a nahG transgene or treated with a phenylalanine ammonia lyase (PAL) inhibitor, but not *sid2* mutant plants, showed enhanced symptoms, suggesting that SA synthesized via PAL, and not via isochorismate synthase (ICS), mediates lesion development (Ferrari et al., 2003). However, studies from Veronese et al. (2004) and Laluk et al. (2011) showed that the loss of SA in nahG plants does not affect Botrytis resistance, while the genetic cross between *bos3* and nahG or *bik1* and nahG restored WT-like susceptibility to *bik1* but not to *bos3*, indicating that high level of SA could in same cases affect resistance to Botrytis, while SA deficiency does not affect the resistance to Botrytis. Instead, SA is dispensable for

flagellin-induced resistance to *B. cinerea* (Laluk et al., 2011).

Recent reports pushed ET to the center of PTI to diverse pathogens, including necrotrophs (Boutrot et al., 2010; Laluk et al., 2011). ET perception promotes EIN2-dependent expression of TFs and ET-responsive genes (Zhao and Guo, 2011) implicated in immunity to necrotrophs. Among these, EIN3 is important for ET signaling and ET-mediated gene expression. EIN3 regulates FLS2 as well as BIK1, two components of early PTI responses. EIN2 is required for flg22-induced PTI to *B. cinerea* and *P. syringae* (Boutrot et al., 2010; Laluk et al., 2011). MYB51-mediated accumulation of callose and secondary metabolites during flg22-PTI is also dependent on ET functions (Clay et al., 2009). In addition, cell wall modifications play a major role in ET-mediated resistance against *B. cinerea*. The accumulation of hydroxycinnamates and monolignols at the cell wall to restrict pathogens was linked to ET (Lloyd et al., 2011). Molecularly, ET regulates expression of genes encoding components of the PAMP receptor complex, diverse TFs, and defense gene expression as well as activation of protein kinases, such as MAPKs and BIK1, exerting its impact at all levels of the signaling hierarchy that modulates disease symptoms and pathogen growth.

Like ET, exogenous application of JA confers resistance to Botrytis (Thomma et al., 1999a), whereas loss of JA responses and JA synthesis compromises defense to fungal necrotrophs (Vijayan et al., 1998; Thomma et al., 1998; Ferrari et al., 2003). In *Arabidopsis*, JA and ET synergistically regulate expression of many immune response genes and resistance (Glazebrook, 2005). The receptor of JA, *Arabidopsis* COI1, is crucial for JA responses (Li et al., 2004) as well as for resistance to necrotrophic pathogens (Thomma et al., 1998; Ferrari et al., 2003; Laluk et al., 2011).

Broadly, GAs may suppress resistance and auxin contributes to resistance, whereas ABA has a complex and context-dependent function (Bari and Jones, 2009). More crucially, there is extensive crosstalk between hormone pathways thought to enable the plant to fine-tune its defenses against specific pathogens (Verhage et al., 2010).

I. 3. 6. Phytoalexins and other secondary metabolites

Secondary metabolites are dispensable chemical agents with a predominant function in aiding plant fitness to broad environmental stimuli (Bennett and Wallsgrove, 1994; D'Auria and Gershenzon, 2005). These organic compounds can be constitutively present in the plant, generated from pre-existing constituents (phytoanticipins), or synthesized *de novo* in response to pathogen ingress (phytoalexins) (VanEtten et al., 1994). Derivatives of indole compounds, glucosinolates, phenylpropanoids, fatty acids, and flavanoids are secondary metabolites that have all been implicated in defense against necrotrophs.

Camalexin, an indole derivative of tryptophan, is considered a characteristic phytoalexin and the most well-described secondary metabolite involved in Arabidopsis defense (Glawischnig, 2007; Rauhut and Glawischnig, 2009). Infection by different microbes induces camalexin synthesis at the site of infection but its antibiotic activity is limited to some pathogens. Camalexin has long been associated with defense against *B. cinerea* (Ferrari et al., 2003; Ferrari et al., 2007; Chassot et al., 2008; Stefanato et al., 2009). Plant infection with necrotrophs (Thomma et al., 1999b; Ferrari et al., 2003) as well as treatment with fungal elicitors, including the *Fusarium* toxin Fumonsin B1 (Stone et al., 2000) and a *Pythium* Nep1-like protein (Rauhut et al., 2009), induced biosynthesis of this metabolite. The Arabidopsis mutants *pad3*, *pad2*, *bos2*, *bos4* and *esa1*, impaired in camalexin synthesis or accumulation, exhibit enhanced susceptibility to *B. cinerea, A. brassicicola* and *P. cucumerina* (Thomma et al., 1999b; Tierens et al., 2002; Ferrari et al., 2003; Veronese et al., 2004; Ferrari et al., 2007). Among these, *PAD3* encodes the P450 monooxygenase responsible for the synthesis of camalexin (Schuhegger et al., 2006). However, some exceptions have been described. Plants harboring loss of function alleles of *UPS1* (*UNINDUCER AFTER PATHOGEN AND STRESS1*), involved in tryptophan biosynthesis, have

reduced camalexin but show wild type levels of resistance to *B. cinerea* (Denby et al., 2005). Moreover, the Arabidopsis *bos3* mutant, despite increased camalexin levels exhibits extreme susceptibility to *B. cinerea* and *A. brassicicola* (Veronese et al., 2004). The variation in camalexin-based resistance to necrotrophs is likely a result of interplay between multiple defense factors and disparities in isolate sensitivity to camalexin (Kliebenstein et al., 2005; Rowe and Kliebenstein, 2008). Interestingly, camalexin is also involved in wounding-, flagellin-, and OG-induced resistance to *B. cinerea*, with the *pad3* mutant displaying compromised protection in response to all three treatments (Ferrari et al., 2007; Chassot et al., 2008).

I. 3. 7. Major transcription regulators of plant immune responses to necrotrophs

Several studies have identified thousands of Arabidopsis transcripts that change in expression following *B. cinerea* infection (Ferrari et al., 2007; Rowe et al., 2010; Birkenbihl et al., 2012; Mulema and Denby, 2012), pointing to a major role for transcription factors (TFs) in coordinating these changes. Indeed, both forward and reverse genetic approaches have identified numerous TFs involved in defense against *B. cinerea*. Two major groups of TFs with roles in defense against *B. cinerea* are the WRKY and ERF families. Among WRKYs, WRKY3, 4, 8, 18, 33, 40, 60, and 70 have been shown to influence *B. cinerea* immunity (Abuqamar et al., 2006; Xu et al., 2006; Lai et al., 2008; Chen et al., 2010; Birkenbihl et al., 2012). In uninfected leaves, WRKY33 is bound in a complex with MAP kinase 4 (MPK4) and MKS1 (Qiu et al., 2008). Infection with *Pseudomonas syringae* or treatment with flg22 activates MPK4, causing the release of WRKY33, which then enters into the nucleus. Chromatin immunoprecipitation (ChIP)-PCR experiments have shown direct binding of WRKY33 to sequences upstream of genes involved in JA signaling (jasmonate ZIM-domain1 [JAZ1] and JAZ5), ET-JA crosstalk (ORA59), and camalexin biosynthesis (PAD3 and CYP71A13) following *B. cinerea* infection (Birkenbihl et al., 2012). ERFs are characterized by a single AP2/ERF DNA binding domain (Nakano et al., 2006) and expression of ERF1, ERF5, ERF6, RAP2.2 and ORA59 influences host susceptibility to *B. cinerea*, with ERF5 a key component of chitin- mediated immunity (Berrocal-Lobo et al., 2002; Pre et al., 2008; Moffat et al., 2012; Son et al., 2012; Zhao et al., 2012).

I. 3. 8. A high resolution temporal transcriptomic analysis revealed a chronology in the Arabidopsis defense to *Botrytis cinerea*

A temporal (every 2 hours post inoculation, HPI) transcriptomic analysis of Arabidopsis leaves during the infection with *Botrytis cinerea* revealed that about one-third of the Arabidopsis genome changes in expression during the first 48 hours post infection, with the majority of gene expression changes in a relatively small time window (c.ca 18 to 30 HPI), when the pathogen has penetrated the leaf epidermis but only very small lesions were present (Windram et al., 2012).

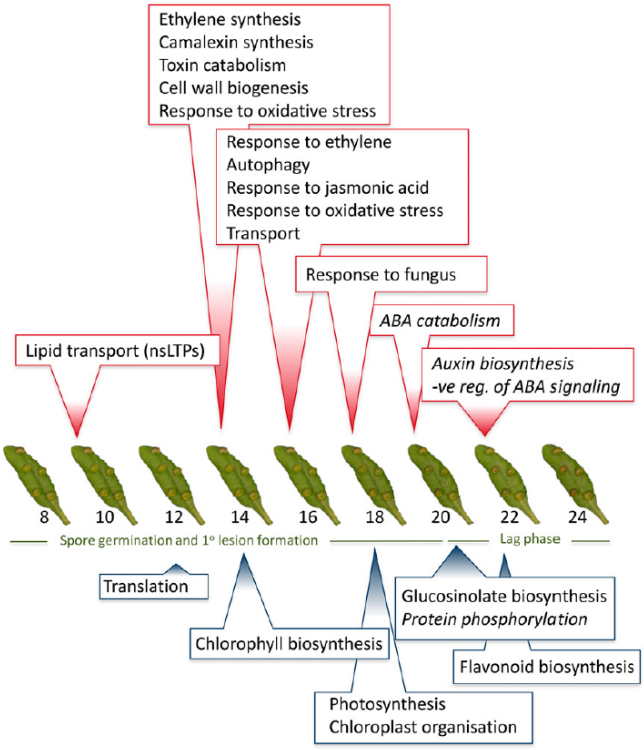

Figure 5. Selected GO Terms overrepresented in clusters of genes differentially expressed after *B. cinerea* infection of Arabidopsis leaves. GO terms are aligned with the time of gradient change and/or time of first differential expression of the cluster, with red boxes containing GO terms from upregulated genes and blue boxes containing GO terms from downregulated genes. Adapted from Windram *et al.* 2012 (2012).

Regarding of hormones synthesis and signaling, genes involved in ethylene biosynthesis, such as *ACS2* and *ACS6*, as well as JA biosynthesis, such as allene oxide synthase (AOS), were up-regulated from 12 to 14 HPI, while the ET- and JA-responsive genes (such as *ERF1*, *ERF4* and *ORA59*) 2 hours later (Figure 5). Interestingly, *ERF4* mediates antagonism between the ET and ABA pathways, with overexpression of *ERF4* leading to decreased sensitivity to ABA (Yang et al., 2005). Moreover, since JA biosynthetic genes are rapidly induced after treatment of seedlings with OGs (Denoux et al., 2008), the early activation of JA and ET biosynthetic genes in response to Botrytis suggest that their expression is mediated by PAMP/DAMP recognition.

After ET (and JA) synthesis, genes involved in auxin biosynthesis (such as *ASA1*) were up-regulated at 22 HPI (Figure 5). At least in roots, *ASA1* and *ASB1* are required for ET-triggered auxin increase leading to the inhibition of Arabidopsis root growth (Stepanova et al., 2005). Thus, the earlier ET production suggest that ET potentially act as a trigger for auxin production also during Botrytis infection (Windram et al., 2012). At the same time, genes involved in the suppression of ABA accumulation and signaling were accumulated. Thus, ABA seems to play a negative role in defense against Botrytis with ABA-deficient mutants in both tomato and Arabidopsis being more resistant to this pathogen (Audenaert et al., 2002; Adie et al., 2007), and ABA-hypersensitive Arabidopsis mutants being more susceptible (Abuqamar et al., 2006). In Arabidopsis, it was demonstrated that the high resistance associated with ABA-deficiency is based on increased accumulation of ROS during the early stages of tissue penetration (Asselbergh et al., 2007). In Arabidopsis, ABA can function as a repressor of SA-dependent signaling (Yasuda et al., 2008) and the JA/ET-dependent signaling integrated by ERF1 and ORA59 (Anderson et al., 2004; Ton et al., 2009), but appears to act positively on the JA responses activated by MYC2 (Lorenzo et al., 2004; Anderson et al., 2004; Yadav et al., 2005; Dombrecht et al., 2007). Interestingly, these two branches of the JA signaling (that integrated by ERF1 and ORA59, and

that integrated by MYC2) act antagonistically on each other (Lorenzo et al., 2004).

Lastly, around 22 HPI SA biosynthetic genes were down-regulated. The role of SA signaling in defense against Botrytis is not completely clear. JA and ET responses are often found to be more important in defense against necrotrophic pathogens, but a study by Ferrari et al. (2003) demonstrated that exogenous application of SA increased resistance while plants expressing the NahG transgene (which reduces SA levels) or treated with a phenylalanine ammonia lyase (PAL) inhibitor had increased susceptibility. SA can be synthesized through the pathway involving PAL (Coquoz et al., 1998) or, alternatively, isochorismate synthase (ICS) (Wildermuth et al., 2001a), and mutants defective in ICS1 had wild-type levels of susceptibility to *B. cinerea* (Ferrari et al., 2003). Moreover, expression profiles by Windram *et al.* (2012) suggest that basal levels of PAL are sufficient for early synthesis of SA and that synthesis of this hormone is down-regulated as infection progresses. These data would point to SA synthesized via PAL having a protective role against *B. cinerea*.

The SA and JA pathways are known to be mutually antagonistic and the earlier JA synthesis during Botrytis infection leads to down-regulation of the SA pathway (Windram et al., 2012). Conversely, increased SA also suppresses the expression of JA-responsive genes (Spoel et al., 2003) normally required for full resistance to necrotrophic pathogens (Thomma et al., 1998; Thomma et al., 1999a). Coherently, the Arabidopsis *bik1* mutant (which is more susceptible to *B. cinerea*) showed increased levels of SA and PR1 expression during Botrytis infection, as well as decreased levels of the JA/ET-responsive genes *ORA59* and *PDF1.2* (Veronese et al., 2006; Laluk et al., 2011), suggesting that when SA increase above a certain threshold levels, it may suppress certain mechanisms required for resistance to Botrytis.

Regarding metabolism, a change in expression of genes encoding enzymes of specific secondary metabolic pathways was also observed during infection. In particular, at 14 HPI two genes involved in camalexin biosynthesis (*CYP79B2* and *TSB2*) were up-regulated. The accumulation of camalexin during biotic stress is a well-known phenomenon, and camalexin levels are inversely correlated with susceptibility of Arabidopsis to *B. cinerea* infection (Denby et al., 2004). TSB2 encodes the tryptophan synthase beta-subunit 2 converting 3-indoylglycerol phosphate to Trp (Last et al., 1991), and CYP79B2 encodes an enzyme responsible for conversion of Trp to indol-3-acetaldoxime (Hull et al., 2000), a metabolic step common to both camalexin and indole glucosinolate biosynthesis. Curiously, PAD3, a cytochrome P450 enzymes that catalyze the last step of camalexin biosynthesis (Zhou et al., 1999), was not present on the arrays.

Two genes involved in cell wall biosynthesis and defense signaling (CeSA1 and CeSA3) were down-regulated during infection. Coherently, the cev1 mutation of CeSA3 has decreased susceptibility to *B. cinerea*, most likely due to overproduction of JA and ET and associated downstream gene expression (Ellis et al., 2002). The rsw1 mutant of CeSA1 also exhibits increased expression of VSP1, suggesting overproduction of JA in this mutant as well. Instead, cell wall-associated genes, including a peptidoglycan binding protein containing a LysM domain (At5g62150), a predicted chitinase (At2g43590) and a member of the pectin methylesterase inhibitor (PMEI) superfamily (At2g45220), were strongly up-regulated at 14 HPI. Chitin is a characteristic component of fungal cell walls; the LysM domain is thought to mediate binding to peptidoglycans and chitins, whereas chitinases can degrade chitin. PMEIs inhibit pectin methylesterases (PMEs), maintaining a high level of methylated pectin in the cell wall, making the wall more resistant to degradation by enzymes, such as endopolygalacturonases, produced by pathogens. Overexpression of two characterized Arabidopsis PMEIs has been shown to confer decreased susceptibility to *B. cinerea* (Lionetti et al., 2007).

PART II - AIM OF THE THESIS

The capability of plants to survive adverse conditions and reach reproductive maturity critically depends on their ability to continually adapt to changes in the environment. Therefore, plants have evolved an array of intricate regulatory mechanisms that involve the generation of signaling molecules mediating the activation of adaptive responses. In particular, the activation of pathogen-specific defense mechanisms upon microbial infection and the acquisition of architectural and physiological adjustments to environmental changes permit survival, development, and reproduction of plants. Plant activity at the cellular level can be classified as growth (cell division and enlargement) and differentiation (chemical and morphological changes leading to cell maturation and specialization). These processes are often affected in response to microorganisms because plants must grow fast enough to compete, yet maintain the defenses necessary to survive in the presence of pathogens or symbiotic organisms. The plant cell wall is a complex extracellular structure that plays important roles in plant growth and development (Humphrey et al., 2007). It is also the first line of defense against pathogens (Ridley et al., 2001). At the early stages of infection, phytopathogenic microorganisms produce enzymes capable of degrading the plant cell wall (Vorwerk et al., 2004); among these enzymes, polygalacturonases (PGs) cleave the α-1,4 glycosidic bonds present between the galacturonic acid units of homogalacturonan, the main component of pectin. 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). These fragments of homogalacturonans with a wide degree of polymerization of 9 to 16 residues called oligogalacturonides or OGs, which accumulate in the plant apoplast, trigger plant defense responses (Galletti et al., 2009). The defense–related biological responses to OGs are well known and documented. OG induce the production of reactive oxygen species, glucanases, chitinases, and phytoalexins, and expression of inducible genes involved in the production of antimicrobial compounds (Cote and Hahn, 1994; Ridley et al., 2001; Ferrari et al., 2007; Galletti et al., 2009). Responses activated by OGs in Arabidopsis can activate defense responses effective against *B. cinerea* and OG-induced protection independently of salicylic acid, ethylene and jasmonic acid (Ferrari et al., 2007), similar to what observed in the case of flg22-induced resistance to *Pseudomonas syringae* (Zipfel et al., 2004). Furthermore, genome-wide transcript profile analysis of Arabidopsis seedlings treated with either OGs or flg22 for 1 hour indicates an extensive overlap of transcriptional changes triggered by these elicitors (Denoux et al., 2008). However, the changes induced by flg22 are more conspicuous, both in terms of fold-change and number of genes affected, and more sustained (after 3 hours of treatment most of the changes induced by OGs at 1 hour drop to basal levels). The OG-induced expression of several defense genes has been shown to be also independent of salicylic acid, ethylene and jasmonic acid (Ferrari et al., 2007; Denoux et al., 2008), and the rapid oxidative burst triggered by OGs is mediated by AtrbohD (Galletti et al., 2008). In addition to these defense events, OGs have also been shown to induce changes in plant growth and development. Indeed, biologically active OGs inhibit root formation (Bellincampi et al., 1993) and increase stomata formation (Altamura et al., 1998b) in tobacco leaf explants. Increasing experimental evidence indicates that thes developmemtal effects may be due to the capability of OGs of antagonizing the action of the phytohormone auxin (indole-3-acetic acid, IAA) (Savatin et al., 2011).

Because maintenance of immunity is costly, plants finely tune immunity and activated it only upon sensing danger signals. Immune response activation is accompanied by a down-regulation of growth processes, a phenomenon called growth-defense tradeoff, and plants that constitutively express defense responses are dwarf (Ferrari et al., 2008). Immunity has therefore evolved so that costs for a defense function do not exceed benefits. Disentangling the interplay between plant immune responses and developmental processes is particularly daunting due to the pleiotropy of

hormones and transduction elements and the cross-talk between them. Moreover, many players that act in both defense- and growth-related pathways are encoded by complex gene families, with a redundant, synergistic or antagonistic action of the different members.

The purpose of this thesis is to shade light on the very early transduction events triggered in Arabidopsis by OGs, the first elicitors for which the antagonism with auxin has been demonstrated, in order to better understand which elements are involved in the defense-related responses and whether OGs are players in the growth-defense trade-off.

Most of the studies concerning plant defence–related signalling pathways focused on PAMP perception and transduction. The PAMPs recognized by plants are multifarious and include proteins, carbohydrates, lipids and small molecules. Most known PRRs require the leucine-rich repeat receptor kinase BAK1 for function and/or its closest homolog BKK1(Chinchilla et al., 2009). As such, BAK1 is a central regulator of plant immunity and consequently the target of several pathogen virulence effector molecules (Shan et al., 2008). Other elements have been described to be required for PAMP signalling, i.e. CDPKs, or to function as amplifiers of PTI after flg22 sensing, i.e. PEPRs.

OGs and PAMPs trigger very similar early and late defense responses. In fact, they can rapidly induce an oxidative burst, phosphorylation of MAPK3 and MAPK6 (Asai et al., 2002; Denoux et al., 2008)and both type of elicitor lead to induced-protection against Botrytis. Furthermore, transcriptome analysis shows that more than 95% of the early OG-regulated genes are also regulated in the same direction by the PAMP flg22 (Denoux et al., 2008). It is than reasonable to assume that PAMPs and OGs could share some elements in their signaling pathways.

In this thesis responses to OGs have been analyzed in KO mutants for some of the elements involved in PAMP signal transduction, i.e. CDPKs, BAK1/BKK1 and PEPR1 and PEPR2.

Knowledge of the mechanisms underlying the ability of plants to defend themselves paves the way to strategies for crop protection (Dangl et al., 2013).

PART III - ROLE OF CA^{2+}-DEPENDENT PROTEIN KINASES (CDPKs) IN OG SIGNALING

III.1. INTRODUCTION

Calcium ions (Ca^{2+}) are second messengers that play a pivotal role in many biological processes in plants and animals. Ca^{2+} activates several signaling pathways both during development and in response to different stimuli, including hormones, light and stress (Sanders et al., 1999; Reddy, 2001; Rudd & Franklin-Tong, 2001; White & Broadley, 2003). A rapid increase in intracellular Ca^{2+} concentration is triggered in the host plant following the exposition to several biotic and abiotic stresses, including pathogen or herbivore attacks, cold, drought and salinity stresses as well as elicitors (Hashimoto and Kudla, 2011). Very rapid apoplastic influx of Ca^{2+} (and H^{+}) induced by PAMPs and DAMPs are a prerequisite for several defense responses (Lecourieux et al., 2006).

III. 1. 1. Decodification of calcium signal and calcium sensor proteins

It is still not well understood how plant cells discriminate among the Ca^{2+} signals produced by different biotic and abiotic stimuli. Studies on both animal and plant cells show that, depending on the type of stimulus, the Ca^{2+} signals may have different spatio-temporal characteristics, including sub-cellular localization, amplitude, duration and frequency, referred to as calcium signatures (Sanders et al., 2002); these features represent a message which is decoded and transmitted by a toolkit of Ca^{2+}-binding proteins (Sanders et al., 1999; Sanders et al., 2002; Harper et al., 2004; Kudla et al., 2010; Dodd et al., 2010; DeFalco et al., 2010). In plants, as in animals, Ca^{2+}-binding proteins, also called sensors, undergo a conformational change that leads to target protein phosphorylation and consequentially downstream cascades activation.

The plant Ca^{2+}-binding proteins belong to complex families of EF hand proteins represented by the calmodulin (CaM) and calmodulin-like protein (CML) family, the family of Ca^{2+}-dependent protein kinases (CDPK), and the calcineurin B-like protein (CBL) family (Luan et al., 2002; Harper and Harmon, 2005; Luan, 2009) (Figure 1). Plants also possess Ca^{2+}-binding proteins that do not rely on EF hand-mediated Ca^{2+} binding. However, with a few exceptions, their function and regulation is not well understood (Reddy and Reddy, 2004).

CaM is highly conserved in all eukaryotes, whereas CML, CDPK and CBL proteins have been identified only in plants and some protozoans (Day et al., 2002; Harper and Harmon, 2005; Batistic and Kudla, 2009) and, notably, are absent from the sequenced eukaryotic genomes of yeast (*Saccharomyces cerevisiae*), nematodes (Harmon et al., 2000), fruitflies (*Drosophila melanogaster*) (Adams et al., 2000) and humans (*Homo sapiens*) (Venter et al., 2001; Lander et al., 2001).

Conceptually, plant Ca^{2+} sensor proteins that function as signaling components have been classified into "sensor relays" and "sensor responders" (Sanders et al., 2002). For example, CaM/CML family members, which have no enzymatic function, alter downstream target activities via Ca^{2+}-dependent protein–protein interactions. Therefore, they represent *bona fide* sensor relay proteins. CBL proteins also belong to sensor relay proteins, due to the lack of any enzymatic activity. However, CBLs specifically interact with a family of protein kinases designated as CBL-interacting protein kinases (CIPKs). Therefore, CBL–CIPK complexes have been considered as bimolecular sensor responders (Hashimoto and Kudla, 2011). In contrast, CDPKs combine a Ca^{2+} sensing function (EF hand motifs) and a responding function (protein kinase activity) within a single polypeptide and have therefore been classified as sensor responders. Consequently, these kinases directly bind calcium, and their calcium-stimulated kinase activities are independent of calmodulins, unlike calcium/calmodulin-dependent protein kinases (CaMKs) (Roberts and Harmon, 1992).

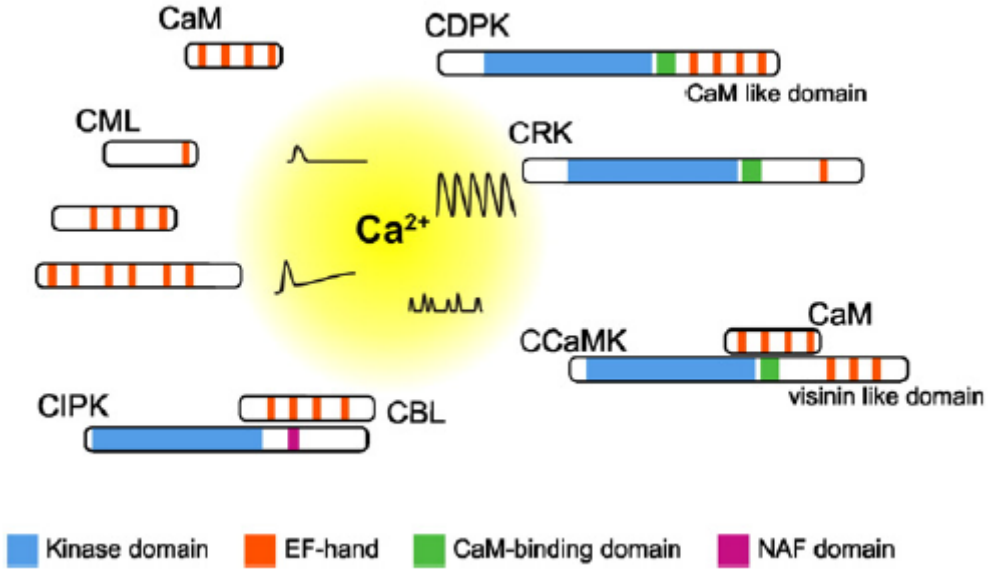

Figure 1. Ca^{2+} sensor proteins in plants. Representative plant Ca^{2+} sensor proteins are shown and functional domains are highlighted. CaMs, CMLs and CBLs harbor EF hand motifs and regulate target proteins. They do not contain any additional functional domains. CBLs interact and modulate the activity of CIPKs, while CDPKs are directly activated by Ca^{2+} binding to the CaM-like domain. CRKs are CDPK related kinases with a degenerated CaM like domain. In contrast to CDPKs, CCaMKs are dual regulated kinases. These proteins bind Ca^{2+} via a visinin like domain, while in addition Ca^{2+}-CaM binds to the regulatory domain of the kinase and mediates further activation. The different Ca^{2+} signatures represented in the center likely mediate a specific activation of these sensors contributing to the decoding of the signal. Adapted from (Batistic and Kudla, 2012).

In the CDPK family, there are three groups of kinases, distinguishable by distinct calmodulin- or visinin-related C-terminal domains (Figure 1). The conventional archetypical CDPKs (also abbreviated as CPKs) contain a regulatory calmodulin-like domain (CaM-LD) with four functional Ca^{2+}-binding EF hands (Harper et al., 1991; Suen and Choi, 1991). The class of CCaMKs is distinct since the members have regulatory domain with only three EF-hands and a higher sequence similarity to VISININ (another EF-hand protein) than to calmodulin (Patil et al., 1995). The CDPK-related kinases (CRKs) instead are characterized by a regulatory domain that has high sequence similarity to the CaM-LD of a CDPK, but with EF-hands that seem to have degenerated and are predicted to no longer bind Ca^{2+}. CRKs are sometimes referred to as CBKs, calmodulin-binding kinases, or plant CaMKs (Ma et al., 2004).

III. 1. 2. CDPK domain structure

Four distinct domains typify CDPK family members: an N-terminal variable domain, a protein kinase domain, an autoinhibitory domain, and a calmodulin-like domain. Based on phylogenetic analysis, it is thought that the *CDPK* gene family arose through the fusion of a CaMK and a calmodulin (Harper et al., 1991; Suen and Choi, 1991; Harmon et al., 2000; Zhang and Choi, 2001). This unique molecular structure allows the direct activation of CDPKs by Ca^{2+}. Unlike the analogous mammalian protein, the multisubunit CaMKII, CDPKs function as monomers (Roberts and Harmon, 1992). Alignments of the predicted amino acid sequences of all 34 Arabidopsis CDPKs reveal a conservation of the kinase (44%–95% identity and 60%–98% similarity), autoinhibitory (23%–100% identity and 42%–100% similarity) and calmodulin-like (27%–97% identity and 50%–98% similarity) domains, whereas the N-terminal variable domain shows little sequence similarity. The kinase domain (264–273 amino acids long) contains all 12 of the highly

conserved subdomains of typical eukaryotic Ser/Thr protein kinases (Hanks and Hunter, 1995). The autoinhibitory domain is a basic amino acid region (31 amino acids long) that functions as a pseudosubstrate (Harmon et al., 1994). The calmodulin-like domain (94–147 amino acids long) contains Ca^{2+}-binding EF hands allowing the protein to function as a Ca^{2+} sensor. Each EF hand consists of a loop of 13 amino acid residues flanked by two α-helices. A single Ca^{2+} molecule is bound to each EF hand via the loop domain (Zhang and Yuan, 1998). The number of EF hands differs depending on the isoform. Most Arabidopsis CDPKs contain four EF hands, whereas a few of them have one, two, or three. The positions where the EF hands are absent also vary. These differences in numbers and positions of EF hands likely yield variations in the allosteric properties of Ca^{2+} binding and the activation threshold. The mechanism by which CDPK activity is regulated is largely controlled through interactions between the kinase, autoinhibitory, and calmodulin-like domains. Under the basal condition of low free Ca^{2+}, the autoinhibitory domain is bound by the kinase domain, keeping substrate phosphorylation activity low. Upon binding Ca^{2+} via the EF hand motifs, CDPKs undergo conformational changes that release the pseudosubstrate from the catalytic site, activating the protein (Harmon et al., 1994; Harper et al., 1994). Little is known about the function of the N-terminal variable domain. It has been proposed that this region contains subcellular targeting information (Schaller and Sussman, 1988; Harper et al., 1994; Hrabak et al., 1996). CDPKs are reported to associate with various membranes (Ellard-Ivey et al., 1999; Martin and Busconi, 2000; Lu and Hrabak, 2002). However, none of the 34 Arabidopsis CDPKs are predicted to be integral membrane proteins. The N-terminal sequence of CDPKs is variable not only in amino acid sequence, but also in length, ranging from 25 (AtCPK11) to nearly 200 (AtCPK2) amino acids in Arabidopsis.

III. 1. 3. Sequence homology and chromosomal distribution of *CDPK* genes

Analysis of the genome sequence of Arabidopsis indicates the presence of 34 *CDPK* genes (Kaul et al., 2000). All 34 Arabidopsis *CDPKs* are highly similar to each other and such high homology may indicate similar functions. Based upon sequence homology, the *CDPKs* of Arabidopsis cluster into four subgroups (I–IV) (Figure 2). Subgroup IV is the least complex, with three members, and subgroup II is the most complex, with 13 members. Sequencing projects indicate the presence of multigene families of CDPKs in other plants, including soybean *(Glycine max*), tomato (*Lycopersicon esculentum*) (Chang et al., 2009), rice (*Oryza sativa*) (Asano et al., 2005), and maize (*Zea mays*) (Estruch et al., 1994).

The 34 Arabidopsis *CDPKs* are distributed among all five chromosomes. Chromosome IV has the most *CDPKs* (11), whereas chromosome III has the least (4). The only region that contains no *CDPKs* is the short arm of chromosome II. Interestingly, one gene cluster on the short arm of chromosome IV contains five genes (AtCPK 21, 22, 23, 27, and 31), all within subgroup II. They are organized in tandem in the same transcriptional orientation, and their amino acid sequences are very homologous. Furthermore, sequence homology also exists in the N-terminal variable domain in this gene cluster (Cheng et al., 2002). These findings suggest that they arose relatively recently by gene duplication and that they may have similar or overlapping functions.

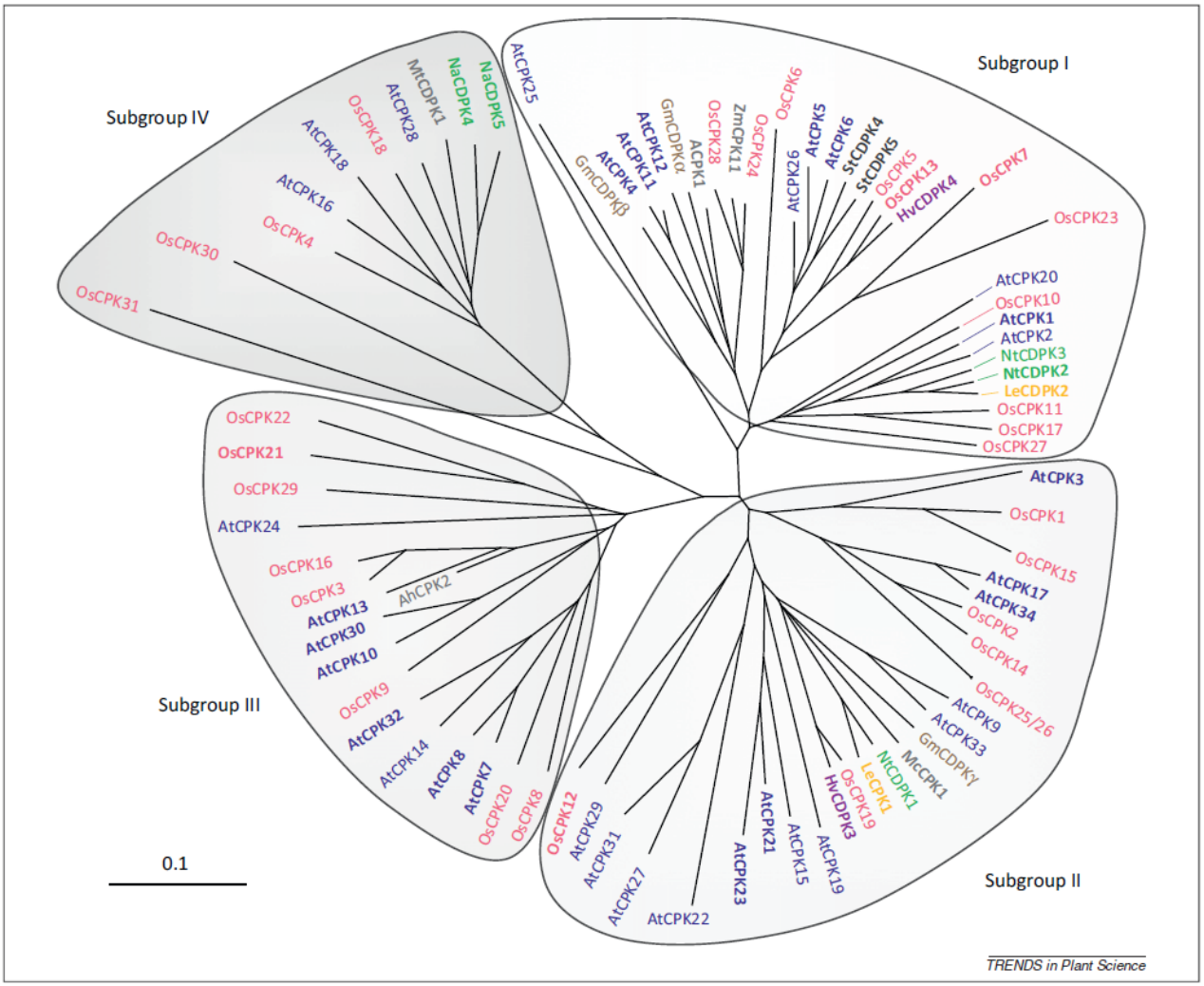

Figure 2. Relation tree of selected plant CDPKs. The unrooted phylogenetic tree was generated by the alignment of the full-length amino acid sequences of CDPKs from Arabidopsis (At, blue), rice (Os, pink), soybean (Gm, brown), potato (St, black), barley (Hv, purple), tobacco (Nt, green), coyote tobacco (Na, green), tomato (Le, yellow) and grapevine (ACPK1, gray), maize (Zm, gray), alfalfa (Mt, gray), ice plant (Mc, gray) and peanut (Ah, gray). The CDPK family is divided into four major subgroups (I–IV). The branched lengths are proportional to divergence and the scale of 0.1 represents 10% change. The CDPKs with known biological functions are highlighted in bold. Adapted from Boudsocq and Sheen (2013).

III. 1. 4. Expression and subcellular distribution

Some CDPKs are expressed in most organs whereas others are specific to some tissues. For example, AtCPK17 and AtCPK34 are preferentially expressed in mature pollen and regulate pollen tube growth (Myers et al., 2009). Differential expression of CDPKs has been observed in response to diverse stimuli, including abscisic acid (ABA), cold, drought, salinity, heat, elicitors and pathogens (Romeis et al., 2001; Ray et al., 2007; Wan et al., 2007; Li et al., 2008).

The subcellular distribution of several Arabidopsis CDPKs has been studied and diverse localizations have been observed, including the cytosol, nucleus, plasma membrane, endoplasmic reticulum (ER), tonoplast, mitochondria, chloroplast, oil bodies and peroxisomes (Lu and Hrabak, 2002; Dammann et al., 2003; Choi et al., 2005; Yu et al., 2006; Zhu et al., 2007; Li et al., 2008; Benetka et al., 2008; Myers et al., 2009; Boudsocq et al., 2010; Zou et al., 2010;

Mehlmer et al., 2010; Coca and Segundo, 2010), indicating that CDPKs have access to a multitude of potential substrates throughout the cell.

III. 1. 5. Regulation of CDPK activity

In addition to Ca^{2+}, reversible phosphorylation also may regulate CDPK kinase activity. Autophosphorylation has been observed in both native and recombinant CDPKs (Saha and Singh, 1995; Chaudhuri et al., 1999; Harmon et al., 2000). CDPK activation may be modulated also by other protein kinases (Romeis et al., 2000; Romeis et al., 2001). Moreover, biochemical analysis has revealed that in the presence of Ca^{2+}, specific phospholipids can enhance *in vitro* substrate phosphorylation by CDPKs from oat (*Avena sativa*), Arabidopsis (AtCPK1), and carrot (*Daucus carota*; DcCPK1) by 2 to 30 times above that observed with Ca^{2+} alone (Schaller et al., 1992; Harper et al., 1993; Binder et al., 1994; Farmer and Choi, 1999). Also 14-3-3 proteins may be regulators of a subset of CDPKs in Arabidopsis. Three 14-3-3 isoforms have been demonstrated to specifically bind and activate AtCPK1 in vitro in the presence of Ca^{2+} (Camoni et al., 1998). Besides AtCPK1, AtCPK24 and AtCPK28 also possess such putative 14-3-3 binding sites (The Arabidopsis Information Resource Patmatch).

III. 1. 6. Physiological functions

CDPKs are implicated in many different processes, from hormone-regulated developmental processes to abiotic and biotic stress signaling [for review see (Cheng et al., 2002; Ludwig et al., 2004; Harper and Harmon, 2005; Tena et al., 2011)]. In this thesis I will focus on latest developments, in which CDPK function was addressed in the context of plant immune response (Figure 3).

III. 1. 7. CDPKs in plant immunity

CDPKs have been demonstrated to participate in most of the signaling events triggered by DAMPs and PAMPs [reviewed in (Boudsocq and Sheen, 2013; Liese and Romeis, 2013)]. Members of the CDPK family, including homologous kinase pairs NtCDPK2/3 of tobacco or StCDPK4/5 of potato, have been shown to participate in the activation of early defence responses (Romeis et al., 2001; Ludwig et al., 2005; Kobayashi et al., 2007; Kobayashi et al., 2012). For example, loss of NtCDPK2 and NtCDPK3 in tobacco resulted in a compromised *Avr9/Cf9* gene-for-gene triggered hypersensitive cell death response (Romeis et al., 2001). The ectopic expression of constitutively active tobacco NtCDPK2VK, barley HvCDPK4VK, potato StCDPK5VK or Arabidopsis AtCPK5, as well as AtCPK6, variants, lacking the regulatory calcium-binding domain and pseudosubstrate segment, in *N. benthamiana*, correlated with the induction of plant defense reactions including ROS production, changes in phytohormone levels (see also below), defense gene expression and cell death development (Ludwig et al., 2005; Kobayashi et al., 2007; Freymark et al., 2007; Dubiella et al., 2013; Asai et al., 2013). Moreover, the overexpression of an Arabidopsis CDPK (AtCPK1) in tomato protoplasts triggered ROS production by stimulating the NADPH oxidase activity (Xing et al., 2001). Notably, the potato StCDPK4 and StCDPK5 are capable of phosphorylating both S82 and S97 in the N-terminal region of the plasma membrane StRBOHB (respiratory burst oxidase homolog B) in a calcium-dependent manner *in vitro* (Kobayashi et al., 2007). Further *in vivo* experiments showed that constitutively active StCDPK5 ectopically coexpressed with StRBOHB in *N. benthamiana* leaves phosphorylated S82, but not S97, in StRBOHB (Kobayashi et al., 2007; Asai et al., 2013). In Arabidopsis there are some conflicting data about the role of CDPKs in the

phosphorylation of RBOHs. While a recent report showed that AtCPK5 phosphorylated N-terminal serine residues (S39, S148, S163 and S347) of AtRBOHD *in vitro* and *in vivo* (Dubiella et al., 2013), in line with the behavior of the potato CDPKs orthologs, in another report, AtCPKac5 and 6, the closest Arabidopsis orthologs of StCPK4 and 5, only displayed weak phosphorylation activity on the cytoplasmic N-terminus of AtRBOHD or AtRBOHF (Gao et al., 2013). On the other hand, AtCPKac1, 2, 4 and 11 strongly phosphorylated the cytoplasmic N-terminus of RBOHD and RBOHF in a calcium-dependent manner *in vitro*. Mutation of S148, but not S133 (corresponding to S97 and S82 in *Solanum tuberosum* RBOHB, respectively), to alanine reduced the RBOHD phosphorylation by CPK2, 4 and 11, indicating S148 as an important phosphorylation site of RBOHD by CPKs (Gao et al., 2013). Consistently, the ROS production triggered by *Pst avrRpm1* or *avrRpt2* infection was reduced in *cpk1 cpk2* double mutant plants (Gao et al., 2013). In Arabidopsis, RBOHD was found in the EFR-, BAK1- and BIK1-including PRR complex in condition of both non-elicitation and elf18-elicitation (Kadota et al., 2014). Moreover, BIK1 interacted with and phosphorylated six residues (S39, T123, S140, S339, S343 and S347) in the N-terminal domain of RBOHD *in vitro* (Kadota et al., 2014). In a comparison between BIK1 and CPK4, CPK5, CPK6 and CPK11 specificity to phosphorylate RBOHD, it was shown that common and distinctive phosphosites exist. In particular, S39, S339 and S343 were phosphorylated specifically by BIK1; S347 was phosphorylated by both BIK1 and CPKs; S133 (corresponding to S82 in the StRBOHB ortholog) and S163, which is a PAMP-induced phosphosite (Benschop et al., 2007), were phosphorylated specifically by CPKs. This seems in contrast with data obtained by Gao and colleagues, which showed that mutation of S133 does not affect the phosphorylation of RBOHD by CPKs, and by Dubiella and colleagues, which showed that CPK5 plays a role in S39 phosphorylation. Further *in vivo* experiments showed that the interaction between RBOHD and BIK1 increased upon PAMPs (flg22 or elf18) perception and that the elf18-triggered phosphorylation of the sites S39 and S343 by BIK1 was independent of calcium (Kadota et al., 2014). Moreover, the flg22-induced S39 phosphorylation was not affected in a *cpk5 cpk6 cpk11* triple mutant (Kadota et al., 2014). In addition, while the residues S133 and S163 (which are CPK-mediated phosphosites) and S347 (which can be phosphorylated by both BIK1 and CPKs) are within predicted CPK-mediated phosphorylation motifs, this is not the case for S39, S339 and S343 (which are BIK1-mediated phosphosites). Instead, the treatment with EGTA (a Ca^{2+} chelator) abolished the elf18-induced phosphorylation of the CPK-mediated site S163 (Kadota et al., 2014). Importantly, the fact that PAMP-induced ROS burst is globally Ca^{2+}-dependent (Segonzac et al., 2011; Kadota et al., 2014) shows that Ca^{2+}-based regulation is also required for the ultimate activation of RBOHD, potentially subsequent to Ca^{2+}-independent, BIK1-mediated phosphorylation. Moreover, Arabidopsis *cpk5* single mutant, but not *cpk6* single mutant, showed reduced flg22-triggered ROS accumulation (Dubiella et al., 2013), in contrast with data obtained by Boudsocq and colleagues, which have not observed any defect in the flg22-triggered ROS production in *cpk5*, as well as *cpk4 cpk6* and *cpk11*, single mutants. Whilst, a reduction in the accumulation of ROS, as well as in the induction of some defense-related genes expression, in response to flg22 was observed in *cpk5 cpk6*, *cpk5 cpk6 cpk11* and *cpk5 cpk6 cpk11 cpk4*^{VIGS} double, triple and quadruple mutants (Boudsocq et al., 2010).

Other than barley, CDPKs mediate the plant immunity in other monocots (e.g. rice). In fact, rice OsCPK13 induced cell death, accumulation of PR-10s and up-regulation of defense-related genes when ectopically expressed in sorghum (Sorghum bicolor) (Mall et al., 2011). On the other hand, rice CDPK12 seems to play a negative role in ROS production, as OsCPK12 overexpressing plants accumulated less H_2O_2 than WT plants under conditions of high salinity, while *oscpk12* and OsCPK12 RNAi plants accumulated more H_2O_2 than WT plants under the same conditions (Asano et al., 2012).

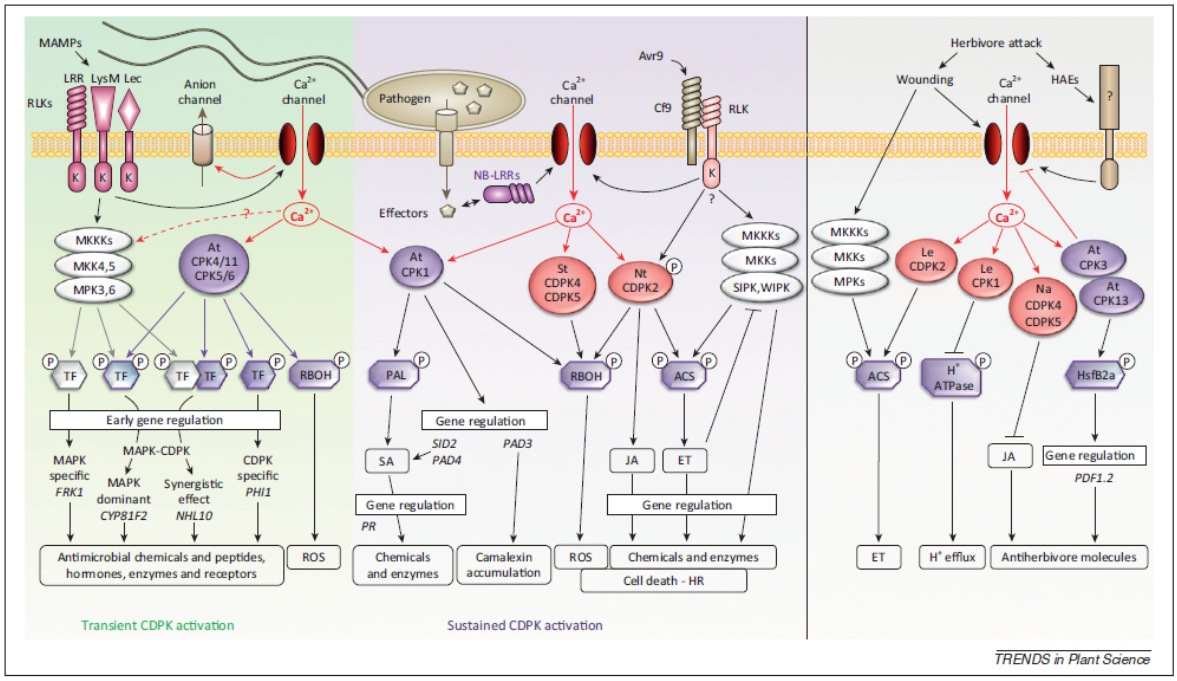

Figure 3. CDPK signaling network in immune responses. Microbe-associated molecular pattern (MAMP) perception by different cell-surface receptor kinases (RLKs) with distinct extracellular domains triggers transient CDPK activation to regulate transcription factors and early gene expression either independently or in coordination with MAPK cascades. Several CDPKs also activate NADPH oxidases (respiratory burst oxidase homologs, RBOHs) to induce early reactive oxygen species (ROS) production. By contrast, the sustained CDPK activation by extracellular (Avr9) or intracellular effector proteins leads to biosynthesis of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) through regulatory gene induction or enzyme activation such as phenylalanine ammonia-lyase (PAL) and ACC synthase (ACS). CDPKs also trigger a prolonged oxidative burst involved in cell death and hypersensitive response (HR). Constitutively active NtCDPK2 inhibits MAPK [salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK)] activation by Avr9–Cf9 in an ET-dependent manner. Herbivores can be sensed through wounding or herbivore-associated elicitors (HAEs) by unknown receptors to activate MAPKs and Ca^{2+} influx. The coregulation of ACS by MAPKs and CDPKs leads to ET production, whereas LeCPK1 inhibits the plasma membrane H^{+}-ATPase to induce extracellular alkalinization. AtCPK3 and AtCPK13 mediate herbivore-induced gene expression by phosphorylating the transcription factor HsfB2a whereas only AtCPK3 negatively regulates Ca^{2+} channels. NaCDPK4 and NaCDPK5 negatively regulate defense against herbivores by inhibiting JA accumulation and subsequent production of defense metabolites. Abbreviations: MKKs, mitogen-activated protein kinase kinases; MKKKs, mitogen-activated protein kinase kinase kinases; MPKs, mitogen-activated protein kinases; TF, transcription factor. Adapted from Boudsocq and Sheen (2013).

III. 1. 8. Role of CDPKs in hormone signaling and gene regulation

The ectopic expression of a constitutive active tobacco NtCDPK2 variant (NtCDPK2-VK), lacking the regulatory calcium-binding domain, in *N. benthamiana* leads to increased levels of jasmonic acid (JA), 12-oxo phytodienoic Acid (OPDA, the JA-precursor) and 1-aminocyclopropane-1-carboxylic acid (ACC, the ethylene precursor) and increased basal expression of JA- and ethylene-responsive genes (*PR1b* and *PR2b*), whereas reduces both salicylic acid (SA) levels and expression of SA-responsive genes (*PR1a* and *PR2a*) (Ludwig et al., 2005). The mechanisms underlying this altered hormone homeostasis are not known yet. A stabilization of the rate-limiting ethylene-biosynthetic enzyme ACC synthase (ACS) by direct phosphorylation have been proposed as a possible mechanism responsible for the increased ethylene levels of plant expressing NtCDPK2-VK (Ludwig et al., 2005), on the basis of previous observations indicating that in a peptide of LeACS2, Ser460 can be phosphorylated *in vitro* by

CDPKs purified from maize ($CDPK_I$ and $CDPK_{II}$) and a soybean recombinant CDPK (CDPKγ) (Sebastia et al., 2004). Subsequently, a direct phosphorylation by LeCDPK2 (the closest homologue of NtCDPK2) has been demonstrated *in vivo* to stabilize LeACS2 (Kamiyoshihara et al., 2010). On the other hand, the ectopic expression of NtCDPK2-VK in *N. benthamiana* leaves, during the biotic Cf9/Avr9 interaction, abrogated the strong activation of the MAPKs SIPK and WIPK whereas further increased the level of ethylene (Ludwig et al., 2005). Notably, Cf9/Avr9-triggered MAPK activation was restored in presence of inhibitors of either ethylene biosynthesis or perception, indicating that high levels of ethylene due to NtCDPK2-VK expression are responsible for the observed inhibition of stimulus-dependent MAPK activation. Because induction of ethylene has been shown to be MAPK-dependent upon expression of gain-of-function MAPK kinase mutants (Yang et al., 2001; Ouaked et al., 2003; Liu and Zhang, 2004), the target of CDPK-triggered MAPK inhibition through ethylene may not necessarily be the MAPK itself but may reside at a component of the signaling cascade further upstream, for example the MAPK kinase. Such a feedback mechanism would be disturbed by the expression of a constitutively active MAPK kinase mutants (Ludwig et al., 2005). Unlike the case of NtCDPK2, overexpression of AtCPK1 in Arabidopsis induces SA accumulation, through the induction of SA regulatory and biosynthesis genes, i.e. *PAD4* and *SID2/ICS1*, and expression of SA-responsive genes, without affecting JA and ethylene biosynthesis (Coca and Segundo, 2010). Interestingly, *in vivo,* AtCPK1 specifically phosphorylates phenylalanine ammonia liase (PAL), which is involved in an alternative pathway leading to SA production (Cheng et al., 2001). Like AtCPK1, AtCPK5, when overexpressed in Arabidopsis, induces SA accumulation, as well as stunted growth, cell death and enhanced expression of the SA-regulated genes *ICS1* and *PR1* and other defense-related genes, such as *NHL10*, *PHI1* and *FRK1*, (Dubiella et al., 2013). Thus, in Arabidopsis, AtCPKs have been so far associated mainly with a positive regulation of the SA pathway (Cheng et al., 2001; Coca and Segundo, 2010; Dubiella et al., 2013). In spite of what has been observed by Dubiella and colleagues, Arabidopsis protoplasts expressing constitutively active CPK5ac (as well as CPK4ac, CPK6ac or CPK11ac) induced the expression of *NHL10* and *PHI1*, but not of *FRK1* (Boudsocq et al., 2010), whereas those expressing the constitutively active MAPKK MKK4a induced the expression of *NHL10* and *FRK1*, but not of *PHI1*, suggesting differential activities of CDPKs and MAPK cascades in plant innate immunity. Coherently, in the same protoplasts, flg22-triggered induction of *NHL10* and *PHI1*, but not of *FRK1*, is affected in *cpk5 cpk6* double mutant, *cpk5 cpk6 cpk11* triple mutant and *cpk5 cpk6 cpk11 cpk4*^{VIGS} quadruple mutant (Boudsocq et al., 2010). These conflicting results could be attributed to the different systems used for the analysis (e.g. plants or protoplasts).

III. 1. 9. Role of CDPKs in the response to pathogens and herbivores

Wounded leaves of Arabidopsis produce ROS within minutes and become more resistant to the infection with the necrotrophic fungus *Botrytis cinerea* (Chassot et al., 2008; L'Haridon et al., 2011). This fast response of the plants is called wound-induced resistance (WIR) and is dependent on calcium, as leaves treated with calcium channels inhibitors or calcium chelators are impaired in ROS production and more susceptible to *B. cinerea* after wounding (Beneloujaephajri et al., 2013). However, the Arabidopsis *cpk5 cpk6* and *cpk11* single, double and triple mutant show normal WIR against *B. cinerea* (Beneloujaephajri et al., 2013), whereas the *cpk5 cpk6* double mutant and *cpk5 cpk6 cpk11* triple mutant are more susceptible to the biotrophic bacterium *Pseudomonas syringae pv. tomato* DC3000 (*Pst* DC3000) and are impaired in flg22-induced protection against *Pst* (Boudsocq et al., 2010). Coherently, i) Arabidopsis plants overexpressing AtCPK5 are more resistant to *Pseudomonas syringae pv. tomato* DC3000 (Dubiella et al., 2013); ii) Arabidopsis *cpk1* single mutants and *AtCPK1* antisense construct expressing plants are more susceptible to *Fusarium*

oxysporum f. sp. matthiolae, *B. cinerea*, *Pst DC3000* and are defective in the expression of SA-responsive genes (PR1, PR2 and PR5), but not of JA- and Et-responsive genes (*PDF1.2*, *HEL* and *PR3*), triggered by *F. oxysporum*, whereas AtCPK1 overexpressing plants exhibited, in addition to the up-regulation of SA levels and SA-regulated gene expression, enhanced resistance to *F. oxysporum*, as well as to *B. cinerea* and *P. syringae* (Coca and Segundo, 2010). Transgenic potato plants expressing the constitutive active StCDPK5-VK under the control of a pathogen-inducible promoter displayed increased resistance to the near-obligate hemibiotrophic oomycete *Phytophthora infestans* and, by contrast, increased susceptibility to the necrotrophic fungus *Alternaria solani*, although they accumulated more H_2O_2 following the infection with both pathogens, suggesting that increased ROS production mediated by StCDPK5-VK confers resistance to near-obligate pathogens, but increases susceptibility to necrotrophic pathogens (Kobayashi et al., 2012). Moreover, in a barley *mlo* mutant background, a constitutive active variant of *Hv*CDPK3 partially compromised the highly effective resistance to *Blumeria graminis* f. sp. *hordei*. However, a similar break of *mlo* resistance was seen upon expression of the junction domain of *HvCDPK4*, supposed to act as a dominant inhibitor of CDPK activity. The observation that expression of constitutive active forms of *Hv*CDPK3 or *Hv*CDPK4 also compromises penetration resistance to the inappropriate wheat *B. graminis* f. sp. *tritici*, suggest antagonistic roles of individual CDPK paralogs in the control of host cell entry during the early phase of powdery mildew pathogenesis (Freymark et al., 2007). In rice, *Os*CPK12 overexpressing plants exhibited an increased susceptibility to both compatible and incompatible blast fungus (*Magnaporthe grisea*) compared with wild type plants, as well as a decreased basal expression level of the pathogenesis-related (PR) genes *OsPR1b* and *PBZ1* (Asano et al., 2012).

During insect feeding wounded tissues constitute entry sites for herbivory elicitors and rapid immune responses, including ROS production and calcium influxes, are triggered (Maffei et al., 2007a; Maffei et al., 2007b). In Arabidopsis, CPKs have been described to be involved in the signaling following insect attack, since *cpk3* and *cpk13* single mutants displayed a lower induction of the defense gene *PDF1.2* in response to *Spodoptera littoralis* compared to WT plants, without affecting ET or JA production (Kanchiswamy et al., 2010). Moreover *cpk3*, but not *cpk13*, accumulated less intracellular calcium following *S. littoralis* exposition or wounding (Kanchiswamy et al., 2010), indicating that AtCPK3, also triggers a negative feed-back on herbivore-induced calcium influx and that CDPKs can play redundant as well as specific functions in plant defense.

III. 1. 10. Calcium signaling in the response to OGs

OGs have been shown to trigger a rapid and transient increase of cytosolic Ca^{2+} in carrot cells that is inhibited by the voltage-gated Ca^{2+} channel antagonists verapamil (Messiaen and Van Cutsem, 1994; Van Cutsem and Messiaen, 1994) as well as in tomato cells, where this response precedes alkalinization of the extracellular medium (Felix et al., 1993). Transient increase of cytosolic Ca^{2+} precedes also H2O2 production in aequorin-transformed tobacco cells (Chandra and Low, 1997; Cessna and Low, 2001). Ca^{2+} transient induced by OGs within 3 min is completely abolished by pretreatment of cells with the Ca^{2+} channel blocker La^{3+}, suggesting an apoplastic origin, and by the protein kinase inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB), suggesting that an upstream phosphorylation event is essential for the generation of the Ca^{2+} signal. Exposure to TBB also abolishes the production of extracellular H_2O_2 (Navazio et al., 2002). The magnitude of the Ca^{2+} increase evoked by OGs is dose-dependent. Although the lag-phase period remains unchanged within the dose range tested, the rise time to the peak of the Ca^{2+} transient gradually increases. H2O2 accumulation displayed the same dose-dependence. Intriguingly Ca^{2+} transients evoked by low concentrations of OGs with a DP of 9–18 resemble those elicited by the highest doses of OGs with a DP of 1–5. OG fractions with DP10, DP11 and DP12 are more active, on a molar basis, in inducing a Ca^{2+} elevation than DP-13 molecules, with responses

very similar to those triggered by the OG mixture with DP9–1 8. OG fractions with DP1, DP2 and DP3 were able to induce a detectable cytosolic Ca^{2+} increase, although of smaller amplitude than the OG mixture of DP1–5. Esterification of the carboxyl group on the galacturonic acid chain or a differential stereo-configuration of the molecule completely abolishes the Ca^{2+} response: fully methylated OGs and oligomannuronides, epimers of OGs, fail to trigger any detectable increase in cytosolic Ca^{2+} (Navazio et al., 2002).

Transcriptomic analysis of Arabidopsis OG-treated cells in the presence or absence of calcium influx inhibitors, i.e. La^{3+} or TBB, show that the absence of a elicitor-triggered Ca^{2+} signal results in a defective regulation of a part of the downstream genes that therefore appear to be Ca^{2+} and calcium sensors dependent (Moscatiello et al., 2006). Calcium signatures have been analyzed in Arabidopsis leaves upon treatment with flg22, elf18 or OGs, showing that, they are characteristic for each elicitor (Aslam et al., 2009). Differences observed in defense-related responses induced by these elicitors, i.e. gene induction (Denoux et al., 2008), may depend therefore on specific and different calcium spikes.

Because these observations clearly indicate an important role of calcium in OG signaling, one of the objectives of my work was to investigate, using a reverse genetic approach, whether CDPKs are involved in the decodification of OG-induced calcium transients.

III. 2. RESULTS

III. 2. 1. Lack of CDPK5/6/11 differentially impairs expression of early defense response genes, but does not affect OG-induced ROS production and MAP kinase activation

Arabidopsis single loss-of-function (KO) mutants of *CPK4*, *CPK5*, *CPK6* and *CPK11* have been shown not to display alterations of flg22-induced responses or pathogen susceptibility, which are instead observed in the double and, more markedly, in the triple and quadruple mutants (Boudsocq et al., 2010). For this reason, the *cpk5 cpk6 cpk11* triple KO mutant was used for this study. Genotypic characterization of this mutant is shown in Figure 1A.

Like flg22, OGs are able to induce an oxidative burst, which is mediated by the NADPH-oxidase AtrbohD in Arabidopsis (Galletti et al., 2011). A defect in flg22-triggered ROS production has been described in the *cpk5 cpk6 cpk11* mutant, as well as in the double mutants (Boudsocq et al., 2010). To determine whether CDPKs play a role also in the OG-triggered oxidative burst, H_2O_2 production was quantified treatments with OGs and flg22, as a control, both in leaf disks obtained from Col-0 and *cpk5 cpk6 cpk11* plants, and in seedlings. As shown in Figure 1B and 1C, OG- and, unexpectedly, flg22-induced H_2O_2 production was not reduced in the triple mutant compared to wild type, indicating that loss of CPK5, CPK6 and CPK11 does not significantly affect the oxidative burst triggered by OGs and flg22.

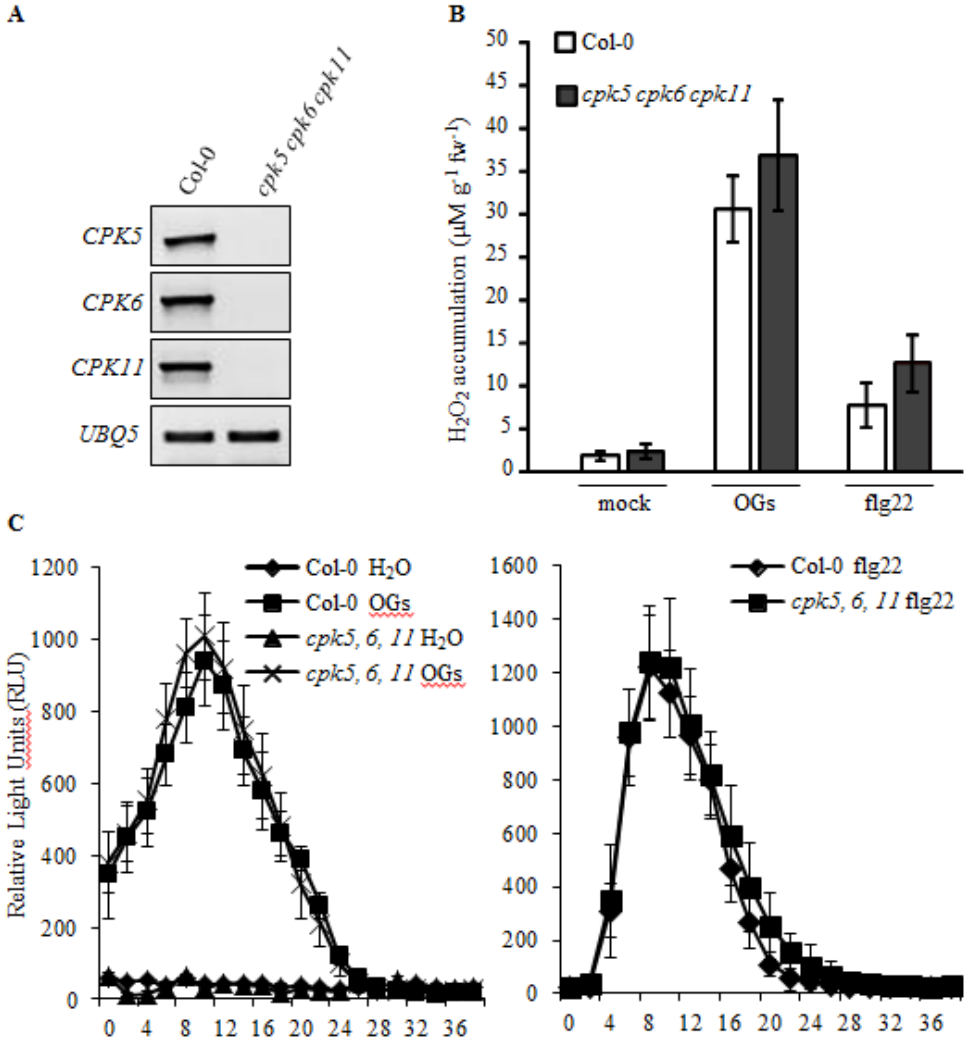

Figure 1. Accumulation of extracellular H_2O_2 in the Arabidopsis *cpk5 cpk6 cpk11* mutant upon treatment with OGs or flg22. A) Genotyping analysis of the *cpk5 cpk6 cpk11* T-DNA insertion mutant. The *CPK4*, *CPK5* and *CPK11* genes were analyzed in Col-0 and triple mutant seedlings, using gene-specific primers flanking the T-DNA insertion. B) Col-0 and *cpk5 cpk6 cpk11* 14-day-old

seedlings were treated for 30 min with H_2O, OGs (100 µg/ml) or flg22 (1 µM). The H_2O_2 concentration in the incubation medium, expressed as µmolar H_2O_2/g fresh weight (fw) of seedlings, was determined by a xylenol orange based assay. Results are average ±sd (n=4). Data are from one of two independent experiments that gave similar results. C) ROS production measured in relative light units (RLUs) in 4-wk-old Col-0 and *cpk5 cpk6 cpk11* leaf discs after elicitation with 200 µg/ml OG, 200 nM flg22 or water. Results are average ±sd (n = 12). Data are from one of three independent experiments that gave similar results.

The MAPK cascade is rapidly (within 5 min) activated by treatment with either flg22 (Zipfel et al., 2006) and OGs (Galletti et al., 2011). Flg22-induced MAPK activation has been shown to be independent of CDPKs, since the phosphorylation of the MAP single kinases MPK3 and MPK6 is not affected in the *cpk5 cpk6 cpk11* mutant (Boudsocq et al., 2010). To assess whether the role of CPK4 CPK5 and CPK11 is dispensable also for the OG-induced activation of MPK3 and MPK6, seedlings were treated for 15 min with OGs or flg22, as a control, and phosphorylation of the MPK3 and MPK6 was determined by western blot analysis using a commercial antibody generated against the human homologs of these MAPKs (α-p44/p42). Levels of phosphorylated MPK3 and MPK6 in *cpk5 cpk6 cpk11* mutant and wild type seedlings were comparable in response to OGs and, as expected, to flg22 (Figure 2). This result shows that CPK5 CPK6 and CPK11 do not play a major role in the regulation of MAPK activation by OGs, similar to what observed with flg22.

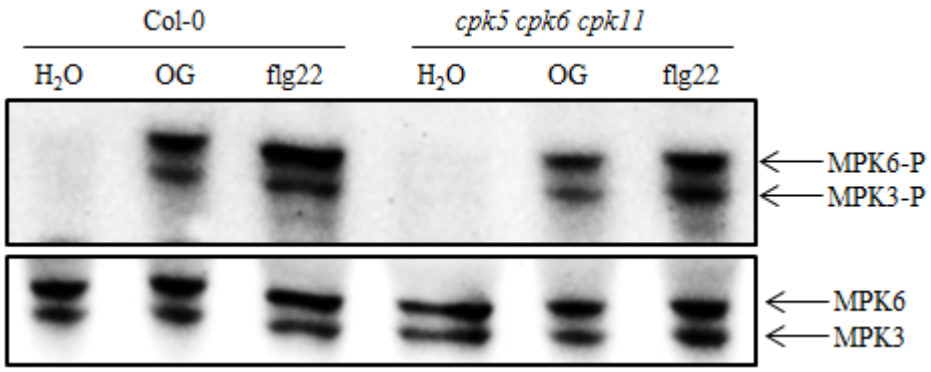

Figure 2. MAP kinases activation in the Arabidopsis *cpk5 cpk6 cpk11* mutant upon treatment with OGs or flg22. Col-0 and *cpk5 cpk5 cpk11* 10-day-old seedlings were treated for 15 min with H_2O, OGs (100 µg/ml) or flg22 (10 nM). Total protein extracts (30 µg) were analyzed by Western blot using α-p44/p42 as a primary antibody (top panel). The identity of individual MAP kinases as determined by size is indicated by arrows. The immunoblot was stripped and probed with a mix of α-MPK3 and α-MPK6 antibodies detecting native MAP kinases to determine equal loading (bottom panel).

The activation of early defense gene expression in response to flg22 is regulated by both the MAPK (Zipfel et al., 2006) and CDPK cascades, and four flg22-activated regulatory programs differentially involving the two cascades have been proposed (Boudsocq et al., 2010). The gene *FRK1* (*FLG22-INDUCED RECEPTOR-LIKE KINASE 1*) is activated, upon treatment of protoplasts with flg22, specifically through the MAPK-mediated pathway, whereas other genes, such as *PHI-1* (*PHOSPHATE-INDUCED 1*), appear to be activated specifically through CDPKs. Genes such as *CYP81F2*, encoding a cytochrome P450 involved in indol-3-yl-methyl glucosinolate catabolism (Clay et al., 2009), are instead activated synergistically by both CDPKs and MAPKs, with a MAPK-dominant effect. Transcript levels of these genes, as well as of *RET-OX,* encoding a protein with homology to reticuline oxidases (Dittrich and Kutchan, 1991) and used as a marker of response to OGs and flg22 in many studies (Galletti et al., 2008; Galletti et al., 2011), were analyzed by qRT-PCR in Arabidopsis seedlings after treatment with OGs or flg22 for 30 and 60 minutes. Wild type seedlings treated with either elicitor showed maximal expression of *PHI-1* at 30 min, with a marked decline at 60 min (Figure 3). In the

same seedlings, expression of *RET-OX* and *FRK1* increased to reach higher expression at 60 minutes, while induced expression of *CYP81F2* was comparable at both 30 and 60 minutes (Figure 3). In the *cpk5 cpk6 cpk11* triple mutant, compared to the wild type, we observed: i) no difference in the basal expression of all genes analyzed; ii) no difference in their induction at 30 minutes after treatment with both OGs and flg22, with the exception of *RET-OX* that displayed a reduced response but only to OGs; iii) a significant reduction of all the four genes in response to both elicitors at 1 hour after treatment (Figure 3). These data indicate that loss of CPK5, CPK6 and CPK11 impairs induction of the OG-triggered early defense gene and confirm their involvement in the gene expression response to flagellin. However, in contrast with the previous reports (Boudsocq et al., 2010), I observed a defective induction of *FRK1,* questioning its dependence solely on the pathway(s) mediated by MAPK3 and MPK6. I also observed that *PHI-1* expression was fully induced in *cpk5 cpk6 cpk11* triple mutant at 30 minutes after elicitor treatment, and was lower only at 1 hour, i.e. at the declining phase of its induction, suggesting that loss of the three CDPKs affects mainly the duration of the elicitor-induced expression of this gene.

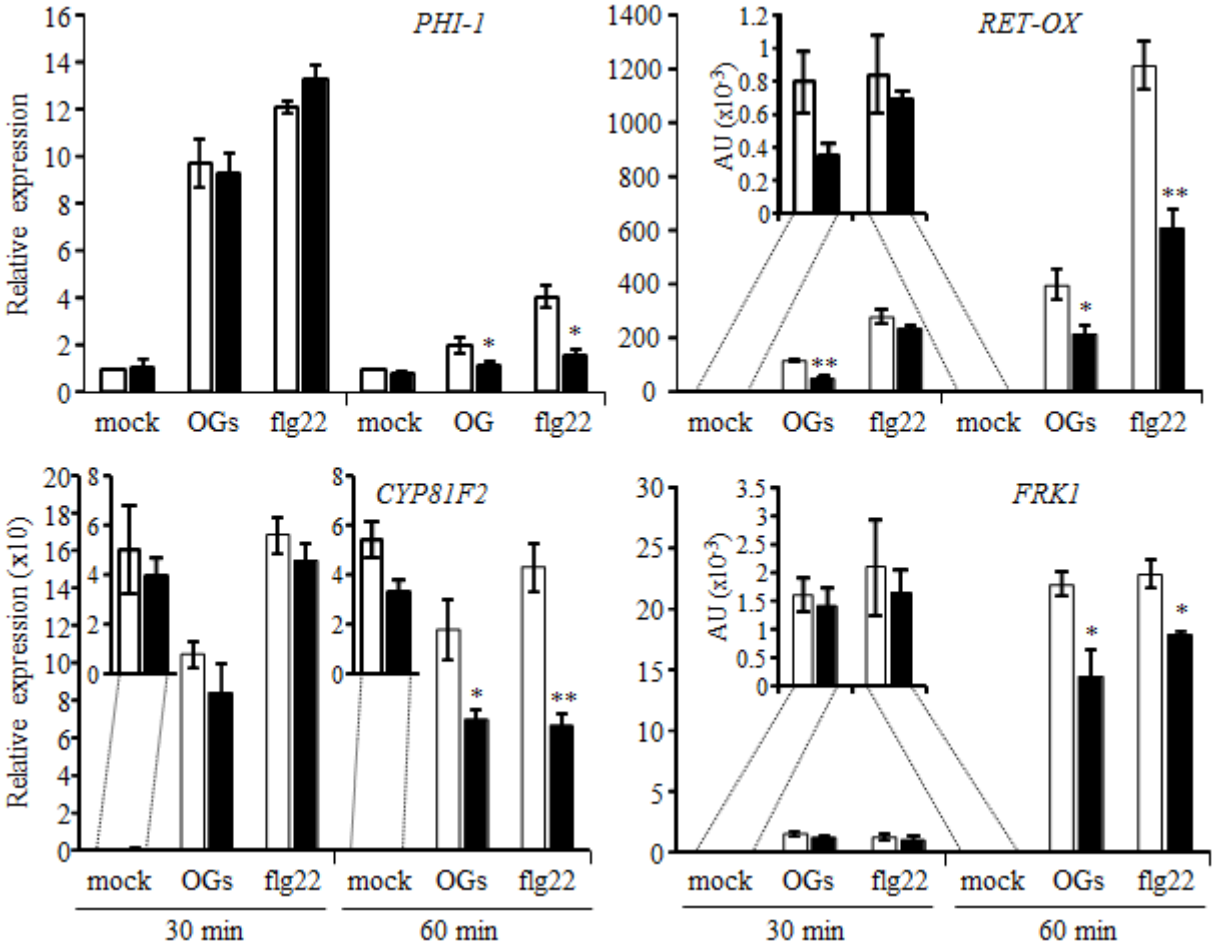

Figure 3. Analysis of early defense-related marker genes induction upon treatment with OGs or flg22 in Col-0 and *cpk5 cpk6 cpk11* mutant. Defense genes induction in response to mock (H_2O), OGs (50 µg/ml) or flg22 (10 nM) of 10-day-old Col-0 and *cpk5 cpk6 cpk11* seedlings. Gene expression of *PHI1*, *RET-OX*, *CYP81F2* and *FRK1*, was measured at the indicated times by quantitative PCR analysis, normalized to *UBQ5* (reference gene) expression, and plotted relative to Col-0 mock treatment expression level. In inset, levels of *RET*-OX, *CYP81F2* and *FRK1* transcripts normalized to *UBQ5* transcripts are expressed Arbitrary Units (AU) $x10^{-3}$. Results are average ±se (n=3). Asterisks indicate statistically significant differences between mutant- and wild type-treated seedling, according to Student's *t* test (*, P<0.05; **, P<0.01).

III. 2. 2. Lack of CDPK5/6/11 differentially impairs expression of late defense response gene, but does not affect OG-induced callose deposition

Because the behavior of the *PHI1* gene suggests that duration of defense responses may be affected in the triple mutants, the role of CPKs in the induction of late immune responses triggered by OGs and flg2 was investigated. For analysis of late elicitor-induced gene expression, I chose the genes *PGIP1* (*POLYGALACTURONASE INHIBITING PROTEIN 1*), *PAD3* (*PHYTOALEXIN DEFICIENT 3*), *PDF1.1* (*PLANT DEFENSIN 1.1*), *PDF1.2* (*PLANT DEFENSIN 1.2*) and *PR-1* (*PATHOGENESIS-RELATED GENE 1*), a marker for salicylic acid responses (Ward et al., 1991). All these genes reach maximal induction at time points between 3 and 12 hours in response to both OGs and flg22 (Ferrari et al., 2007; Denoux et al., 2008). A time-course analysis of transcript accumulation was performed in *cpk5 cpk6 cpk11* and wild type seedlings treated with OGs or flg22 for 3, 6, 12 and 24 hours. Four different expression profiles were observed in the mutant compared to wild type: i) no difference either in the basal expression or in the elicitor-triggered induction (*PGIP1*, Figure 4A); ii) lower basal and induced expression in response to OGs, but not to flg22 (*PAD3*, Figure 4B); iii) lower basal and induced expression in response to both OGs and flg22 (*PDF1.1* and *PDF1.2*, Figure 4C); iv) a higher expression both in the absence and in the presence of elicitors, with a lower fold-induction with respect to the mock treatment in response to both OGs and flg22 (*PR-1*, Figure 4D). Collectively, these results indicate that loss of CPK5, CPK6 and CPK11 leads to altered basal expression and elicitor-triggered induction of defense response genes, with a different role depending on the gene, and suggests that the defense response gene up-regulation likely occurs through multiple pathways, some dependent and other independent from the three CDPKs. Furthermore, the three kinases appear to act both as positive (*PAD3*, *PDF1.1* and *PDF1.*2) and negative regulators (*PR1*) of basal expression of defense-response genes.

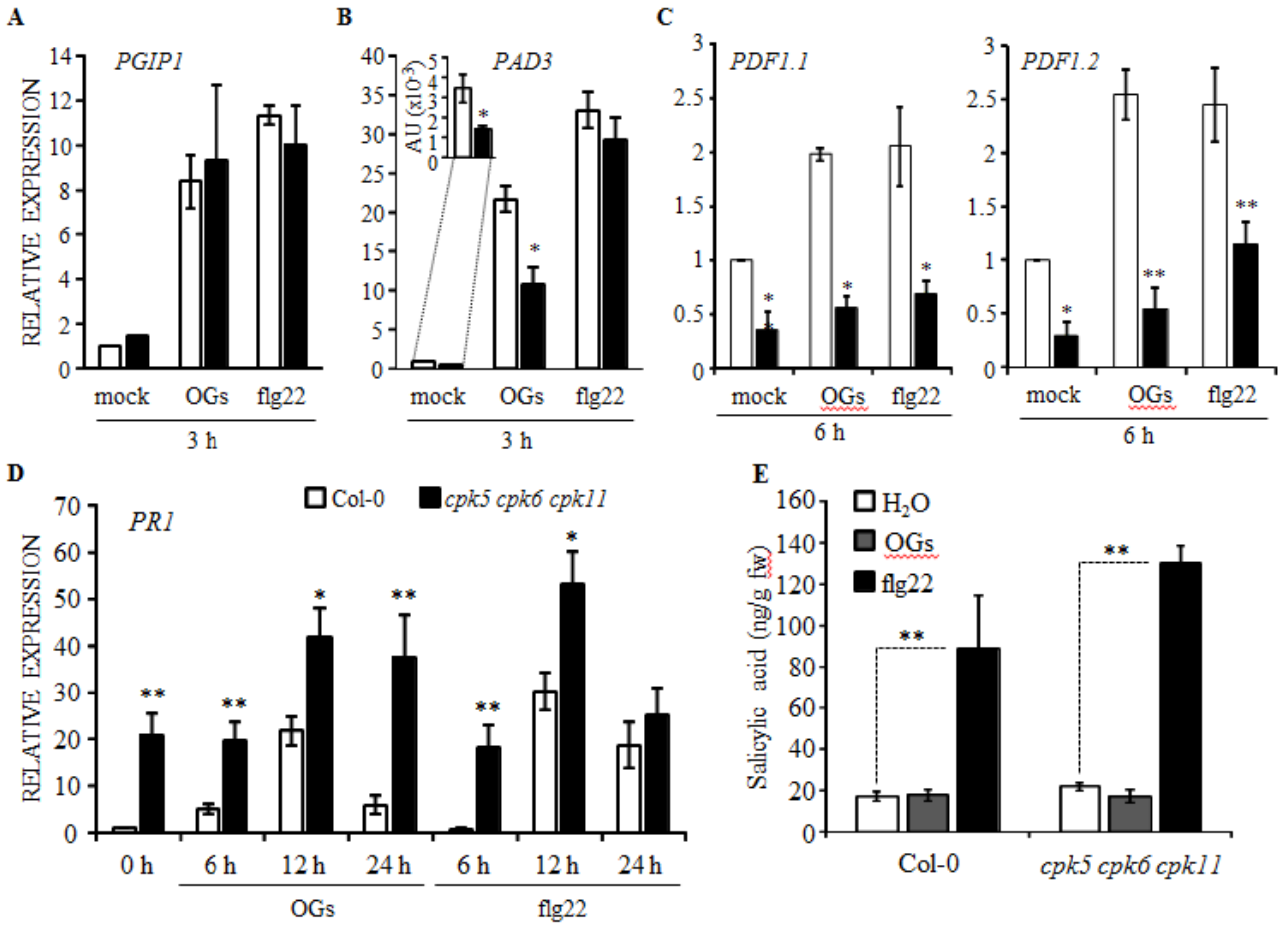

Figure 4. Analysis of late defense-related marker genes induction and SA production upon treatment with OGs or flg22 in Col-0 and *cpk5 cpk6 cpk11* mutant. A) Defense genes induction in response to mock (H_2O), 50 µg/ml OGs or 10 nM flg22 of 10-day-old Col-0 and *cpk5 cpk6 cpk11* seedlings. Gene expression of *PGIP1*, *PAD3*, *PDF1.1*, *PDF1.2* and *PR-1*, was measured at the indicated times by quantitative PCR analysis, normalized to *UBQ5* (reference gene) expression, and plotted relative to Col-0 mock treatment expression level. In inset, mock-treated mutant and wild type seedlings transcript of *PAD3* related to *UBQ5* is shown in AU ($x10^{-3}$). Results are average ±se (n=3). Asterisks indicate statistically significant differences between mutant- and wild type-treated seedling, according to Student's *t* test (*, P<0.05; **, P<0.01). B) Salicylic acid production expressed in ng/g fresh weight (fw) in response to OGs or flg22. 14-day-old Col-0 and *cpk5 cpk6 cpk11* seedlings were treated with mock (water), OGs (100 µg/ml) or flg22 (10 nM) for 8 h. Salicylic acid was extracted as described in (Pan et al., 2010e) and measured by HPLC-MS spectrometry. Results are average ±se (n=4). Asterisks indicate statistically significant differences between elicitor (flg22 or OGs) and mock (H_2O)-treated seedlings, according to Student's *t* test (**, P<0.01).

Both flg22 and OGs are able to induce callose deposition in Arabidopsis seedlings and rosette leaves as a late response (Gomez-Gomez et al., 1999; Denoux et al., 2008). Callose accumulation, measured as number of callose deposits, was therefore analyzed in response to infiltrated OGs and flg22 in *cpk5 cpk6 cpk11* adult leaves. I did not find significant differences between wild type and mutant plants either in response to OGs or to flg22 (Figure 5). This result indicates that CPK5, CPK6 and CPK11 do not play a major role in elicitor-triggered callose deposition. Moreover, as OG- and flg22-induced callose accumulation is dependent on *At*RbohD-mediated extracellular H_2O_2 accumulation (Zhang et al., 2007; Galletti et al., 2008; Luna et al., 2011), it is in agreement with the observation that lack of CPK5 CPK6 and CPK11 does not impair OG- and flg22-induced H_2O_2 production.

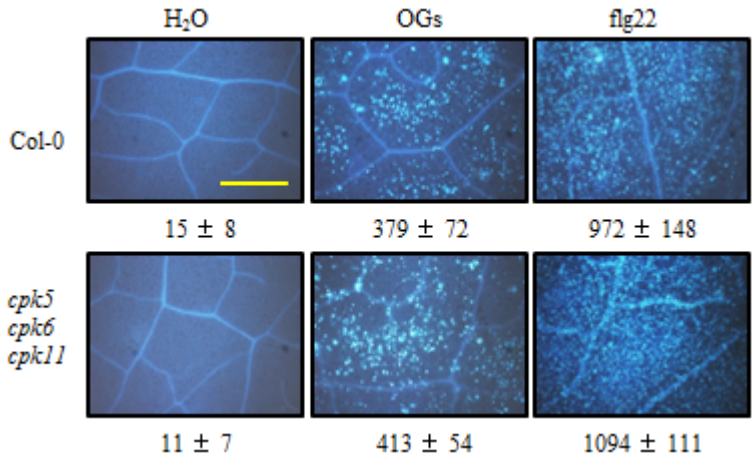

Figure 5. Callose accumulation upon treatment with OGs or flg22 in Col-0 and *cpk5 cpk6 cpk11* mutant. Leaves from Arabidopsis wild-type and *cpk5 cpk6 cpk11* plants were infiltrated with water, OGs (200 µg/ml) or flg22 (1 µM); excised leaves were stained 24 h later with aniline blue for callose visualization. The number below each image indicates the average number of callose deposits 6 ±se of six different leaf samples from at least five independent plants (three microscopic fields of 0.1 mm^2 for each leaf). Images show representative leaves for each treatment. All images are at the same scale; scale bar = 1 mm (10X magnification). This experiment was repeated twice with similar results.

III. 2. 3. Basal resistance and elicitor-induced protection against *Botrytis cinerea* is impaired in the *cpk5 cpk6 cpk11* mutant

In order to establish if the defense response defects observed in the *cpk5 cpk6 cpk11* mutant affect basal or elicitor-induced resistance to pathogens, the involvement of CPKs in the response of Arabidopsis plants to *Botrytis cinerea* and in protection against this fungus induced by OG or flg22 (Ferrari et al., 2007; Galletti et al., 2011) was investigated.

Adult *cpk5 cpk6 cpk11* and wild type plants were sprayed with OGs, flg22 or water, and excised leaves were drop-inoculated with *B. cinerea* conidia after 24 h. In water-pretreated leaves, the average of disease lesion diameter, measured 48 hours upon infections, was approximately 40% larger in mutant plants compared to WT, indicating a higher susceptibility of the mutant plants. Moreover, unlike the wild type, *cpk5 cpk6 cpk11* plants displayed neither OG- nor flg22-induced protection against Botrytis (Figure 6A). These results were corroborated by qRT-PCR analysis of the fungus β-tubulin transcripts in the infected wild type and mutant leaves, performed to evaluate the extent of fungal growth (Figure 6B). In infected wild type leaves that had been pre-treated with either OGs or flg22, levels of β-tubulin transcript were lower at 48 hours than in water-pretreated infected leaves, whereas no difference was observed at 24 h (not shown). This result indicates that elicitor-induced protection affects, in addition to symptom development, fungal growth, but only later during infection. In the *cpk5 cpk6 cpk11* mutant, β-tubulin transcripts levels were comparable for all the pre-treatments (water, OGs and flg22) and were much higher than in wild type plants (Figure 6B). Thus, both symptom development and growth of the fungus confirm that the *cpk5 cpk6 cpk11* mutant plants are more susceptible to Botrytis and are defective in elicitor-induced protection. These results clearly indicate that loss of CPK5, CPK6 and CPK11 impairs both basal resistance to Botrytis and the immunity against this necrotrophic fungus induced by both OGs and flg22.

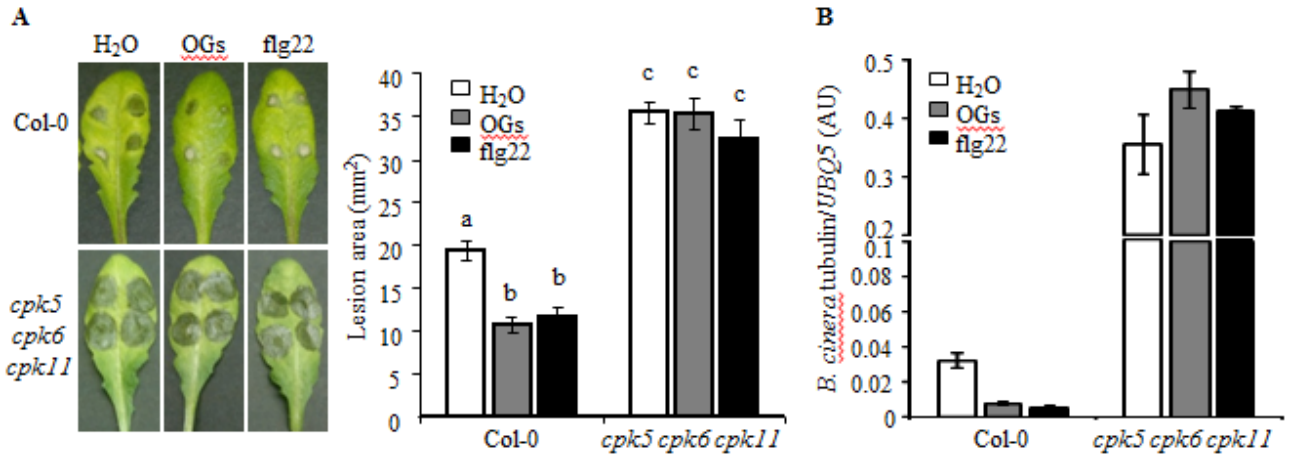

Figure 6. Elicitor-induced protection against *Botrytis cinerea* in Col-0 and *cpk5 cpk6 cpk 11* mutant. Four-week old plants were sprayed with OGs (200 µg/ml), flg22 (1 µM) or water; after 24 h, leaves were inoculated with *B. cinerea* spores ($5x10^5$ conidia/ml). A) Lesion areas were measured 48 h after inoculation (hpi). Results are average ±sd (n=12). Letters indicate P<0.01 by one-way ANOVA with Tukey's HSD test. Data are from one of three independent experiments that gave similar results. B) Botrytis tubulin expression was analyzed 48 hpi. Results are average of three technical replicates ±sd. The experiment was repeated three times with similar results.

III. 2. 4. Camalexin accumulation is not impaired in the *cpk5 cpk6 cpk11* mutant

In the attempt to elucidate the basis of the defective resistance to Botrytis of the *cpk5 cpk6 cpk11* mutant, different mechanisms that are known to be relevant for resistance to this fungus were considered. I first analyzed cuticle permeability, which is known to influence susceptibility to pathogens. In particular, a higher cuticle permeability leads to a higher basal resistance to Botrytis (Bessire et al., 2007), whereas a reduced permeability leads to higher susceptibility to the fungus (Bourdenx et al., 2011). However, cuticle permeability of mutant and wild type plants, measured by the chlorophyll leaching assay, was comparable (Figure 7A).

Then, the expression of the *PAD3* gene, which encodes the cytochrome CYP71B15 that catalyzes the last step of camalexin biosynthesis and is required for OG-induced protection against *B. cinerea* (Ferrari et al., 2007) was analyzed. I found that levels of *PAD3* transcripts, examined by qRT-PCR in water-, OG- and flg22-sprayed leaves, were lower in the *cpk5 cpk6 cpk11* mutant plants than in wild type (Figure 7B), suggesting that an impaired basal expression and response to the elicitors may cause the enhanced basal susceptibility and the lack of protection against *B. cinerea*. Because camalexin has been implicated in local *B. cinerea* resistance (Ferrari et al., 2003) and inversely correlated with susceptibility with this fungus (Denby et al., 2004), it was investigated whether a defect in camalexin production during the infection might be responsible for the enhanced basal susceptibility as well as for lack of protection against *B. cinerea* of the mutant. Camalexin levels were therefore measured in infected leaves of water- and OG-sprayed plants. In both type of leaves and in both wild type and *cpk5 cpk6 cpk11* mutant, camalexin was not detectable at earlier time point, whereas it was measurable at 20 hours post-inoculation in both wild type and mutant plants. Camalexin levels were higher in infected OG-pretreated leaves than in infected water-pre-treated ones in both genotypes (Figure 7C), confirming our previous observation that pre-treatment with OGs prompts tissue to produce camalexin either more rapidly and/or at higher levels in response to *B. cinerea* (Savatin *et al.* 2014, in publication). In response to each treatment, however, camalexin levels were similar in wild type and mutant plants (Figure 7C), suggesting that the priming effect still occurred in the mutant. These observations suggest that camalexin production, although necessary, is

not sufficient both for basal resistance and OG-induced protection against *B. cinerea*, and that mechanisms other than camalexin accumulation and relevant for the response to Botrytis are defective in the mutant.

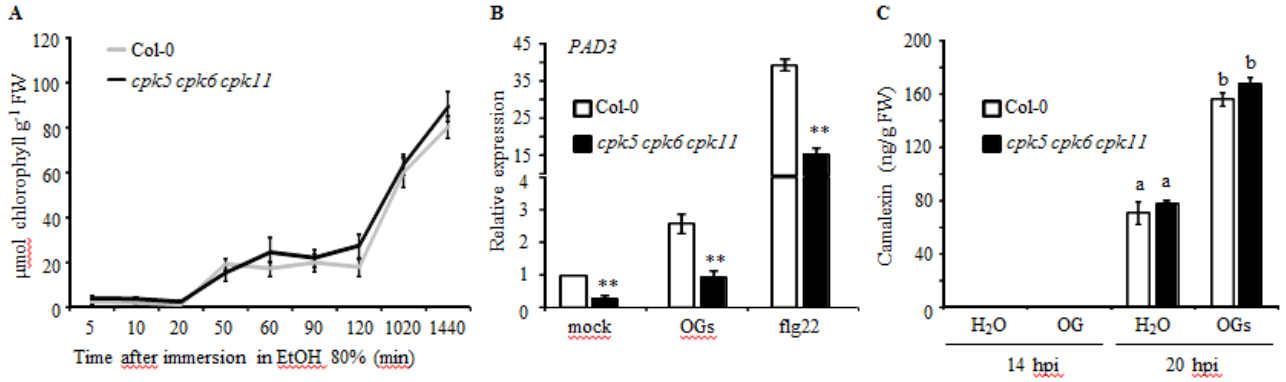

Figure 7. A) Analysis of cuticle permeability. Leaves from Col-0 and *cpk5 cpk6 cpk11* mutant 4-week-old plants were cut at the petiole, weighed and immersed a solution of 80% ethanol. Chlorophyll was extracted at 5, 10, 20, 50, 60, 90, 120, 1020, 1440 min after immersion. The chlorophyll content, expressed as micromolar g^{-1} fresh weight was determined by measuring absorbance. **B) Analysis of *PAD3* expression in leaves.** 4-week-old Col-0 and *cpk5 cpk6 cpk11* plants were sprayed with mock (water), OGs (200 µg/ml) or flg22 (1 µM) and 3 h after leaves were cut at the petiole and gene expression was measured by quantitative PCR analysis, normalized to *UBQ5* (reference gene) expression, and plotted relative to Col-0 mock treatment expression level. Results are average ±se (n=2). Asterisks indicate statistically significant differences between mutant- and wild type-treated leaves, according to Student's *t* test (**, P<0.01). **C) Analysis of camalexin accumulation during *B. cinerea* infection.** Four-week-old Col-0 and *cpk5 cpk6 cpk11* plants were sprayed with mock (water) or OGs (200 µg/ml) and 24 h after spray leaves were inoculated with B. *cinerea* spores ($5x10^5$ conidia/ml). The camalexin (expressed in ng/g fresh weight) was extracted at the indicated hour post infection (hpi) as described in Pan *et al.* 2010 (Pan et al., 2010d) and measured by HPLC-MS spectrometry. Results are average ±se (n = 4). Letters indicate P<0.01 by one-way ANOVA with Tukey's HSD test.

III. 2. 5. Expression of ethylene-regulated genes during *Botrytis cinerea* infection is impaired in the *cpk5 cpk6 cpk11* mutant

Ethylene (Et) production is one of the defense related responses activated upon pathogen and elicitor sensing (Chague et al., 2006; Han et al., 2010), and mutants impaired in the synthesis of this hormone are more susceptible to Botrytis (Han et al., 2010; Galletti et al., 2011; Akagi et al., 2011; Li et al., 2012; Zhang et al., 2013). Moreover, flg22-induced PTI to Botrytis requires an intact ET pathway (Laluk et al., 2011). To understand whether ethylene mediated signaling plays a role in the increased susceptibility of the mutant, I first analyzed the expression of the genes that are regulated by Et, during Botrytis infection. Genes such as *ORA59* (*OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59*), *ERF1* (*ETHYLENE RESPONSE FACTOR 1*) and *ERF5* (*ETHYLENE RESPONSE FACTOR 5*), all encoding transcription factors (Fujimoto et al., 2000; Lorenzo et al., 2003; Pre et al., 2008), and *PDF1.1*, *PDF1.2* and *PR4* (*PATHOGENESIS-RELATED 4*), encoding defense proteins (Broekaert et al., 1995; Bertini et al., 2012), respond to Et with different kinetics. Moreover, *ORA59* and *ERF5* are known to be required for basal resistance to Botrytis (Pre et al., 2008; Moffat et al., 2012). In addition, *PR-1*, and *PAD3* were analyzed. The expression of these genes was examined in mutant and wild type leaves 24 and 48 hours after Botrytis inoculation. Basal expression of all genes, except for *ERF5*, was considerably lower in *cpk5 cpk6 cpk11* compared to wild type plants (Figure 8), whereas pathogen-induced expression was reduced for *ORA59*, *PDF1.1* and *PDF1.2* (Figure 9A), increased for *PR-1* and *PAD3*, and unaltered for *ERF1* and *ERF5* (Figure 10). Notably, in agreement with the notion that the transcription factor

ORA59 controls the transcription of *PDF1.2* (Pre et al., 2008), I found that, in response to the fungus, expression of *ORA59* is induced earlier than that of *PDF1.2* and that, in the mutant, the strong reduction of the pathogen-induced expression of *ORA59* is accompanied by a strong reduction of expression of *PDF1.2*. Taken together, these results reveal that an impairment in the mutant of the expression of specific Et-regulated genes, such as *ORA59* and *ORA59*-regulated genes, during Botrytis infection correlates with an enhanced susceptibility to this fungus, further supporting the crucial role of *ORA59* in defense. They also suggest that Et-regulated responses activated by Botrytis are complex and unlikely to be regulated through a single pathway.

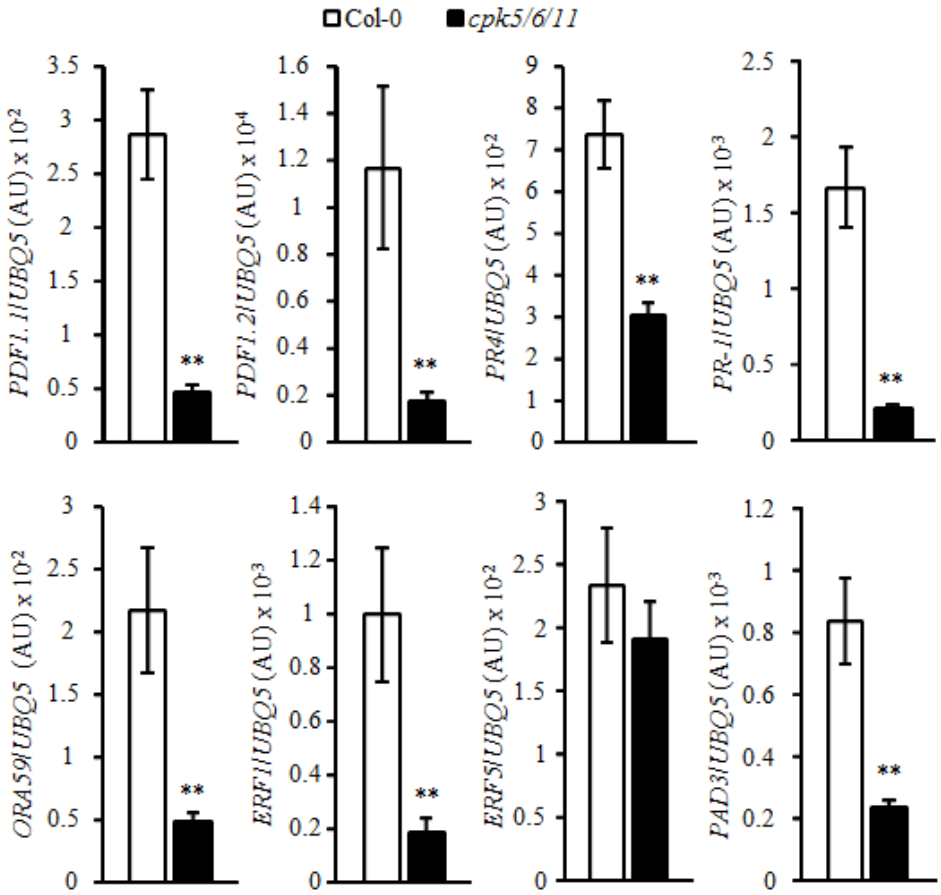

Figure 8. Analysis of gene expression in untreated Col-0 and *cpk5 cpk6 cpk11* mutant leaves. Gene expression (expressed as arbitrary units, AU) of *PDF1.1*, *PDF1.2*, *PR-4*, *PR-1*, *ORA59*, *ERF1*, *ERF5* and *PAD3*, was measured in leaves from 4-week-old Col-0 and cpk5 cpk6 cpk11 mutant plants by quantitative PCR analysis and normalized to *UBQ5* (reference gene). Results are average ±sd (n=2). Asterisks indicate statistically significant differences between mutant- and wild type-treated leaves, according to Student's *t* test (**, P<0.01).

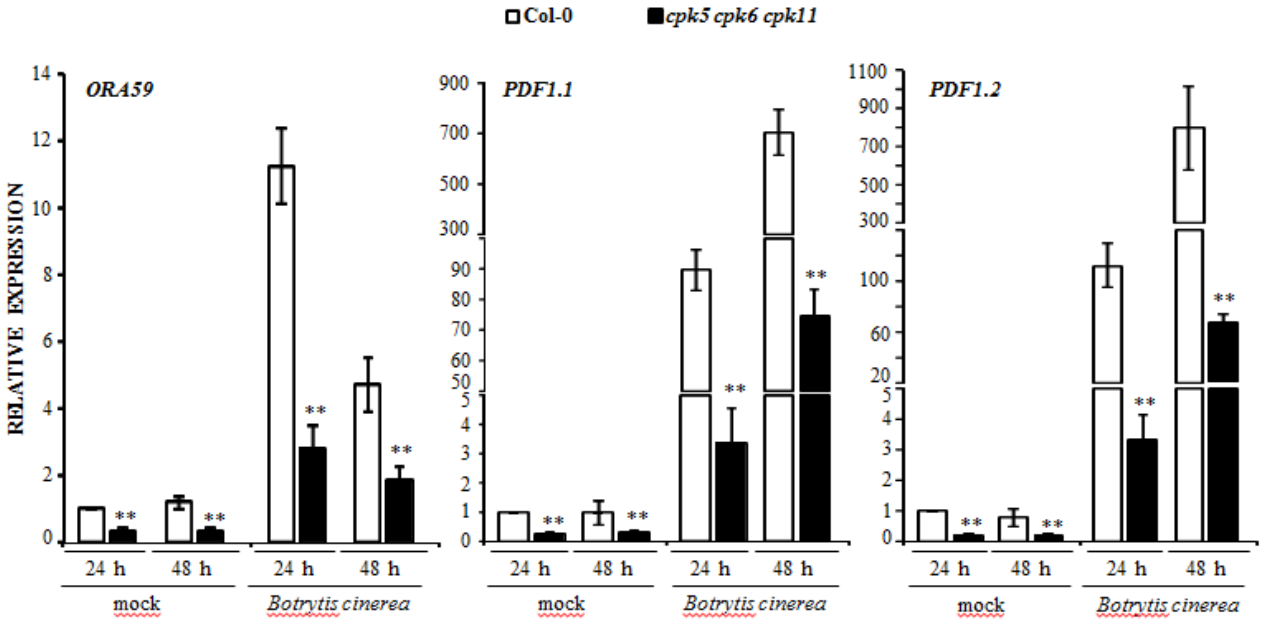

Figure 9. Analysis of gene expression during *B. cinerea* infection in Col-0 and *cpk5 cpk6 cpk11* mutant. Leaves from Col-0 and *cpk5 cpk6 cpk11* mutant 4-week-old plants were inoculated with *B. cinerea* spores ($5x10^5$ conidia/ml) or mock (potato dextrose broth, PDB). Gene expression of *ORA59*, *PDF1.1*, and *PDF1.2* was measured 24 h and 48 h post inoculation (hpi) by quantitative PCR analysis, normalized to *UBQ5* (reference gene) expression, and plotted relative to Col-0 mock treatment expression level. Results are average ±se (n=3). Asterisks indicate statistically significant differences between mutant- and wild type-treated leaves, according to Student's *t* test (**, P<0.01).

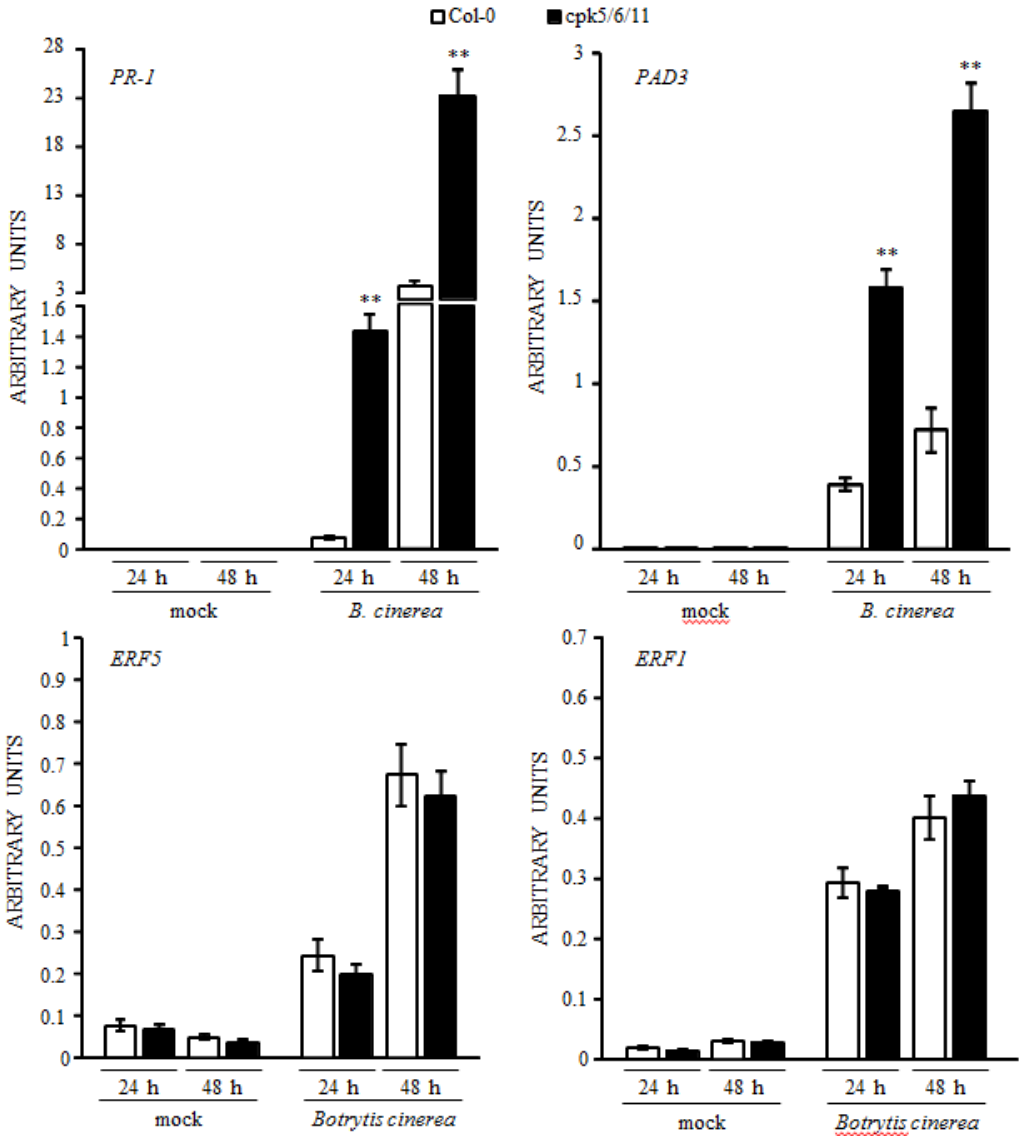

Figure 10. Analysis of gene expression during *B. cinerea* infection in Col-0 and *cpk5 cpk6 cpk11* mutant. Leaves from Col-0 and *cpk5 cpk6 cpk11* mutant 4-week-old plants were inoculated with *B. cinerea* spores ($5x10^5$ conidia/ml) or mock (potato dextrose broth, PDB). Gene expression of *PR-1*, *PAD3*, *ERF5* and *ERF1* was measured 24 h and 48 h post inoculation (hpi) by quantitative PCR analysis and normalized to *UBQ5* (reference gene) expression. Results, indicated as arbitrary units (AU), are average ±se (n=3). Asterisks indicate statistically significant differences between mutant- and wild type-treated leaves, according to Student's *t* test (**, P<0.01).

III. 2. 6. Response to ethylene, but not to jasmonic acid, is impaired in the *cpk5 cpk6 cpk11* mutant

The defective Et-regulated gene expression observed in the mutant during *B. cinerea* infection may reflect a defective response to Et and/or a reduced production of Et produced in the mutant. Moreover, because all the genes examined are regulated also by JA, with a synergistic effect when the jasmonic acid (JA) and Et are applied in combination (see scheme in Table 1), a defect in JA signaling may contribute to their altered regulation. I therefore investigated whether the response of these genes to the two hormones is defective in the triple mutant. Expression of the genes examined above was analyzed by qRT-PCR in two-week-old seedlings treated with methyl JA (MeJA), ethephon (E), an Et-releasing chemical (Lawton et al., 1994), or both for 8 hours. Induced expression of all genes was

significantly lower in mutant seedlings respect to WT in all treatments. Intriguingly, while basal expression of the defense response-genes *PDF1.1*, *PDF1.2* and *PR4* was decreased in the *cpk5 cpk6 cpk11* mutant, that of the transcription factor-encoding genes *ORA59*, *ERF1* and *ERF5* was not altered (Figure 11). These results indicate that regulation of JA/Et-responsive genes by both Et and JA is impaired in the triple mutant.

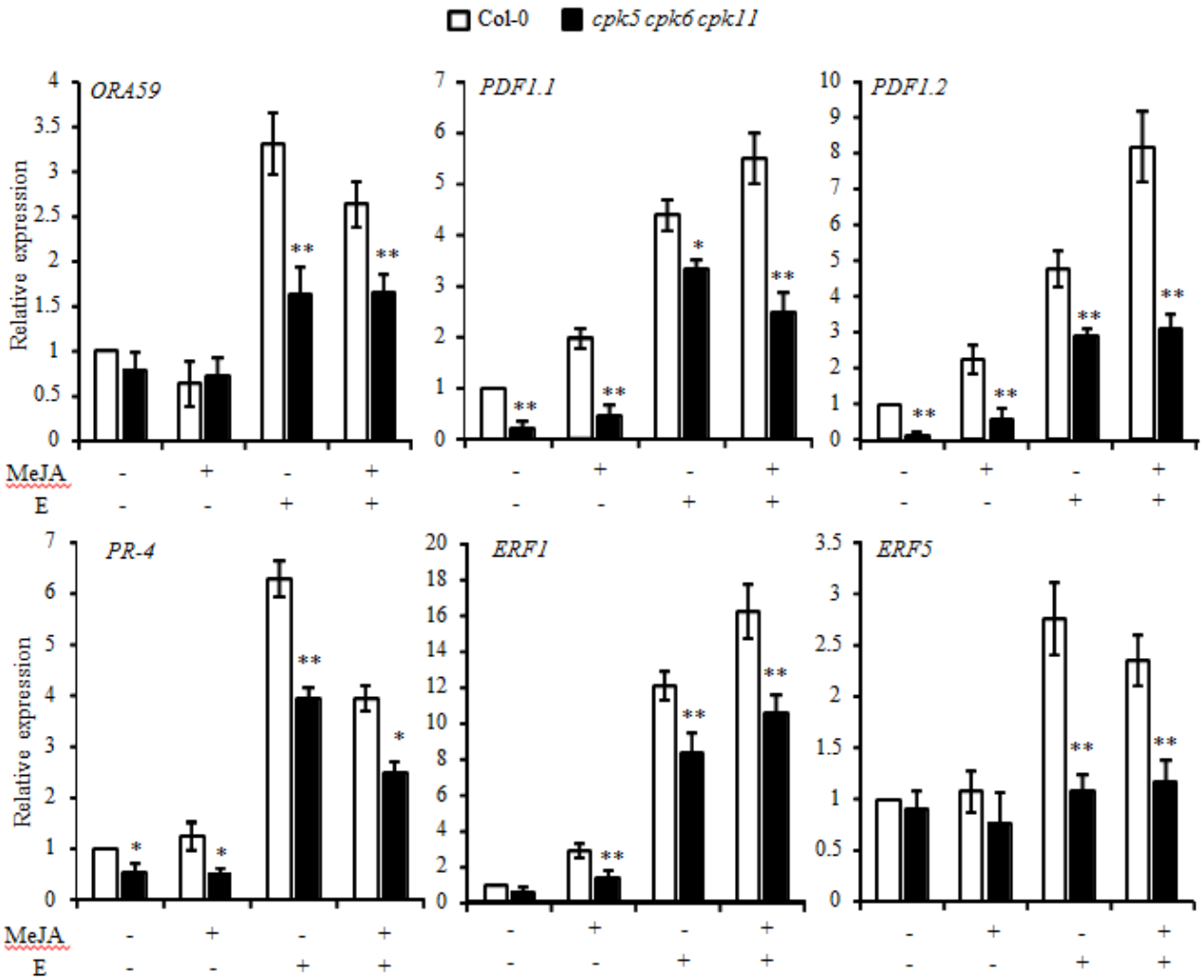

Figure 11. Analysis of gene expression in Col-0 and *cpk5 cpk6 cpk11* mutant upon treatment with methyl jasmonate, ethephon or both. Fourteen-day-old Col-0 and *cpk5 cpk6 cpk11* seedlings were treated with mock (DMSO + $NaPO_4$ 0.5 mM), 50 µM methyl jasmonate (MeJA), 1 mM Ethephon (E) or both (MeJA+E) for 8 h. Gene expression of *ORA59*, *PDF1.1*, *PDF1.2*, *PR-4*, *ERF1* and *ERF5* was measured by quantitative PCR analysis, normalized to *PEX4* (reference gene) expression, and plotted relative to Col-0 mock treatment expression level. Results are average ±se (n=3). Asterisks indicate statistically significant differences between mutant- and wild type-treated seedlings, according to Student's *t* test (*, p<0.05; **, P<0.01).

Mutants in ET signaling display a defective induction of the ET-JA-responsive genes also when treated with JA alone and *vice versa* (Lorenzo et al., 2003; Pre et al., 2008), suggesting that signaling pathways of both hormones must be intact for a proper regulation by each hormone. I therefore assessed whether JA signaling was also defective in the *cpk5 cpk6 cpk11* triple mutant. The induction of the *THI2.1* (*THIONIN 2.1*) and *VSP2* (*VEGETATIVE STORAGE PROTEIN 2*) genes, described to be specifically regulated by JA and not by Et (Epple et al., 1995; Berger et al., 1995; Benedetti et al., 1995) was analyzed in wild type and mutant seedlings treated with MeJA or E, used as a control, for 8 h. As expected, both genes were up-regulated by MeJA treatment only; no difference was observed between Col-0 and mutant

seedlings, except for the basal expression (Figure 12), suggesting that JA-specific signaling is not affected by the lack of CPK5, CPK6, and CPK11. Thus, the lower induction of the Et/JA-regulated genes observed in *cpk5 cpk6 cpk11* triple mutant seedlings can be likely explained by the defect in ethylene signaling.

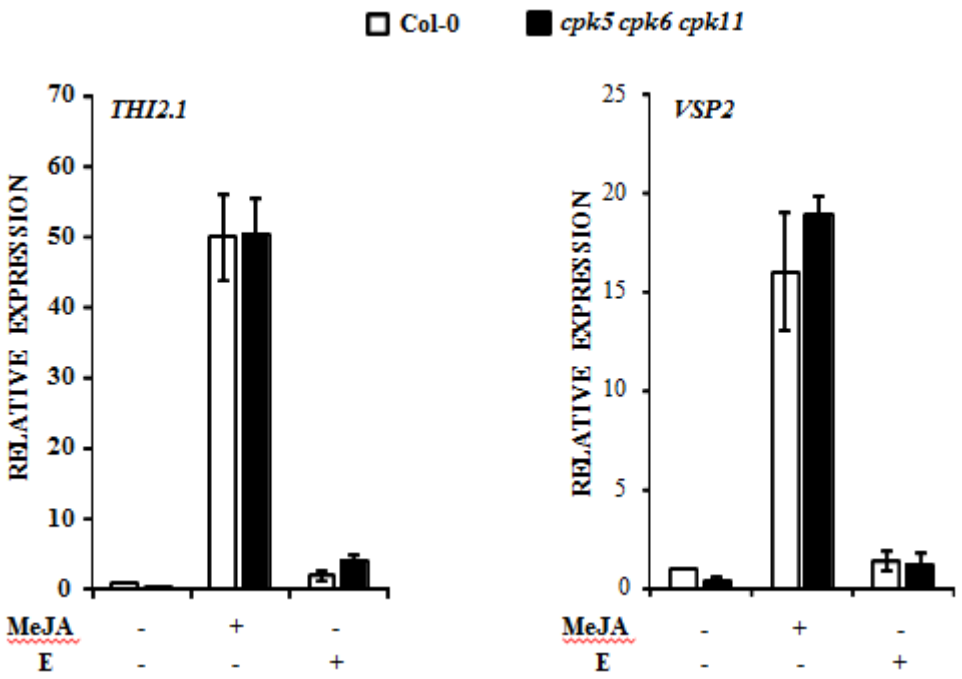

Figure 12. Analysis of gene expression in Col-0 and *cpk5 cpk6 cpk11* mutant upon treatment with methyl jasmonate or ethephon. Fourteen-day-old Col-0 and *cpk5 cpk6 cpk11* seedlings were treated with mock (DMSO + $NaPO_4$ 0.5 mM), 50 µM methyl jasmonate (MeJA) or 1 mM Ethephon (E) for 8 h. Gene expression of *THI2.1* and *VSP2* was measured by quantitative PCR analysis, normalized to *PEX4* (reference gene) expression, and plotted relative to Col-0 mock treatment expression level. Results are average ±se (n=3).

		Regulation by [b]		
Gene	Function [a]	JA	ET	JA+ET
ORA59 [c]	TF	(1 h)	(1 h)	(1 h)
PDF1.1 [c]	D	(1 h)	(8 h)	(8 h)
PDF1.2 [d]	D	(1 h)	(8 h)	(8 h)
PR-4 [c]	D	(4 h)	(8 h)	(24 h)
ERF1 [e]	TF	(8 h)	(8 h)	(8 h)
ERF5 [f]	TF	(12 h)	(6 h)	(12 h)
THI2.1 [g]	D	(8 h)		
VSP2 [d]	?	(8 h)		

Table 1. List of genes induced in response to ethylene and jasmonate synergistically or in response to only jasmonate. [a]: TF, transcription factor; D, defense gene. [b]: Arrows indicate up-regulation,with the larger size indicating the synergistic effect of the two

hormones; numbers in parenthesis indicate the time of maximal induction. [c]: (Pre et al., 2008). [d]: (Jung et al., 2007). [e]: (Lorenzo et al., 2003). [f]: (Son et al., 2012) 2012. [g]: (Epple et al., 1995).

To further support these conclusions, I analyzed an additional Et regulated response, i.e. the root growth inhibition response induced, in the light and in the dark, using the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) (Kende, 1993). The *cpk5 cpk6 cpk11* mutant evidenced a reduced root growth inhibition in both conditions compared to wild type seedlings (Figure 13A and 13B), confirming the impairment in Et signaling and indicating that CPK5, CPK6 and CPK11 mediate also growth responses mediated by this hormone. On the other hand, *cpk5 cpk6 cpk11* seedlings grown in the presence of JA displayed a root growth inhibition response similar to that of the wild type (Figure 13C). Taken together these data demonstrate that the lack of CPK5, CPK6, and CPK11 affects responsiveness to ethylene, but not to JA, in Arabidopsis.

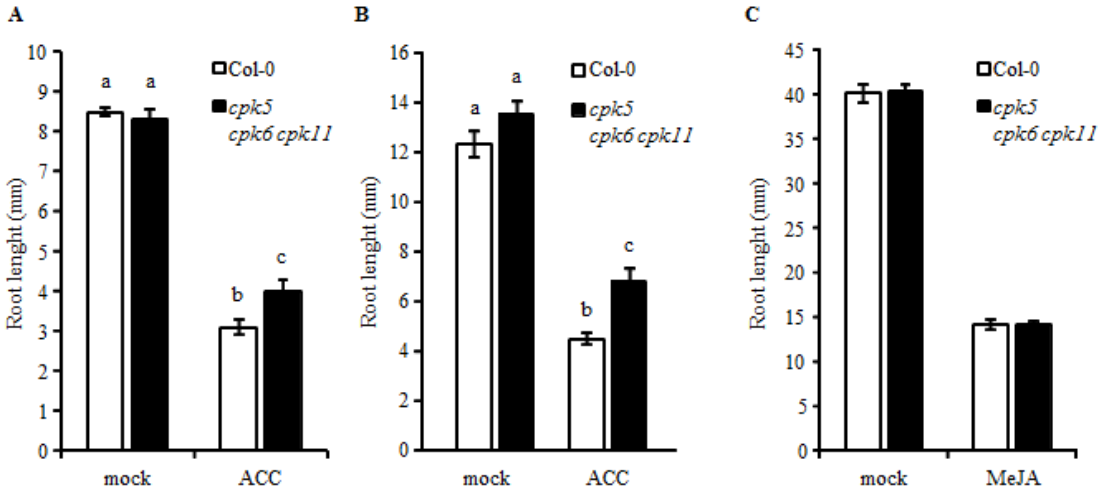

Figure 13. Analysis of root growth inhibition induced by ACC or MeJA in Col-0 and *cpk5 cpk6 cpk11* mutant. A) Col-0 and *cpk5 cpk6 cpk 11* seedlings were growth for 5 days under light condition (photoperiod 16/8) on Murashige and Skoog (MS) agar and then transferred on Murashige and Skoog (MS) agar with 500 nM ACC or mock (water). After 5 additional days root length was measured by Image J software. Results are average ±se (n=15). Letters indicate P<0.05 by one-way ANOVA with Tukey's HSD test. B) Col-0 and *cpk5 cpk6 cpk 11* seedlings growth for 8 d in the dark on Murashige and Skoog (MS) agar with 10 µM ACC or mock (water). Root length was measured by Image J software. Results are average ±se (n=15). Letters indicate P<0.01 by one-way ANOVA with Tukey's HSD test. C) Col-0 and *cpk5 cpk6 cpk 11* seedlings growth for 8 d under light condition (photoperiod 16/8) on Murashige and Skoog (MS) agar with 50 µM MeJA or mock ($NaPO_4$ 0.5 mM). Root length was measure by Image J software. Results are average ±se (n=15).

III. 2. 7. Ethylene production in response to Botrytis and OGs is impaired in *CPK5 CPK6 CPK11* mutant

I next assessed if ET production induced by Botrytis is also altered in the triple mutant. Ethylene production was measured by gas chromatography, in WT and mutant leaves at 20, 40 and 60 hours post-inoculation with *B. cinerea* conidia. In leaves of both types of plants, hormone levels were detectable only at 40 hours post-inoculation, and increased after 60 hours. *cpk5 cpk6 cpk11* leaves, however, produced significantly lower ET levels than WT leaves at both 40 and 60 hpi, being approximately 3-fold lower at 60 hours (Figure 14A). Jasmonic acid, instead was not significantly produced during the early phase of infection in both type of plants (data not shown).

In infected leaves of water- and OG-sprayed plants salicylic acid levels were also measured. In wild type leaves, salicylic acid levels were higher in infected OG-pretreated leaves than in infected water-pre-treated ones at both 14 and 20 hpi (Figure 15A). In water-sprayed *cpk5 cpk6 cpk11* leaves salicylic acid levels were higher than those of wild type. However, salicylic acid levels were similar in water- and OG-sprayed *cpk5 cpk6 cpk11* plants (Figura 15A). These results indicate that, besides camalexin synthesis, SA accumulation is also primed by OGs, and provide further evidence that the priming effect is affected in the mutant.

Because elicitation by OGs is thought to play an important role during Botrytis infection (Aziz et al., 2004; Ferrari et al., 2007), I investigated whether the triple mutant shows a defective ethylene production response to OGs. The capability of OGs with a DP of 10-15 to induce ethylene production in Arabidopsis has never been described, although microarray data indicate that OG treatment does induced Et biosynthetic genes (Denoux et al., 2008). In fact, leaf strips of both tobacco and Arabidopsis have been shown not to produce ethylene in response to OGs (Brutus et al., 2010). Because ethylene production may be suppressed in the presence of the wound response (Leon et al., 2001), likely to occur in the leaf strips, I decided to perform the analyses in intact tissues, using seedlings. Ethylene production in the triple mutants was determined either in the absence and in the presence of OGs. Notably, basal production of the hormone did not differ between the two genotypes (Figure 14B), whereas OG-induced ethylene was reduced in mutant seedlings by more than 45% compared to the wild type (Figure 14B), indicating that loss of CPK5, CPK6, and CPK11 affects OG-induced Et production.

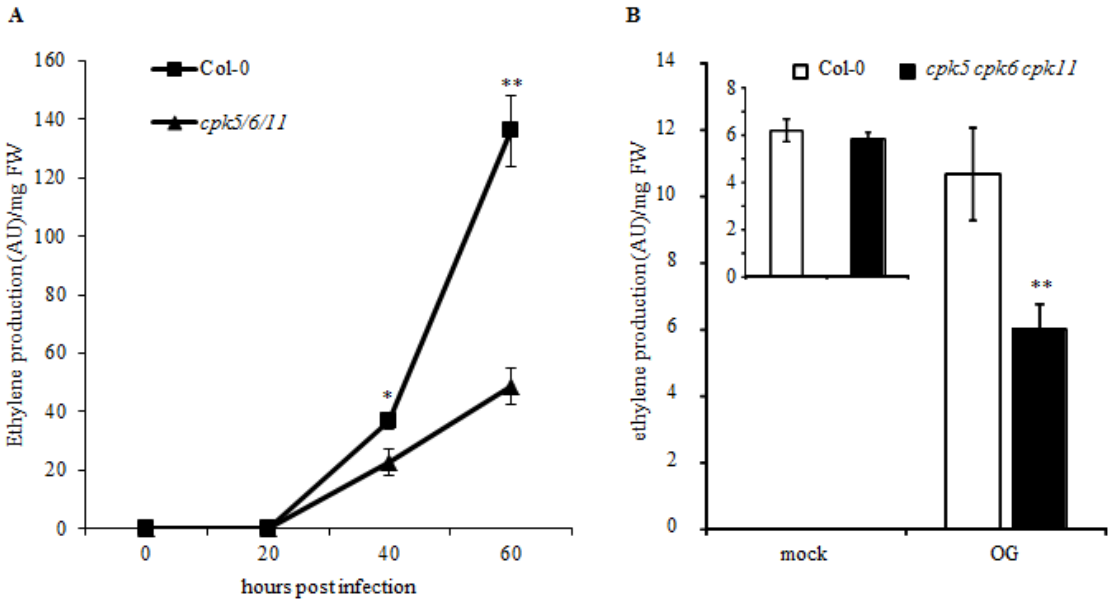

Figure 14. Analysis of ethylene production in response to Botrytis and OGs in Col-0 and *cpk5 cpk6 cpk11* mutant. A) Leaves from Col-0 and *cpk5 cpk6 cpk11* 4-week-old plants were cut from the petiole and placed in 10 ml flasks (one leaf/flask) containing 1.5 mL 0.8% agar. Leaves were inoculated with *B. cinerea* spores ($5x10^5$ conidia/ml). Ethylene production was measured at the indicated times by gas chromatography analysis. Results are average ±se (n=4). Asterisks indicate statistically significant differences between mutant- and wild type-treated leaves, according to Student's *t* test (*, p<0.05; **, P<0.01). B) Ten-day-old Col-0 and *cpk5 cpk6 cpk11* seedlings were treated inside flasks with mock (H_2O) or OG (50 µg/ml) and sealed. Ethylene production was measured after 4 h by gas chromatography analysis. In inset the basal ethylene production was measured after 12 h in mock-treated seedlings. Results are average ±se (n = 7). Asterisks indicate statistically significant differences between mutant- and wild type-treated seedlings, according to Student's *t* test (**, P<0.01).

Whether jasmonic acid and salicylic acid production is also affected in the *cpk5 cpk6 cpk11* mutant was determined after treatment with OGs and flg22. As shown in Figure 15B, OGs- and flg22-treated Col-0 leaves displayed jasmonic acid levels about three times higher than those of the mock-treated seedlings; a similar elicitor-triggered production was observed in the mutant. In the wild type seedlings treated with OGs, SA levels were not increased above basal levels (see Figure 4E), whereas they increased upon flg22 treatment, in agreement with previous reports (Tsuda et al., 2008).

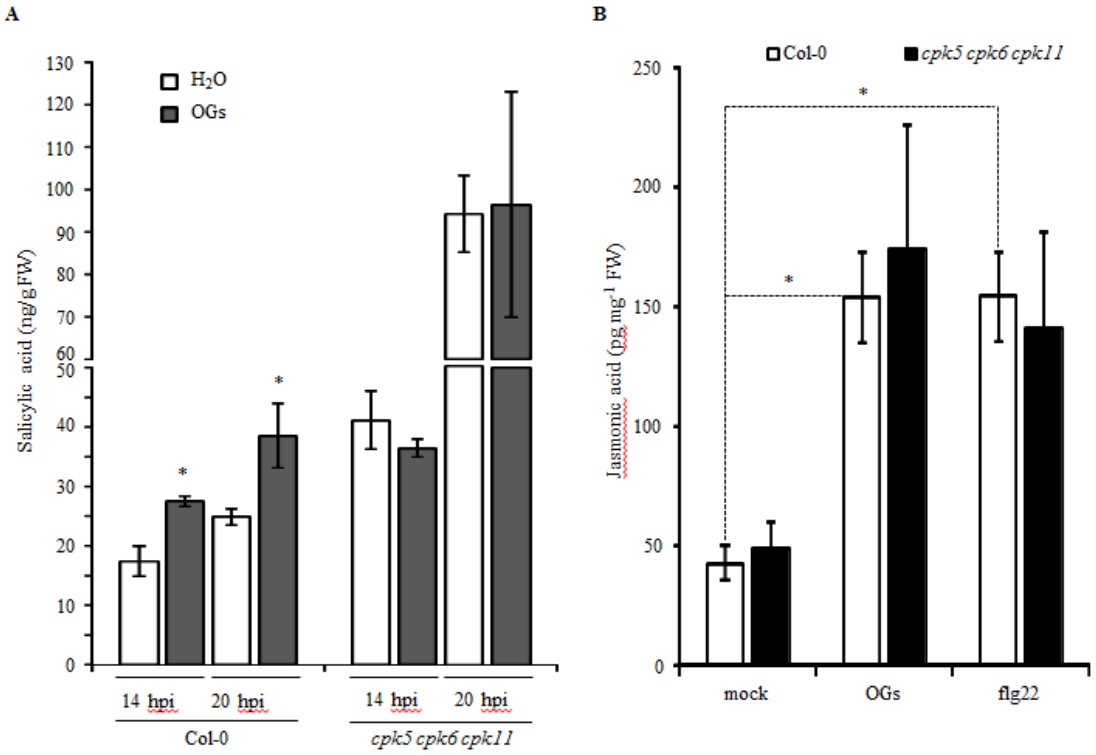

Figure 15. Analysis of SA production in response to Botrytis and JA production in response to OGs and flg22 in Col-0 and *cpk5 cpk6 cpk11* mutant. A) Four-week-old Col-0 and *cpk5 cpk6 cpk11* plants were sprayed with mock (water) or OGs (200 µg/ml) and 24 h after spray leaves were inoculated with B. *cinerea* spores ($5x10^5$ conidia/ml). The salicylic acid (expressed in ng/g fresh weight) was extracted at the indicated hour post infection (hpi) as described in Pan *et al.* 2010 (Pan et al., 2010c) and measured by HPLC-MS spectrometry. Results are average ±se (n = 4). Asterisks indicate statistically significant differences between mutant- and wild type-treated leaves, according to Student's *t* test (*, P<0.05). B) Four-week-old Col-0 and *cpk5 cpk6 cpk11* plants were sprayed with mock (water), OGs (200 µg/ml) or flg22 (1 µM). Jasmonic acid (expressed in ng/g fresh weight) was extracted after 24 h as described in Pan *et al.* 2010 (Pan et al., 2010b) and measured by HPLC-MS spectrometry. Results are average ±se (n = 3). Asterisks indicate statistically significant differences between mutant- and wild type-treated leaves, according to Student's *t* test (*, P<0.05).

Thus, a reduced ethylene production and a defective Et response likely explain the increased susceptibility of the mutant, and suggest that camalexin is not sufficient to protect against *B. cinerea* when ethylene signaling and accumulation are compromised.

III. 2. 8. OG-induced protection to Botrytis requires ethylene signaling

So far, my results suggest that, although the mutant is not affected in the priming response to OGs (see results on camalexin accumulation, Figure 7C), it exhibits a defective OG-induced protection against Botrytis.

The defect observed in OG-induced ethylene biosynthesis may account for its defective OG-induced immunity. However, previous analyses, performed using the *ein2-1* ethylene signaling mutant, suggested that OG-induced

protection is independent of ethylene (Ferrari et al., 2007). On the other hand, the *ein2-5* mutation leads to a defective flg22-induced protection against Botrytis (Laluk et al., 2011). While the *ein2-1* mutation might result in a partially functional EIN2, the *ein2-5* mutation is represented by a frame-shift in the predicted eighth transmembrane-spanning α-helix that would likely cause more disruption of function (Alonso et al., 1999). I therefore decided to reevaluate the contribution of ethylene signaling in OG-induced protection, using the *ein-2-5* mutant. Indeed I found that OG-induced protection is compromised in the *ein2-5* mutant (Figure 16). Thus the defect in ethylene production and signaling may provide an explanation to the altered OG-induced protection observed in the CPK mutant.

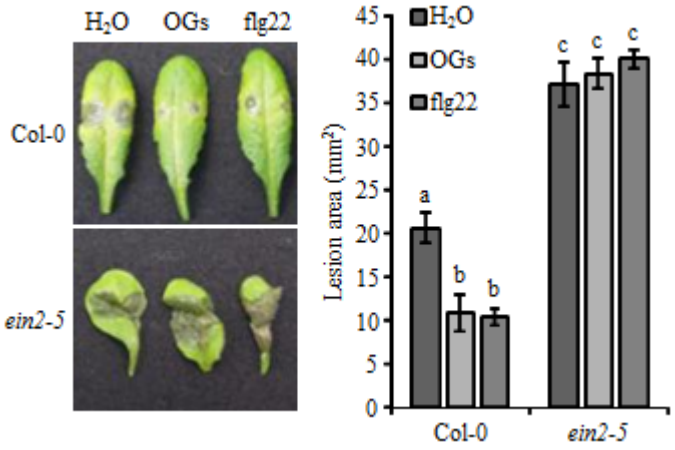

Figure 16. Elicitor-induced protection against *Botrytis cinerea* in Col-0 and *ein2-5* mutant. Four-week old plants were sprayed with OGs (200 µg/ml), flg22 (1 µM) or water; after 24 h, leaves were inoculated with *B. cinerea* spores ($5x10^5$ conidia/ml). A) Lesion areas were measured 48 h after inoculation (hpi). Results are average ±sd (n=12). Letters indicate P<0.01 by one-way ANOVA with Tukey's HSD test. This experiment has to be confirmed with one or more replicates.

III. 3. DISCUSSION

Plant ability to adapt to changes in the environment is the main trait that permits their survival. Indeed, plants, as sessile organisms, are incessantly exposed to adverse conditions, both abiotic and biotic. In particular, plants are continually exposed to microbes, some of which establish a symbiotic relationship (e.g. mycorrhizae and *Rhizobium*), whereas others are able to induce pathogenic effects by penetrating tissues surface directly or through wounds or natural openings such as stomata and pores used for gas exchange. Due to the absence of an adaptive immune system, plants rely on a so-called "innate immune system", that depends on efficient pathogen sensing and rapidly mounted defence responses, analogous to that found in animals (Nurnberger et al., 2004; Gomez-Gomez, 2004). The first line of defense against pathogen infections is the cell wall, a complex extracellular structure that plays important roles also in plant growth and development (Humphrey et al., 2007). In order to breach this polysaccharide rich network, phytopathogenic microorganisms synthetize and secrete in the infection site hydrolytic enzymes capable of degrading different cell wall components (Vorwerk et al., 2004). Once cell wall is overcome, pathogens must face plasma membrane, where a large arsenal of trans-membrane PRRs is ready to sense and recognize invaders, through direct binding PAMPs or DAMPs, and consequently to activate immune responses by promptly switching on different and parallel signal transduction pathways. Once a PRR is activated by ligand binding defense responses must be quickly activated in order to be effective against invasion attempts as soon as possible. Indeed plants are able to mount defense responses, i.e. oxidative burst, in a few minutes after elicitor sensing and this is possible because of signaling is performed through rapid post-translational modification(s), i.e. phosphorylation, of a large numbers of pre-synthetized signal transduction elements.
Most of the studies concerning plant defence–related signalling pathways focused on PAMP perception and transduction. Most known PRRs require the leucine-rich repeat receptor kinase BAK1 for function and/or its closest homolog BKK1(Chinchilla et al., 2009), which therefore act as central regulator of plant immunity. Consequently, they are the target of several pathogen virulence effector molecules (Shan et al., 2008). CDPKs are also required for PAMP signalling.
Because there is a large overlap between responses elicited by OGs and PAMPs (Asai et al., 2002; Denoux et al., 2008; Galletti et al., 2008; Galletti et al., 2011)and both type of elicitor lead to induced-protection against Botrytis, it is than reasonable to assume that PAMPs and OGs could share some elements in their signaling pathways.
In this part of the work, responses to OGs have been analyzed in loss-of-function mutants of CDPKs.

III. 3. 1. CDPK5/6/11 are required for both basal and OG-triggered PTI against *Botrytis cinerea*

PAMPs and DAMPs perception lead to defense responses activation which ultimately confer an induced resistance against further pathogen attacks (Zipfel et al., 2004; Kunze et al., 2004; Ferrari et al., 2007). This complex process requires both early, i.e. MAPK6 activation (Galletti et al., 2011), and late, i.e. camalexin production (Ferrari et al., 2007) responses. OGs, as well as PAMPs, have been shown to trigger a fast and transient elevation in cytosolic free Ca^{2+} in Arabidopsis (Moscatiello et al., 2006), which likely bound and activate transduction elements containing EF-hand domains. In the case of flg22-induced signal transduction, CDPK5/6/11 together with CDPK4 have been shown to be activated by a Ca^{2+}-dependent phosphorylation and to be required for PAMP-triggered responses such as oxidative burst, gene induction and induced protection against Pseudomonas (Boudsocq et al., 2010). Here I show that, when infected with the necrotrophic fungus *Botrytis cinerea*, the *cpk5/6/11* triple KO mutant display both an enhanced susceptibility and the lack of both OG- and flg22-induced protection. To understand the mechanism(s) affected by the

absence of CDPK5/6/11 that could explain this behavior, defense-related responses triggered by OGs and flg22, used as control, where analyzed on the triple *cpk5/6/11* KO mutant. Results obtained revealed that, in response to OGs, these CDPKs are dispensable for the oxidative burst and MAPK3 and MAPK6 activation, but are differentially required for the full induction of both early and late elicitor-induced genes. The lack of CDPK5/6/11 affects the basal expression of several defense-related genes such as *PDF1.1*, *PDF1.2*, *ORA59* and *PAD3*. In particular, *ORA59* have been demonstrated to be induced by Botrytis infection, to be required for basal resistance against this fungus and to regulate the expression of *PDF1.2* by directly binding its promoter (Pre et al., 2008). I found that, in Col-0 leaves and 12 h after Botrytis inoculation, the *ORA59* expression is highly induced while *PDF1.2* reaches, together with *PDF1.1*, its maximal induction later, i.e. after 24 h. *cpk5/6/11* triple mutant leaves lack the induction of *ORA59*, *PDF1.2* and *PDF1.1* in response to Botrytis. The defective basal expression and induction of these defense-related genes may lead to the enhanced susceptibility to *B. cinerea* observed in the triple mutant. A reduced basal expression was detected also for *PAD3*, the cytochrome CYP71B15 that catalyzes the last step of camalexin biosynthesis. It is known that the *pad3* loss-of function mutant plants lack both the basal and OG-induced resistance protection against *B. cinerea* (Ferrari et al., 2007). I therefore analyzed whether elicitor-induced expression of *PAD3* is affected in the *cpk5/6/11* triple mutant leaves. Levels of *PAD3* transcripts in H_2O-, OG- and flg22-sprayed leaves were lower in the mutant plants than in WT, suggesting that an impaired response to the elicitors likely causes the lack of protection against *B. cinerea*. Analysis of camalexin levels at 20 h post-inoculation, i.e. during the early phase of camalexin production (Ferrari et al., 2003), showed 1) a higher amount of camalexin in the WT OG-pretreated leaves compared to the water (mock) pre-treated leaves, and 2) unexpectedly, camalexin levels similar to those of the WT leaves in the triple mutant. These results reveal that pretreatment with OGs prompts ("primes") tissue to produce camalexin either more rapidly and/or at higher levels in response to *B. cinerea*, but that CDPK5/6/11 are not required for this priming effect. Furthermore, camalexin production is not affected in the triple mutant indicating that 1) the lower expression of *PAD3* is likely sufficient and 2) it is necessary but not sufficient for bot basal resistance and elicitor-induced protection against Botrytis.

I show in this thesis that lack of the CDPK5/6/11 expression affects responses to ethylene such as gene induction and root growth inhibition. Ethylene have also well documented roles in plant immunity and, indeed, plants with abolished synthesis or signaling of this hormone are more susceptible especially when challenged with necrotrophic pathogen, i.e. Botrytis (Thomma et al., 1999a; Ferrari et al., 2003; Ren et al., 2008; Galletti et al., 2011; Laluk et al., 2011; Akagi et al., 2011). Thus, diminished ethylene-dependent immune responses may contribute to the enhanced susceptibility to Botrytis observed in the triple mutant. Moreover, it may also influence the basal expression of the defense-related genes. In a recent paper it was demonstrated that another null mutant for the expression of BIK1, a very important signaling molecule (Zhang et al., 2010; Lu et al., 2010), displays 1) low sensibility to ethylene, 2) diminished defense-related genes basal expression, 3) enhanced susceptibility to Botrytis and, importantly, 4) lack of flg22-induced PTI against this fungus (Veronese et al., 2006; Laluk et al., 2011). The requirement of ethylene signaling in OG-triggered resistance against Botrytis was so far excluded because the *ein2-1* mutant displays a wild type similar behavior in response to the elicitor although it enhances susceptibility (Ferrari et al., 2007). However, some differences, also regarding defense responses, were documented among the two allelic mutants *ein2-1* and *ein2-5* (Alonso et al., 1999). In order to definitively exclude a possible involvement of ethylene in OG-induced resistance, I tested the *ein2-5* mutant. This mutant was not protected by OG pretreatment. This result indicates that ethylene signaling is required for OG-induced-PTI as in the case of PAMPs. Furthermore the *cpk5/6/11* triple mutant, in response to OGs, accumulates a significant lower amount of ethylene indicating that, beside displaying a defective ethylene signaling, it lacks also

ethylene synthesis in response to this DAMP, suggesting once more the importance of this hormone in the OG-related PTI.

PART IV - ROLE OF SERKs IN OG SIGNALING

IV. 1. INTRODUCTION

IV. 1. 1. The receptor-like kinase BAK1/SERK3 is a central regulator of plant development and immunity

The Arabidopsis genome encode over 600 receptor-like kinases (RLKs) (Kaul et al., 2000; Shiu and Bleecker, 2001), of which over a third contain leucine-rich repeats (LRRs) in the extracellular domain and indeed are referred as LRR-LRKs (Shiu and Bleecker, 2001). These are further categorized into 13 subfamilies based on the copy number and arrangement of the LRR motifs (Shiu and Bleecker, 2001). The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family consists of five LRR-RLKs belonging to subgroup II (Hecht et al., 2001) that contain five LRRs in their extracellular domain and display similarity to the previously described DcSERK protein that marks embryogenic competence in carrot (*Daucus carota*) tissue cultures (Schmidt et al., 1997). The main feature distinguishing SERK proteins from other RLKs is the proline-rich domain, containing two tandemly repeats of the Ser-Pro-Pro (SPP) motif, located between the LRRs and the trans membrane domain. After identifying the first member, the presence of the SPP domain together with precisely five LRRs was used as a criterion for the identification of the four other SERK genes (SERK2–SERK5) among the numerous LRR-RLK encoding genes in the Arabidopsis database (Hecht et al., 2001). Sequence analysis of the different SERK proteins indicates that they arose through gene duplication events that generated two ancestral precursors, SERK1-SERK2 and SERK3-SERK4-SERK5. Those precursors further duplicated and mutated to generate the five current SERK members (Hecht et al., 2001; He et al., 2007).

In Arabidopsis, the SERK family is involved in several independent pathways, including BR responses, male sporogenesis, immunity and cell death control (Figure 1).

A yeast two-hybrid screen and a genetic screen for suppressors of a weak *bri1* phenotype revealed that SERK3, also known as BRASSINOSTEROID INSENSITIVE1 (BRI1)-ASSOCIATED KINASE1 (BAK1), is a co-receptor of BRI1 (Li and Nam, 2002; Nam and Li, 2002). It has also been shown that BRI1 forms heterodimers with BAK1 in living cells (Russinovaa et al., 2004) and that the interaction is dependent on the presence of brassinosteroids (BRs) (Wang et al., 2005). Loss of BAK1 causes a semi-dwarfed phenotype and a reduced sensitivity to brassinosteroids (Li et al., 2002). Besides BAK1, two other members of the SERK family, SERK1 and SERK4, also known as BAK1-LIKE1 (BKK1), have also been reported to be involved in BR signaling (Karlova et al., 2006; He et al., 2007; Albrecht et al., 2008). Interestingly, double *bak1 bkk1* mutants exhibit a seedling-lethality phenotype due to constitutive defense-gene expression, callose deposition, reactive oxygen species (ROS) accumulation, and spontaneous cell death (Chinchilla et al., 2007; He et al., 2007). A null mutant of a novel RLK gene showed constitutive defense response, cell death, and seedling lethality phenotypes similar to that of bak1 bkk1 double mutant. Using a co-immunoprecipitation approach followed by a proteomic analysis, it was found that this RLK interacts with BAK1 *in vivo*. The RLK was therefore named as BAK1-interacting receptor-like kinase 1 (BIR1) (Gao et al., 2009). These data demonstrate that BAK1 and BKK1 have dual physiological roles: positively regulating a BR-dependent plant growth pathway, and negatively regulating a BR-independent cell-death pathway.

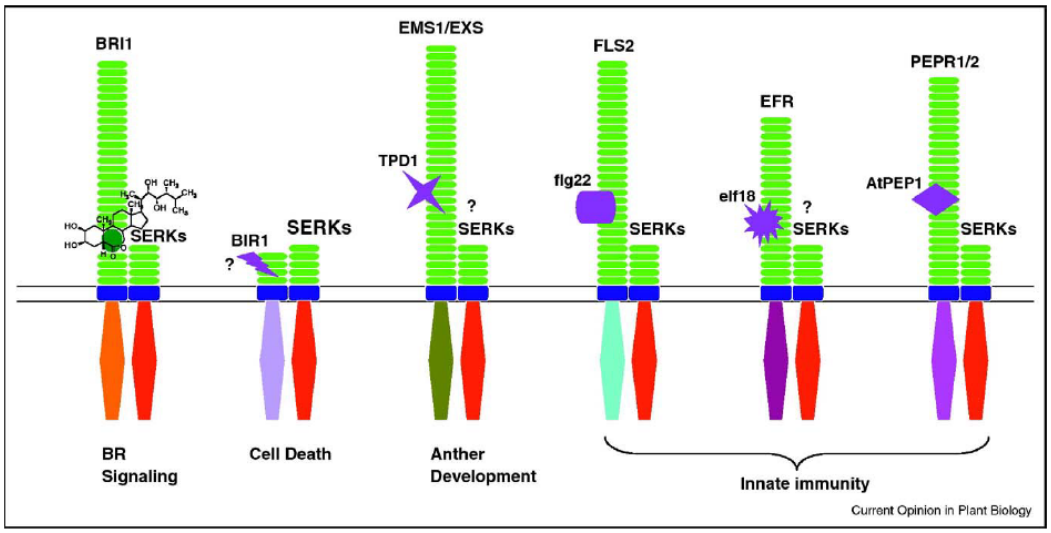

Figure 1. SERKs interact with multiple ligand-binding LRR-RLKs and control multiple developmental and defense-related signaling pathways.

Several studies have highlighted the role of BAK1 in plant immunity. The loss of BAK1 causes, independently of BR signaling, bacterial infection-induced cell death and enhanced susceptibility to infection by necrotrophic fungi, including *Alternaria brassicicola* and *Botrytis cinerea*, as well as biotrophic pathogens, such as *P. syringae* (Kemmerling et al., 2007; Heese et al., 2007). Moreover, in *A. thaliana* it was demonstrated that BAK1 rapidly interacts with FLS2, in a ligand dependent manner (Chinchilla et al., 2007; Heese et al., 2007). However, BAK1 is not involved in flg22 binding (Chinchilla et al., 2007). Coherently, in *A. thaliana* the loss of BAK1 function affects diverse early and late flg22-triggered responses, including, ROS production, MAPK activation, defense-gene induction and growth inhibition (Chinchilla et al., 2007). It was further shown that BAK1 kinase activity is required for flg22-inducible plant responses, and that BAK1 likely phosphorylate itself as well as FLS2 within 15 s upon flg22 treatment (Schulze et al., 2010; Schwessinger et al., 2011). A model of ligand-induced interaction between FLS2 and BAK1 is shown in Figure 2. Phosphorylation by BAK1 of U-Box E3-ubiquitin ligases PUB12 and PUB13 activates proteasome-dependent degradation of FLS2, suggesting that BAK1 may not only be involved in pattern signaling, but may also determine temporary desensitization of the system and PRR turnover (Lu et al., 2011).

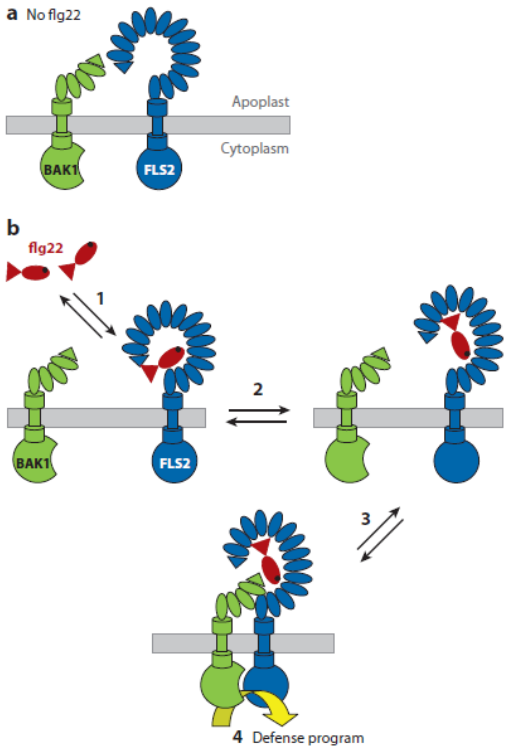

Figure 7. Model for the ligand-induced interaction between FLAGELLIN-SENSING 2 (FLS2) and BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1). (*a*) FLS2 and BAK1 do not interact in the absence of flg22. (*b*) Upon binding of flg22, FLS2 changes its conformation, allowing protein-protein interaction between the extracellular domains of FLS2 and BAK1. This interaction brings the intracellular protein kinase domains of FLS2 and BAK1 in close proximity and initiates signaling, e.g., by transphosphorylation. Adapted from (Boller and Felix, 2009).

Besides flagellin, analyses of responses to more individual PAMPs in isolated leaf cells and in whole seedlings supported the important role of BAK1 in elf18, HrpZ, peptidoglycan (PGN), and lipopolysaccharide (LPS) but not in chitin or necrosis-inducing *Phytophthora* protein 1 (NPP1) signaling (Chinchilla et al., 2007; Shan et al., 2008). Likewise, in *Nicotiana benthamiana* virus-induced gene silencing of the BAK1 homolog affects responses to flg22, the CSP22 peptide derived from bacterial cold-shock protein, and the oomycete elicitor INF1 but not to chitin (Heese et al., 2007). These results suggest that BAK1 functions in many but not all PAMP-signaling responses. Besides with FLS2, BAK1 has been shown to interact physically with the EF-Tu receptor EFR and the AtPeps receptors PEPR1 and PEPR2 (see next section), in both cases in a ligand (elf26 and AtPep1, respectively)-dependent manner (Postel et al., 2010; Schulze et al., 2010; Schwessinger et al., 2011). Furthermore, BAK1 and EFR or PEPR1 are phosphorylated *in vivo* in response to elf26 or AtPep1 signals, respectively (Schulze et al., 2010). In line with these findings, elf18- as well as AtPep1- and AtPep2-inducible responses are reduced in *bak1* genotypes (Chinchilla et al., 2007; Krol et al., 2010). These results indicate that BAK1 mediates signaling not only to PAMPs but also to (peptide) DAMPs signaling. It has to be noted that in the case of responses mediated by BRI1, FLS2, EFR and PEPRs, the *bak1* null mutant allele only displays a subtle phenotype as compared to null mutant alleles of these main receptor/s (Li and Nam, 2002; Nam and Li, 2002; Chinchilla et al., 2007; Krol et al., 2010). These studies suggest functional redundancy with other members of the SERK family.

Recently, a novel mutant allele of *BAK1 (bak1-5*, obtained by chemical mutagenesis*)* has been described that is strongly impaired in PTI signaling but, unlike the *bak1* insertional mutant previously characterized (Li and Nam, 2002; Nam and

Li, 2002) displays a wild-type-like BR signaling capacity (Schwessinger et al., 2011). The *bak1-5* carries a single missense mutation in the 10th exon of *BAK1* that leads to a C408Y change in the subdomain VIa of the cytoplasmic kinase preceding the catalytic loop. Interestingly, *bak1-5* is a semi-dominant allele of *BAK1* regarding PAMP responsiveness, since *bak1-5* x Col-0 heterozygous F1 plants showed an intermediate phenotype between wild type Col-0 and *bak1-5*, both in the PAMP-triggered seedling growth inhibition and ROS production (Schwessinger et al., 2011). Unlike the *bak1 bkk1* double KO mutant (He et al., 2007), *bak1-5* does not display uncontrolled cell death and post-embryonic lethality when combined with the null *bkk1* allele (Schwessinger et al., 2011). Recently, it has been shown that, in contrast to the RD kinase BRI1, the non-RD kinases FLS2 and EFR have very low kinase activity, and neither of them is able to trans-phosphorylate BAK1 *in vitro*. Furthermore, kinase activity for all partners is completely dispensable for the ligand-induced heteromerization of FLS2 or EFR with BAK1 *in planta*, revealing another pathway specific mechanistic difference. The specific suppression of FLS2- and EFR-dependent signaling in *bak1-5* is not due to a differential interaction of BAK1-5 with the respective ligand-binding RK but requires BAK1-5 kinase activity (Schwessinger et al., 2011). These results demonstrate that plant growth, innate immunity, and cell death are differentially regulated through phosphorylation by BAK1. This may reflect important differences in the molecular mechanisms underlying the regulation of ligand-binding RD and non-RD RKs.

The lack of both BAK1 and BKK1 elements totally compromises the responses to flg22 and elf18 as well as to the peptide DAMP *At*Pep1 (Roux et al., 2011), indicating that BAK1 and BKK1 cooperate redundantly to regulate multiple PRR-mediated signaling pathways. Other members of the SERK family, i.e. SERK1 and SERK2, play a redundant and essential function in tapetum specification and pollen development during male sporogenesis in Arabidopsis, independently of BR signaling (Albrecht et al., 2005; Colcombet et al., 2005; Albrecht et al., 2008). These observations show that BAK1 and its paralogs function as co-receptors not only in brassinolide signaling and development, but also in PAMP signaling and innate immunity.

IV. 1. 2. Possible involvement of the SERK element in OG signaling

The receptor-like kinase BAK1/SERK3, henceforth indicated as BAK1, belongs to the SERK family and is a central regulator of innate immunity in plants (Heese et al., 2007), being essential, likely as a co-receptor, for signal transduction of many PAMPs such as flg22, elf18, HrpZ, peptidoglycan (PGN), and lipopolysaccharide (LPS) (Shan et al., 2008). However, BAK1 is not involved in response to chitin or NPP1 (Shan et al., 2008). Accordingly, the *bak1-4* KO mutant (salk_116202) is dramatically altered in its response to flg22 and elf18 treatments (Chinchilla et al., 2007). Co-immunoprecipitation analyses showed that the closest BAK1 homologs, BKK1/SERK4 (henceforth indicated as BKK1) and SERK5 provide partially overlapping activity in MAMP- and BR-signaling (He et al., 2007; Shan et al., 2008), indicating a functional redundancy. The *bak1-4 bkk1-1* double mutant was shown to be seedling lethal, with the two elements play independent roles in BR signaling and the suppression of cell death (He et al., 2007). Unlike *bak1-4 bkk1-1* mutant, *bak1-5* is not impaired in BL responses and does not display uncontrolled cell death when combined with the null *bkk1-1* allele (Schwessinger et al., 2011). Moreover, the lack of both these SERK elements totally compromised the responses to flg22 and elf18 as well as to the damage-associated molecular pattern *At*Pep1 (Roux et al., 2011), indicating that BAK1 and BKK1 cooperate genetically to regulate multiple PRR-mediated signaling pathways.

In this part of the work, the role of BAK1 and BKK1 in OG signaling was investigated, by analyzing responses to OGs in the *bak1-5* and *bkk1* single mutants as well as in the *bak1-5 bkk1-1* double mutant.

IV. 2. RESULTS

III. 2. 1. The *bak1-5* single mutant is not affected in the OG-induced defense responses

An early response to PAMP and DAMP signals is the activation of MAPK cascades. In Arabidopsis, upon stimulation with flg22, a transient phosphorylation of the single kinases AtMPK3, AtMPK6 and AtMPK4 is observed, with a lag phase of ~1–2 min and peaking after 10–15 min (Asai et al., 2002; Bethke et al., 2009). In my experiments, Col-0 wild type and the *bak1-5* mutant were treated with OGs or flg22 (as a control), for 15 minutes. AtMPK3, AtMPK6 and AtMPK4 phosphorylated forms were detected by Western blot analysis using a commercial antibody generated against the human homologs of these MAPKs (α-p44/p42). While *bak1-5* showed a reduced phosphorylation of all three MAPKs in response to flg22 compared to the wild type, it exhibited normal MAP kinases activation in response to OGs (Figure 1).

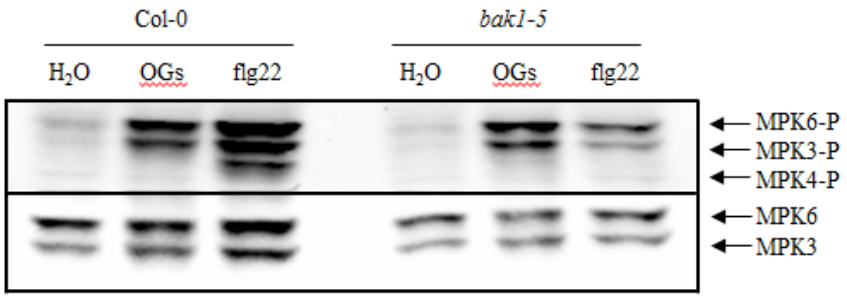

Figure 1. MAP kinases activation in the Arabidopsis *bak1-5* mutant upon treatment with OGs or flg22. Col-0 and *bak1-5* 10-day-old seedlings were treated for 15 min with H_2O, OGs (100 µg/ml) or flg22 (10 nM). Total protein extracts (30 µg) were analyzed by Western blot using α-p44/p42 as a primary antibody (top panel). The identity of individual MAP kinases as determined by size is indicated by arrows. The immunoblot was stripped and probed with a mix of α-MPK3 and α-MPK6 antibodies detecting native MAP kinases to determine equal loading (bottom panel).

The oxidative burst production, mediated by membrane-linked NADPH oxidases (i.e. RbohD), is one of the very early defense related responses triggered by pathogen attack and elicitor sensing (Lamb and Dixon, 1997). The H_2O_2 production upon OG or flg22 treatments was therefore quantified both in in leaf disks obtained from Col-0 and *bak1-5* adult plants and in seedlings. Results showed that OG-triggered H_2O_2 production was not reduced in the *bak1-5* mutant compared to wild type, both in seedlings (Figure 2A) and in adult plants (Figure 2B), indicating that BAK1 does not play a major role in the oxidative burst triggered by OGs. Instead, flg22-induced H_2O_2 production was affected in the *bak1-5* mutant, as expected.

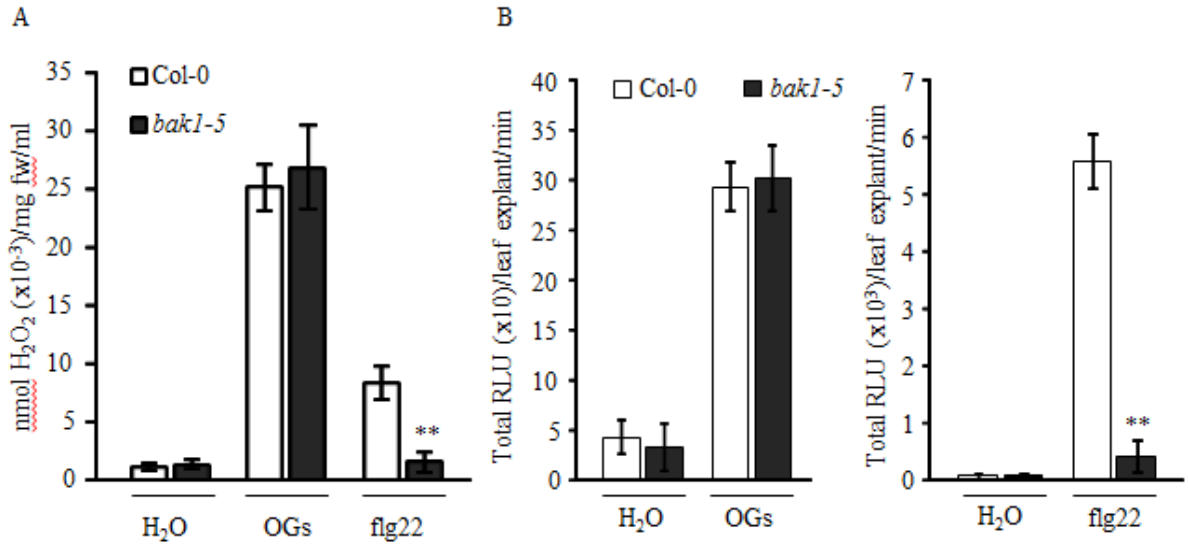

Figure 2. Accumulation of extracellular H_2O_2 in the Arabidopsis *bak1-5* mutant upon treatment with OGs or flg22. A) Col-0 and *bak1-5* 14-day-old seedlings were treated for 30 min with H_2O, OGs (100 µg/ml) or flg22 (1 µM). The H_2O_2 concentration in the incubation medium, expressed as nanomolar H_2O_2/mg fresh weight (fw) of seedlings/ml, was determined by a xylenol orange based assay. Results are average ±sd (n=4). Data are from one of two independent experiments that gave similar results. B) Total ROS production over a period of 30 min represented as relative light units (RLUs) in 4-wk-old Col-0 and *bak1-5* leaf discs after elicitation with 200 µg/ml OG or 1 µM flg22. Results are average ±sd (n=12). Data are from one of two independent experiments that gave similar results.

Induction of *WRKY33*, *RET-OX*, *CYP81F2*, *FRK1* (after 1 h of treatment) and *PAD3*, *PGIP1* (after 3 h of treatment) defense-related genes was also analyzed in the *bak1-5* mutant. In agreement with what observed with kinase activation and ROS production, the *bak1-5* mutant showed a normal response to OGs, and a defective one to flg22, also in terms of defense genes induction (Figure 3).

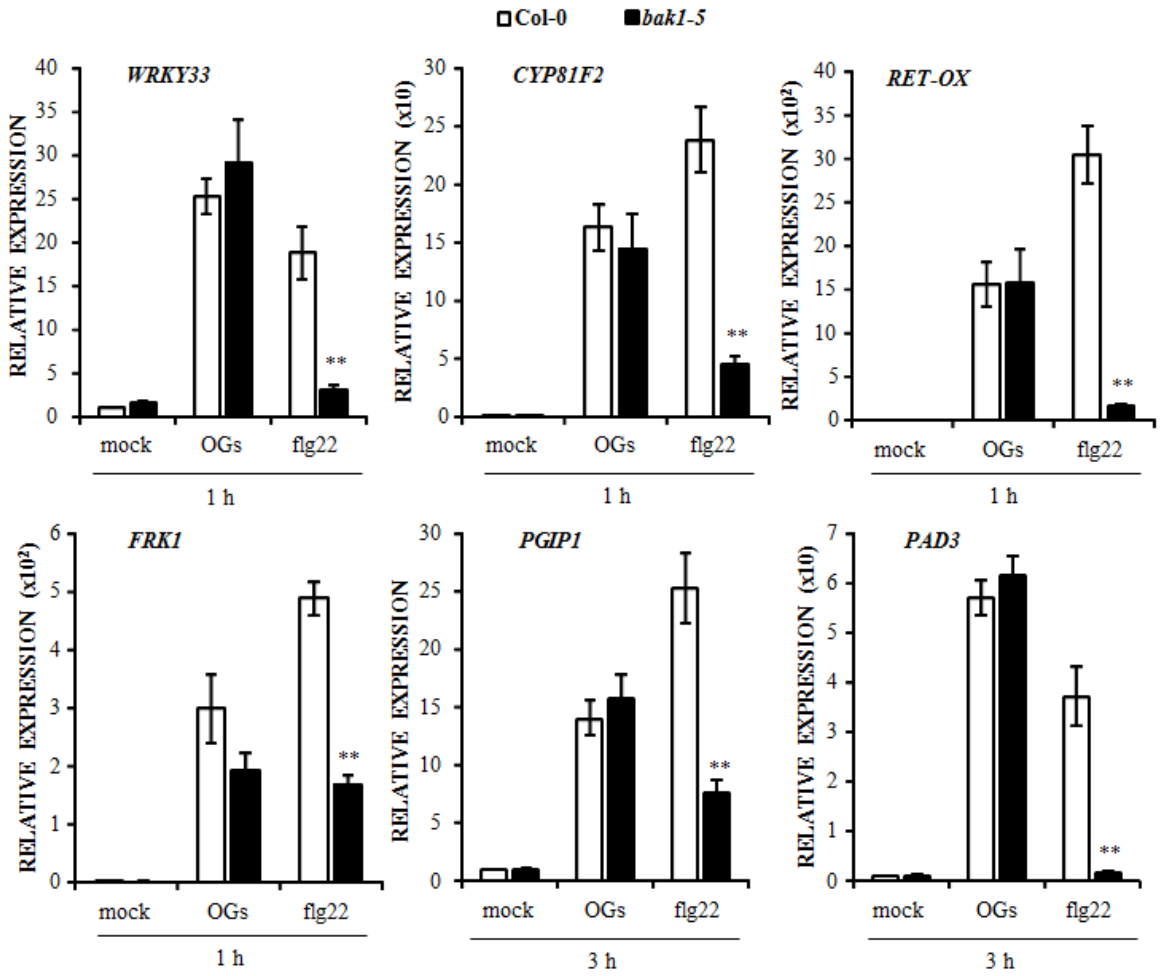

Figure 3. Analysis of defense-related marker genes induction upon treatment with OGs or flg22 in Col-0 and *bak1-5* mutant. Defense genes induction in response to mock (H_2O), 100 µg/ml OGs or 10 nM flg22 of 10-day-old Col-0 and *bak1-5* seedlings. Gene expression of *WRKY33*, *CYP81F2*, *RET-OX*, *FRK1*, *PGIP1* and *PAD3* was measured at the indicated times by quantitative PCR analysis, normalized to *UBQ5* (reference gene) expression, and plotted relative to Col-0 mock treatment expression level. Results are average ±sd (n=3). Asterisks indicate statistically significant differences between mutant- and wild type-treated plants, according to Student's *t* test (**, P<0.01).

Protection against *Botrytis cinerea* induced by OGs or flg22 was also analyzed in Col-0 and *bak1-5* mutant line. The *bak1-5* mutant has been described to be more susceptible to *B. cinerea* infection (Zhang et al., 2013). I have confirmed this results and also observed that OGs, but not flg22, induced protection against fungal infection in the mutant line (Figure 4). Taken together, these data demonstrate that BAK1 is dispensable for the OG responses in Arabidopsis.

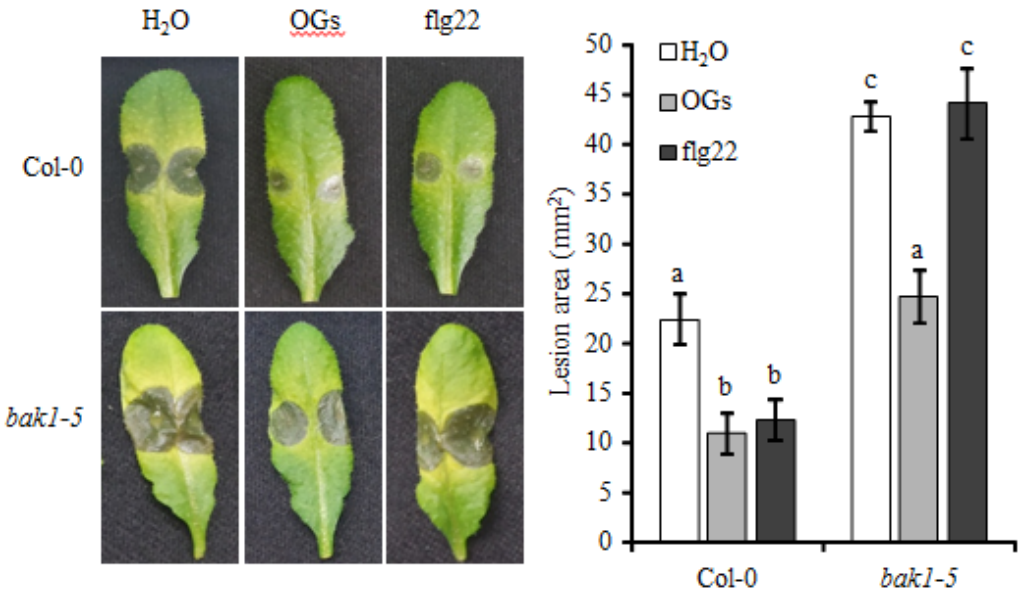

Figure 4. Elicitor-induced protection against *Botrytis cinerea* in Col-0 and *bak1-5* mutant. Four-week old plants were sprayed with OGs (200 µg/ml), flg22 (1 µM) or water; after 24 h, leaves were inoculated with B. *cinerea* spores ($5x10^5$ conidia/ml). Lesion areas were measured 48 h after inoculation (hpi). Results are average ±sd (n=12). Letters indicate P<0.01 by one-way ANOVA with Tukey's HSD test. Data are from one of two independent experiments that gave similar results.

IV. 2. 2. The *serk4*/*bkk1-1* mutant is not affected in the immunity triggered by OGs

I next analyzed whether SERK4 is required for response to OGs. Seedlings of the *serk4* (*bkk1-1*) loss-of-function mutant were treated with OGs or flg22 for 15 minutes and analyzed for MAPK activation. When treated with OGs or flg22, no difference could be observed in response to both elicitors, between the mutant and wild type, in the phosphorylation of AtMPK3 and AtMPK6 (Figure 5A). After OG or flg22 treatment, also the H_2O_2 production, measured in leaf discs of adult plants, was not affected in the *bkk1-1* mutant compared to wild type (Figure 5B). In agreement with these observations, induction of the defense genes *CYP81F2*, *RET-OX*, *FRK1* (after 1 h of treatment) and *PAD3* (after 3 h of treatment) was not affected in the *bkk1-1* mutant line following elicitation with OGs or flg22 (Figure 5C). These results rule out a major role of SERK4 in OG signaling; however, they do not exclude a possible redundant role of BAK1 and BKK1.

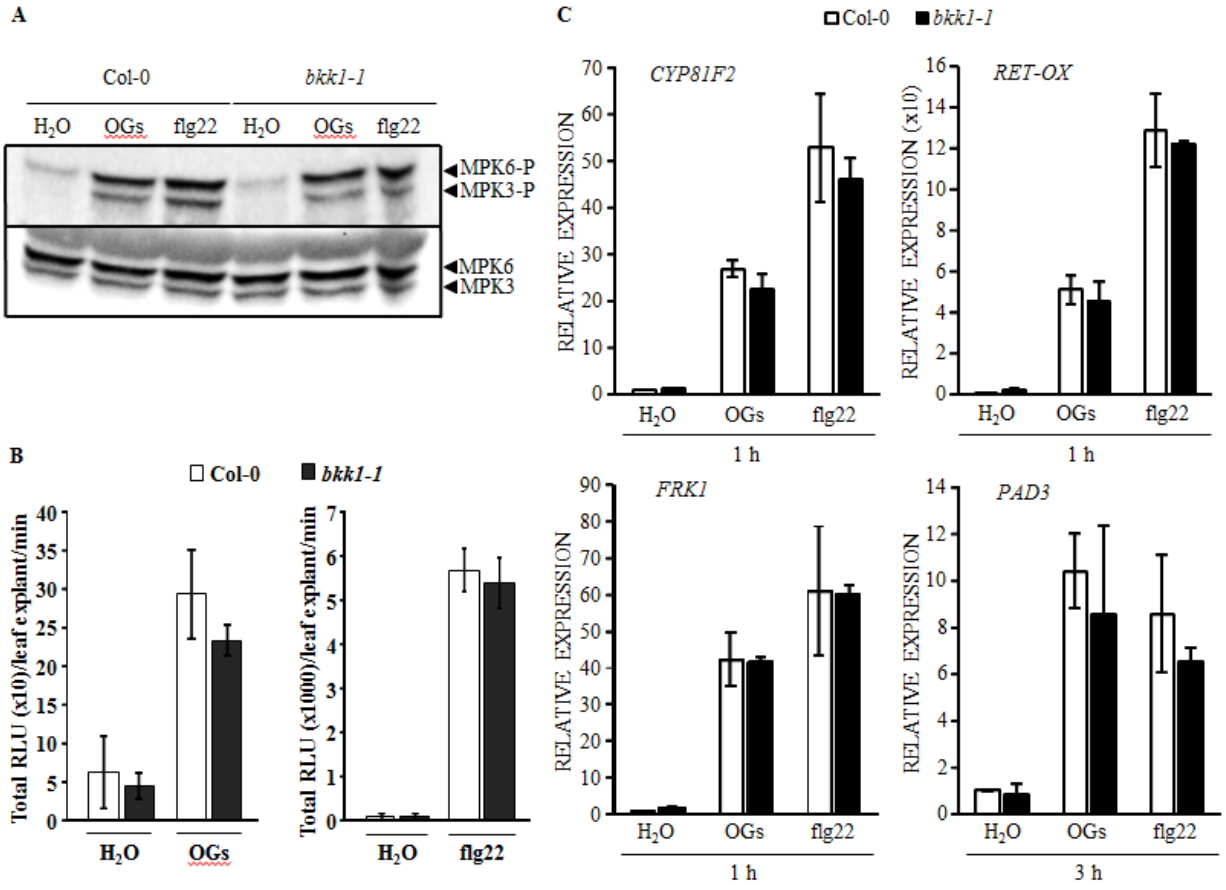

Figure 5. Elicitor-triggerd defense responses in *bkk1-1*. A) MAP kinases activation in ten-day-old Col-0 and *bkk1-1* seedlings upon 15 minutes of treatment with OGs (100 µg/ml), flg22 (10 nM) or water. 30 µg of total protein extracts were analyzed by Western blot using as primary antibody α-p44/p42 (top panel). The identity of individual MAP kinases as determined by size is indicated by arrows. The immunoblot was stripped and probed with a mix of α-MPK3 and α-MPK6 antibodies detecting native MAP kinases to determine equal loading (bottom panel). The experiment was repeated two times with similar results. B) Total ROS production over a period of 40 min represented as relative light units (RLUs) in 4-week-old Col-0 and *bkk1-1* leaf discs after elicitation with 200 µg/ml OG or 1 µM flg22. Results are average ±sd (n=12). Data are from one of two independent experiments that gave similar results. C) Defense genes induction in response to mock (H_2O), 100 µg/ml OGs or 10 nM flg22 of 10-day-old Col-0 and *bkk1-1* seedlings. Gene expression of *CYP81F2*, *RET-OX*, *FRK1* and *PAD3* was measured at the indicated times by quantitative PCR analysis, normalized to *UBQ5* (reference gene) expression, and plotted relative to Col-0 mock treatment expression level. Results are average ±sd (n=3).

IV. 2. 3. BAK1/SERK3 and BKK1/SERK4 act redundantly in OG-triggered ROS production, induction of defense gene expression, ethylene biosynthesis and protection against *Botrytis cinerea*, but are dispensable for MPK3 and MPK6 activation

In order to test whether BAK1 and BKK1 play redundant role in the OG-triggered immunity, the *bak1-5 bkk1-1* double mutant was analyzed. First, the phosphorylation level of MAP kinases was evaluated in the double mutant seedlings treated for 15 min with mock (water), OGs or flg22. While *bak1-5 bkk1-1* showed a dramatic reduction in the phosphorylation of all three MAPKs in response to flg22 compared to the wild type, confirming literature data, it exhibited normal MAP kinases activation in response to OGs (Figure 6A). This result indicates that BAK1 and BKK1

are not indispensable for in the OG-induced phosphorylation of MPK3 and MPK6. Interestingly, OGs, unlike flg22, did not activate the MPK4 kinase, in these experimental conditions.

Next, the H_2O_2 production upon OG or flg22 treatments was quantified in leaf disks obtained from Col-0 and *bak1-5 bkk1-1* adult plants. Contrary to what was observed for MAPKs, a substantial defect in the ROS production induced by OGs was detected in the *bak1-5 bkk1-1* mutant compared to Col-0 wild type leaf disks (Figure 6B), indicating that BAK1 and BKK1 act redundantly for the OG-triggered H_2O_2 accumulation. Moreover, the double mutant completely lacked the flg22-triggered oxidative burst, confirming literature data.

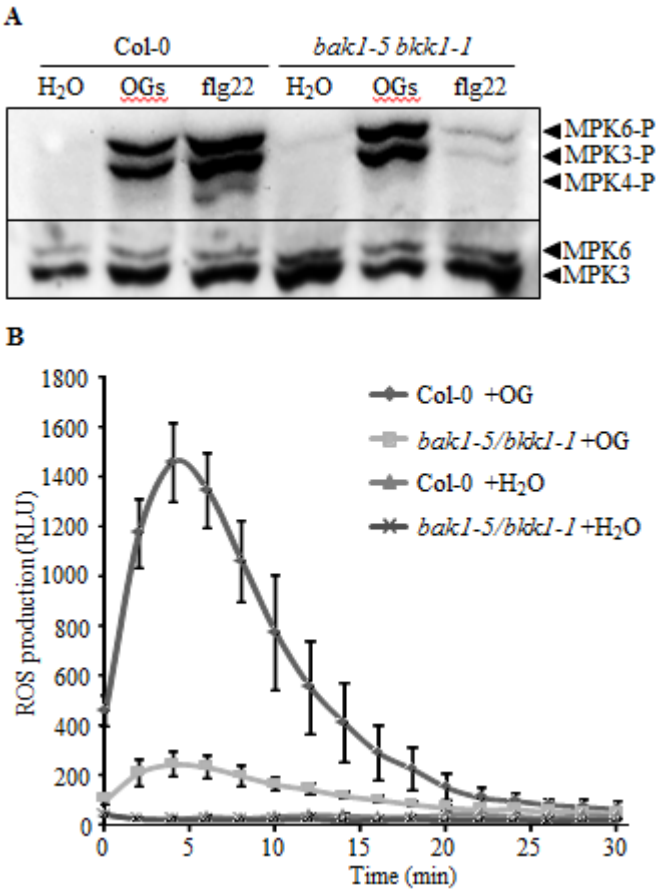

Figure 6. MAP kinases activation and ROS production in the Arabidopsis *bak1-5 bkk1-1* mutant upon treatment with OGs or flg22. A) Col-0 and *bak1-5 bkk1-1* 10-day-old seedlings were treated for 15 min with H_2O, OGs (100 µg/ml) or flg22 (10 nM). Total protein extracts (30 µg) were analyzed by Western blot using α-p44/p42 as a primary antibody (top panel). The identity of individual MAP kinases as determined by size is indicated by arrows. The immunoblot was stripped and probed with a mix of α-MPK3 and α-MPK6 antibodies detecting native MAP kinases to determine equal loading (bottom panel). The experiment was repeated twice with similar results. B) ROS production measured in relative light units (RLUs) in 4-wk-old Col-0 and *bak1-5 bkk1-1* leaf discs after elicitation with 200 µg/ml OG or water. Results are average ±sd (n = 12). Data are from one of four independent experiments that gave similar results.

To investigate whether BAK1 and BKK1 were involved in OG-induced early defense gene expression, transcript levels of some defense genes, including *PHI1*, *RET-OX*, *CYP81F2* and *FRK1*, known to be induced early after both OG and flg22 treatment (Denoux et al., 2008; Galletti et al., 2011), were analyzed. Seedlings were treated for 30 and 60 minutes with OGs or, for comparison, flg22. In response to flg22, the expression of all defense marker genes was largely or completely impaired in the *bak1-5 bkk1-1* mutant compared to Col-0 seedlings, as expected. Instead, in response to

OGs, only *FRK1* gene showed a significant defect of induction in the mutant compared to wild type, both after 30 min and after 60 min of treatment (Figure 7). Also the basal expression level of *FRK1* was affected in the mutant, while both basal expression and OG-triggered induction of the other defense genes were normal.

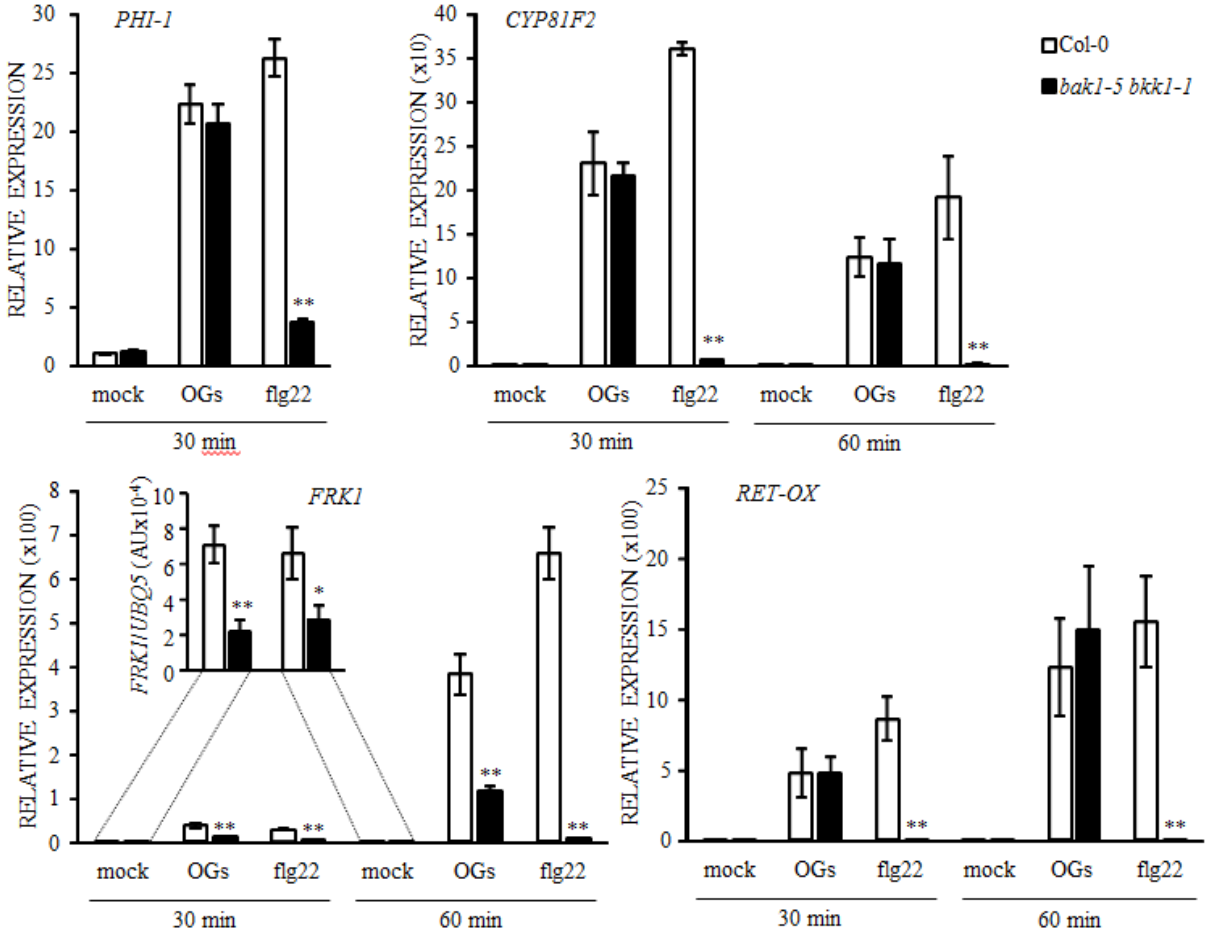

Figure 7. Analysis of defense-related marker genes induction upon treatment with OGs or flg22 in Col-0 and *bak1-5 bkk1-1* mutant. Defense genes induction in response to mock (H_2O), 50 µg/ml OGs or 10 nM flg22 of 10-day-old Col-0 and *bak1-5 bkk1-1* seedlings. Gene expression of *PHI1*, *CYP81F2*, *RET-OX* and *FRK1*, was measured at the indicated times by quantitative PCR analysis, normalized to *UBQ5* (reference gene) expression, and plotted relative to Col-0 mock treatment expression level. In inset, *FRK1* transcripts related to *UBQ5* are shown in mock-treated mutant and wild type seedlings. Results are average ±se (n=2). Asterisks indicate statistically significant differences between mutant- and wild type-treated seedling, according to Student's *t* test (*, P<0.05; **, P<0.01).

An increase in ethylene biosynthesis can be measured in seedlings within 5 h of treatment with flg22 or AtPeps (Bartels et al., 2013) or starting from 12 h of treatment with *B. cinerea* (Han et al., 2010). OGs are known to induce the expression of the ACS2, ACS6 and ACS7 genes in seedlings after one hour of treatment (Denoux et al., 2008), and the results shown in the previous section (paragraph III.2.7) show that OG treatment lead to the accumulation of ethylene. Ethylene production was therefore measured by gas chromatography also in *bak1-5 bkk1-1* as well as in Col-0 wild type seedlings, treated with water (mock), OGs or flg22 in sealed vials for 4 h. As the Figure 8A shows, OGs and flg22 triggered a comparable ET production in the wild type seedlings. In *bak1-5 bkk1-1* seedlings, ET levels produced in

response to both OGs and, as expected, flg22 were greatly lower than those in wild type, indicating that BAK1 and BKK1 are also required for the OG-induced ethylene accumulation. In these experimental conditions, n ethylene production was detected in the mock treatment.

Finally, elicitor-induced protection against *B. cinerea* was also assayed in this mutant. Adult *bak1-5 bkk1-1* and wild type plants were sprayed with OGs, flg22 or water, and excised leaves were drop-inoculated with *B. cinerea* conidia after 24 h. In water-pretreated leaves, the average of disease lesion diameter, measured 48 hours upon infections, was approximately 50% larger in mutant plants compared to WT, in agreement with the evidence that the *bak1-5* single mutant showed already a greater susceptibility (Zhang et al., 2013). Unlike the wild type, *bak1-5 bkk1-1* plants displayed neither OG- nor flg22-induced protection against Botrytis (Figure 8B), indicating that BAK1 and BKK1 are both necessary for the OG- and flg22-induced immunity against *B. cinerea*.

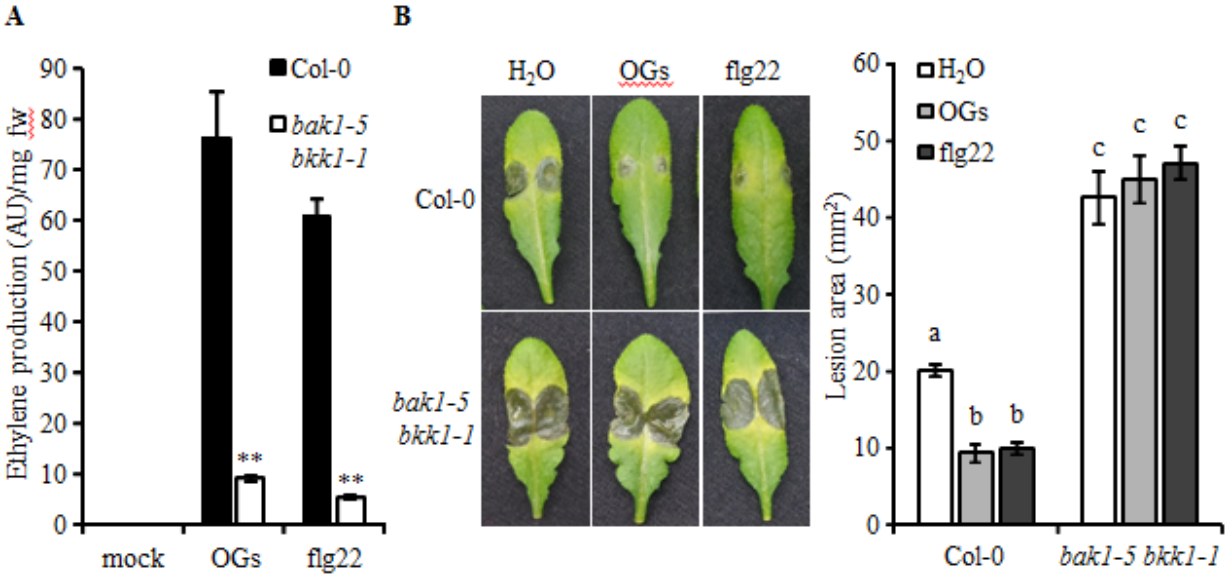

Figure 8. Elicitor-triggered defense responses in *bkk1-1*. A) Ethylene production in response to OGs or flg22. 14-day-old Col-0 and *bak1-5 bkk1-1* seedlings were treated with mock (water), OGs (50 µg/ml) or flg22 (1 µM) in vials and sealed for 4 h. Ethylene production expressed in arbitrary units (AU) was measured by gas chromatography. Results are average ±se (n=6). Asterisks indicate statistically significant differences between mutant- and wild type-treated seedlings, according to Student's *t* test (**, P<0.01). B) OG and flg22-induced protection against *Botrytis cinerea* in Col-0 and *bak1-5 bkk1-1* mutant. Four-week old plants were sprayed with OGs (200 µg/ml), flg22 (1 µM) or water; after 24 h, leaves were inoculated with B. *cinerea* spores ($5x10^5$ conidia/ml). Lesion areas were measured 48 h after inoculation (hpi). Results are average ±sd (n=12). Letters indicate P<0.01 by one-way ANOVA with Tukey's HSD test. Data are from one of two independent experiments that gave similar results.

IV. 3. DISCUSSION

Among the five members of the Arabidopsis SERK gene family, SERK3, better known as BAK1, functions in many (flg22, elf18, PGN, LPS) but not all MAMP-signaling responses. For instance, chitin and NPP1 signaling pathways are not affected by the lack of this co-receptor (Shan et al., 2008). Recent evidence indicates that the closest BAK1 homolog, BKK1 (SERK4), provides partially overlapping activity in both BR-dependent (He et al., 2007) and BR-independent signaling pathways (Albrecht et al., 2008), e.g. cell death control and, importantly, in the MAMP-signaling pathways (He et al., 2007; Roux et al., 2011). The *bak1 bkk1* double mutant is seedling lethal (He et al., 2007), while the double mutant *bak1-5bkk1* is not, likely because the EMS-induced mutation in *bak1-5* do not affect BR-dependent signaling pathways (Schwessinger et al., 2011). Analyses performed on the *bak1-5 bkk1-1* double mutant clearly indicate that these two proteins have redundant functions in flg22- and elf18-triggered signal transduction (Roux et al., 2011). Because nothing is known about the involvement of BAK1 and SERK3 in OG signaling, responses to OGs were analyzed in *bak1-5* and *bkk1-1* single mutants as well as in the *bak1-5 bkk1-1* double mutant. Results obtained on the single mutants indicate that the individual lack of BAK1 or BKK1 does not affect PTI induced by OGs Thus, it may be expected that either both together or neither of them may be involved in the OG signaling. These possibilities were tested by analyzing the *bak1-5 bkk1* double mutant. It was observed that ethylene production, the expression of *FRK1* and the oxidative burst induced in seedlings by OGs, were decreased. OG-induced resistance to Botrytis was also defective in adult double mutant plants. The results shown in the previous section indicate that ethylene is important for this last response (see paragraph III.2.8). ET, together with JA, is considered an essential hormone in the immunity against necrotrophic pathogens (Thomma et al., 1998; Glazebrook, 2005), and mutants impaired in the synthesis and signaling of this hormone are more susceptible to *Botrytis cinerea* (Thomma et al., 1999a; Ferrari et al., 2003; Ren et al., 2008; Galletti et al., 2011; Laluk et al., 2011; Akagi et al., 2011). A defect in ethylene production may therefore explain the defective protection of the *bak1-5* and *bkk1-1* double mutant.

Thus, my results show that BAK1 and BKK1 function redundantly in the activation of a part of the OG-triggered defense responses, and that OG responses that are affected by the loss of BAK1 and BKK1 are required for PTI against pathogens, i.e. Botrytis. Noteworthy, requirement of BAK1 and SERK4 in PAMP and OG signaling is different, because almost all flg22- and elf18- triggered responses are nearly abolished in *bak1-5 bkk1*, while only part of them is affected in the case of OGs, but also because PAMP-induced PTI relies mainly on BAK1 function, while dysfunction of either element is compensated by the other in OG signaling.

PART V - THE ROLE OF *At*PEP RECEPTORS IN OG SIGNALING

V. 1. INTRODUCTION

V. 1. 1. *Arabidopsis thaliana* peptides (*At*Peps): a second class of DAMPs

Recently a novel class of endogenous peptide elicitors that activate plant immunity has been identified from Arabidopsis (Yamaguchi et al., 2006; Huffaker et al., 2006; Bartels et al., 2013). The first peptide discovered, called *At*Pep1was isolated from leaves utilizing an elicitor-induced alkalinization activity assay in Arabidopsis suspension-cultured cells (Huffaker et al., 2006). *At*Pep1 is 23 amino acid (aa) long and derives from the C-terminus of a 92 aa precursor protein, *At*PROPEP1, hereon indicated as PROPEP1. It is believed that the PROPEP1 is cleaved to release the *At*Pep1, even though the enzymes involved in the proteolytic cleavage have not been identified yet. *PROPEP1* belongs to a gene family of eight members (Huffaker et al., 2006; Bartels et al., 2013). Seven paralogs, *PROPEP1-5, PROPEP7* and *8* are located on chromosome V, while *PROPEP6* is found on chromosome II (National Center of Biotechnology Information Arabidopsis genome database). All paralogous proteins are characterized by a low overall amino acid sequence identity, except for the C-terminus conserved *At*Pep motif SSG-x_2-G-x_2-N (Figure 1) (Huffaker et al., 2006; Bartels et al., 2013). The PROPEP full-length amino acid sequence is highly charged and lacks a signal sequence, indicating that it is not synthesized through the secretory pathway, but on cytoplasmic ribosomes, and exported to the extracellular space via an unconventional secretion system (Yamaguchi and Huffaker, 2011; Ding et al., 2012). Microarray data showed that *PROPEP2* and *PROPEP3*, and to a lesser extent *AtPROPEP1*, are strongly induced by treatments with pathogens, such as the fungus *B. cinerea*, the oomycete *P. infestans* and the bacterium *P. syringae*, and various PAMP and DAMP elicitors, including NPP1, HrpZ, flg22 and OGs (Craigon et al., 2004; Toufighi et al., 2005; Denoux et al., 2008), indicating a possible function of these three genes in plant immunity. Interestingly, only *At*Pep1 strongly induces the expression of *PROPEP1*, while *PROPEP2* and *PROPEP3* are strongly induced by all *At*peps. Instead, the other *PROPEP* genes do not seem to be induced by any of the AtPeps (Huffaker et al., 2006; Huffaker and Ryan, 2007), suggesting an amplification loop restricted only to *PROPEPs* induced by pathogens and their derived elicitors.

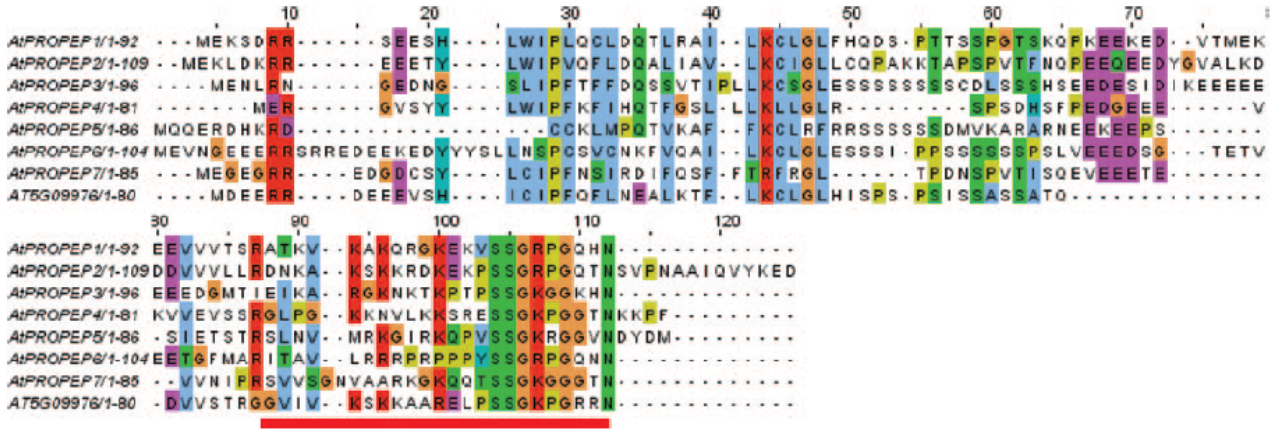

Figure 1. Alignment of the eight Arabidopsis *PROPEPs*. ClustalW alignment of the amino acid sequences of all identified Arabidopsis *PROPEPs* including AT05G09976. Colouring is based in the Clustal colour scheme. Adapted from (Bartels et al., 2013).

Of the eight Arabidopsis *At*Pep paralogs, also AtPep5 was biochemically isolated and shown to be active; the others were synthesized and their ability to activate alkalinization was confirmed (Yamaguchi et al., 2006; Huffaker et al., 2006; Bartels et al., 2013). All eight *At*Peps trigger a set of responses reminiscent of PTI, including induced resistance

against subsequent infections with virulent *Pseudomonas syringae* bacteria, revealing great functional redundancy (Huffaker et al., 2006; Huffaker and Ryan, 2007; Krol et al., 2010; Qi et al., 2010; Yamaguchi et al., 2010; Ma et al., 2012; Bartels et al., 2013; Tintor et al., 2013). In addition to the classical PTI-associated responses, recent data proved that treatment with *At*Pep1 led to an increase in cytosolic cGMP concentration, suggesting that *At*Peps activate cGMP-dependent signaling pathways (Ma et al., 2012).

On the other hand, bi-clustering analysis of *PROPEP*s based on 278 expression profiles of biotic stress treatments revealed that the transcriptional regulation of *PROPEP*s is most likely non-redundant and indicated an association of *PROPEP1*, *2*, and *3* with plant defense, whereas the other *PROPEP* genes appear to be differentially associated with processes ranging from abiotic stress resistance to development and reproduction (Bartels et al., 2013). Also tissue-specific differences and different subcellular patterns highlighted potentially non-redundant properties of the precursors. The analysis of *PROPEP* promoter sequences fused with β-glucuronidase (GUS) reporter gene revealed diverse spatial and temporal expression patterns, felling into two distinct groups. One group, inducible by wounding and comprising the promoters of *PROPEP1*, *2*, *3*, *5*, and *8*, showed expression in the roots, flowers and slightly in the leaf vasculature. The other group, containing the promoters of *PROPEP4* and *7*, was not inducible by wounding and the basal expression was restricted to the root tips (Bartels et al., 2013). Thus, *PROPEP1*, *2*, and *3* might play specific roles in the immune response of the root, which is supported by the report that constitutive expression of *PROPEP1* led to an induced resistance against the oomycete root pathogen *Pythium irregulare* (Huffaker et al., 2006). In addition, analysis of Arabidopsis plants constitutively expressing PROPEPs::YFP (yellow fluorescent protein) fusion proteins revealed a distinct localization of PROPEP proteins, with PROPEP3::YFP present in the cytosol; however, but, in contrast to previous predictions (Yamaguchi and Huffaker, 2011; Ding et al., 2012), PROPEP1::YFP and PROPEP6::YFP localized to the cytoplasmic side of the tonoplast, providing evidence for a potential role of PROPEP1 and 6 associated with the vacuole, unlike *At*Peps and PEPRs (Bartels et al., 2013).

V. 1. 2. Role of second messengers in *At*Peps-triggered immunity

PROPEP genes are differentially induced by ethylene, jasmonate and salicylic acid, in line with the non-redundant role of the precursor genes. *PROPEP1* is induced in response to ethephon (an ethylene precursor) (Huffaker et al., 2006). In response to JA, only *PROPEP1* and *PROPEP2* are highly expressed, with *PROPEP4* being moderately expressed. *PROPEP3*, *PROPEP5* and *PROPEP6* appear to be unaffected by JA treatment. In plants sprayed with SA, only *PROPEP2* and *PROPEP3* were expressed over basal levels (Huffaker et al., 2006; Huffaker and Ryan, 2007). *At*Peps treatment induced the expression of the Et- and JA-responsive gene *PDF1.2*, the SA-responsive gene *PR1* and ROS production (Huffaker et al., 2006; Huffaker and Ryan, 2007). The expression of *PDF1.2* in leaves is most strongly induced by *At*Pep1 and *At*Pep2, and the induction by each requires a functional JA/Et pathway and H_2O_2, since it is lost in *ein2-1*, a mutant incapable of perceiving ethylene (Guzman and Ecker, 1990), in *fad3 fad7 fad8*, a triple mutant incapable of synthetizing JA (McConn and Browse, 1996), and upon treatment with diphenylene iodonium (DPI, an inhibitor of NADPH oxidase). With the exception of *At*Pep4, the expression of *PR-1* is strongly induced by all *At*Pep peptides, and the induction by each requires a functional SA pathway and H_2O_2, since has been shown to be blocked in mutants including *npr1-1*, a SA signaling pathway mutant (Cao et al., 1994), and *sid2-2*, a SA biosynthetic mutant (Wildermuth et al., 2001b), as well as by DPI. Accordingly, plants overexpressing *AtPROPEP1* or *AtPROPEP2* show induced expression of *PDF1.2* and *PR1* and are more resistant to a root pathogen (*P. irregulare*) than the wild type

(Huffaker et al., 2006; Huffaker and Ryan, 2007). Besides phytohormones and H_2O_2, another important second messenger essential for *At*Peps signaling is calcium, since it was shown that the expression of *PDF1.2*, as well as *MPK3* and *WRKY33*, triggered by *At*Pep2 or *At*Pep3 is abolished in presence of a Ca^{2+} channel blocker (Gd^{3+}) (Qi et al., 2010).

The elicitor activity of *At*Peps is species-specific; however, *PROPEP* orthologs have been identified in numerous plant species of diverse families (Figure 2), suggesting that a similar defense mechanism may be found throughout the plant kingdom (Huffaker et al., 2006). For example, Pep1 seems to be conserved in both dicots and monocots, because *Zm*Pep1 has also been shown to regulate defense gene expression in maize (Huffaker et al., 2011).

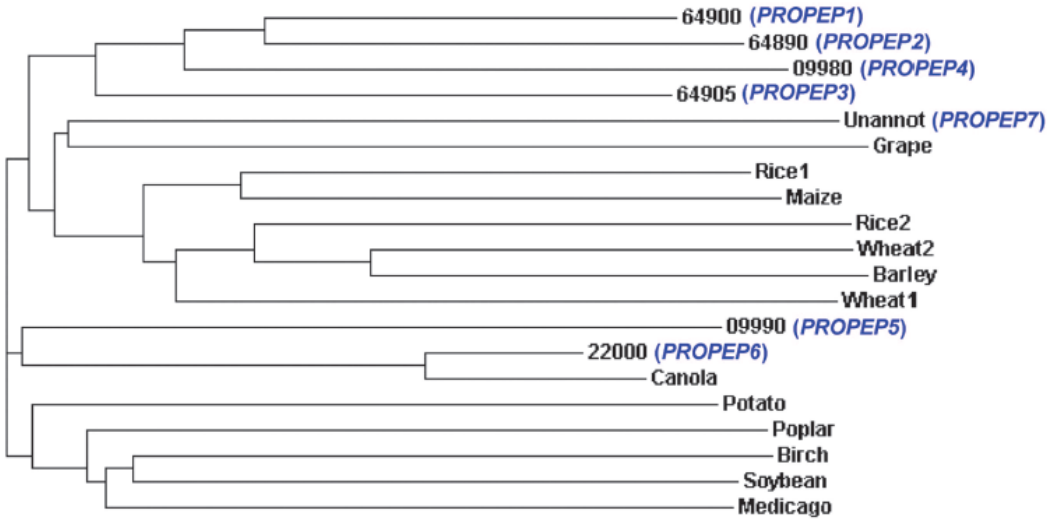

Figure 2. A cladogram showing the relationships of *PROPEP1* (At5g64900) paralogs and orthologs estimated from their amino acid identities and similarities. GenBank accession numbers are as follows: for dicot genes, canola (*Brassica napus*) CD816645; potato (*Solanum tuberosum*) CV505388; poplar (*Populus balsamifera*) CV23975; medicago (*Medicago sativa*) BI311441; soybean (*Glycine max*) CD401281; and grape (*Vitis vinifera*) CF604664; for monocot genes, rice1 (*Oryza sativa*) CF333408; rice2 AK111113, wheat1 (*Triticum aestivum*) AL809059; wheat2 BF201609, maize (*Zea mays*) DN215793; and barley (*Hordeum vulgare*) BQ763246. Adapted from (Huffaker et al., 2006).

The chemical and physiological properties of the *At*Pep1 family members, their precursor proteins, and their genes are strikingly similar to the properties of the 18-aa peptide signal systemin, its precursor pro-systemin, and its gene, which are components of the signaling pathway for defense against herbivorous pests of the Solanaceae family (Pearce and Ryan, 2003). Both *At*Pep1 and tomato systemin are cleaved from the C-terminus of precursor proteins that are induced by JA and lack leader peptides. Both precursors are small, highly positively charged proteins, and each produces peptides that activate defense genes. Tomato plants constitutively expressing *prosystemin* exhibit enhanced resistance toward an herbivore (Pearce and Ryan, 2003), whereas *Arabidopsis* plants constitutively expressing the *PROPEP1* are more resistant to a pathogen (Huffaker et al., 2006). The similarities mentioned above between systemin and *At*Pep1 support a hypothesis that the major role for receptor-mediated defense signaling peptides in plants is to amplify signaling that is initiated by wounding and elicitors to mount a rapid, strong defense against invaders (Pearce and Ryan, 2003; Schilmiller and Howe, 2005).

V. 1. 3. AtPep receptor 1 (AtPepR1) and AtPep receptor 2 (AtPepR2) mediate Arabidopsis peptides plant immunity

An *At*Pep1-binding protein, named *At*PepR1 (*At*Pep Receptor 1), was isolated from the surface of Arabidopsis suspension-cultured cells using radiolabelled *At*Pep1 peptide. *At*PepR1 is a 170 Kda LRR receptor-like kinase of subfamily XI (LRR-RLK XI), containing an extracellular domain with 26 Leucine-Rich Repeats (LRRs), a trans-membrane region and an intracellular protein kinase domain (Yamaguchi et al., 2006). The ectopic expression of *At*PepR1 in tobacco suspension-cultured cells caused the alkalinization of the cell medium after treatments with *At*Pep1 (Yamaguchi et al., 2006). All of the synthetic *At*Pep peptides derived from the sequences of the six paralogues competed for binding of radiolabelled *At*Pep1, suggesting that the receptor may be responsible for defense signaling by all of the *At*Pep peptides (Yamaguchi et al., 2006). The AtPepR1 coding sequence contains a region homologous to the cytosolic guanylate cyclase (GC) domain of *At*BRI1 (Kwezi et al., 2007; Qi et al., 2010) and it was shown that the cytoplasmic domain of AtPepR1 functions as a GC in vitro (Qi et al., 2010). Channels formed by CNGC2 polypeptides conduct Ca^{2+} (among other cations) and are activated by cAMP as well as cGMP (Leng et al., 1999). Furthermore, application of AtPep3 to Arabidopsis leaves expressing a Ca^{2+}-dependent chemiluminescent protein aequorin results in cytosolic Ca^{2+} elevation, *At*PepR1- and CNGC2-dependent, since both *pepr1*-aeq and *defense no death 1* (*dnd1*)-aeq, which lacks a functional CNGC2 coding sequence (Clough et al., 2000), are significantly impaired in *At*Pep3-dependent cytosolic Ca^{2+} elevation compared to wild type-aeq plants. Accordingly, AtPep3-triggered root growth inhibition is completely abolished in *pepr1* mutant. PepR1 and its ligand *At*Pep3 may act upstream of CNGC2 and it was speculated that PepR1 could activate CNGC2 through its GC activity. However, a consistent AtPep3-dependent cGMP elevation in leaves has been never observed.

Phylogenetic analysis among the LRR RLK XI subfamily of *Arabidopsis* (Shiu et al., 2004) showed that At1g17750, designated as *PepR2*, is the most closely related gene to *PepR1*, with 64% identity and 76% amino acid similarity (Yamaguchi et al., 2010). The At1g17750 gene encodes a predicted protein with 1088 amino acid residues (119 kD) and all the characteristic domains of an LRR-RLK. The N terminus contains a hydrophobic secretion signal followed by an extracellular domain with 25 tandem copies of a 24-residue LRR (residues 101 to 699). The LRR domain is flanked by two pairs of Cys residues. A single transmembrane domain (residues 741 to 761) is predicted to separate the extracellular domain from an intracellular Ser-Thr kinase domain (residues 794 to 1080) in which all important subdomains, including guanilyl cyclase catalytic domain (Kwezi et al., 2007) and residues for catalysis, are conserved (Yamaguchi et al., 2010). Results obtained from competition and alkalinization assays using transgenic tobacco cells as well as analysis of defenses response triggered by *At*Pep1-8 in *pepr1* and *pepr2* single mutants and *pepr1 pepr2* double mutants indicated that PEPR1 binds to Pep1-8 and that PEPR2 binds to Pep1 and Pep2 (Yamaguchi et al., 2006; Yamaguchi et al., 2010; Bartels et al., 2013).

The Pep1-triggered induction of defense genes expression, including the early genes *PROPEP1*, *MPK3*, *WRKY22*, *WRKY29*, *WRKY33*, *WRKY53*, *WRKY55*, and the late genes *PDF1.2* and *PR1*, is partially affected or unaffected in the *pepr1* and *pepr2* single mutants, while was totally abolished in the *pepr1 pepr2* double mutant compared to wild type plants, indicating that PepR1 and PepR2 have a redundant role in the immunity induced by Pep1. Moreover it was shown that Pep1-6 induce defense against Pst DC3000 as strong as flg22 and that PEPR1 and PEPR2 receptors are required redundantly for Pep1-mediated defense but not flg22-mediated defense. Furthermore, PepR2 contributes to the *At*Pep1-triggered increase of intracellular Ca^{2+} concentration in wild type-aeq leaves, since Ca^{2+} burst the elevation is impaired in *pepr2*-aeq null mutant (Ma et al., 2012). Like PEPR1 and BRI1, also PEPR2 has a catalytic GC region within the intracellular kinase domain (Kwezi et al., 2007; Ma et al., 2012). Application of Pep1 to intact seedling roots

resulted in a time-dependent elevation of *in planta* [cGMP], detected by a fluorescence indicator of cGMP (FlincG) (Ma et al., 2012). Moreover, fluorescence of FlincG-expressing plant roots pretreated with a GC inhibitor and then with Pep1 was similar to water controls; also the AtPep3- triggered Ca^{2+} elevation was completely blocked in presence of the inhibitor. In contrast to GC inhibitor effects on Pep-dependent Ca^{2+} elevation, flg22-dependent Ca^{2+} elevation was not much affected by inhibitor pretreatment (Ma et al., 2012). This was expected, as FLS2 does not have a cytosolic GC domain. PEPR1 and PEPR2 are induced by wounding and MeJa but not by MeSa and ACC (Yamaguchi et al., 2010). They are also differentially induced by DAMPs (AtPep and OGs) and PAMP (flg22 and elf18) (Zipfel et al., 2004; Zipfel et al., 2006; Denoux et al., 2008; Yamaguchi et al., 2010). Moreover, the analysis of *PEPR* promoter sequences fused with *GUS* revealed that both receptors are expressed in the vascular tissue of roots and leaves, in stems but not in root tips and flowers, indicating that the *PEPR1/2* promoter-mediated expression partially overlaps with that of *PROPEP1*, *-2*, *-3*, *-5*, and *-8*, and pointed out potential new, unknown roles for at least PROPEP4 and -7 independent of PEPRs (Bartels et al., 2013).

Like FLS2, PEPR1 and PEPR2 are involved in stress induced HR hypersensitive response, since loss of function of either FLS2 or PEPR receptors impaired the HR cell death to an avirulent pathogen (*P. syringae avrRpt2*), suggesting a functional interaction between flagellin and Pep signaling (Ma et al., 2012).

V. 1. 4. Involvement of *At*Peps in the amplification of PAMP-triggered immunity

It has been hypothesized that AtPeps might act as an amplifier of PTI since detection of MAMPs like flg22 or elf18 rapidly induces the expression of *PROPEPs* and *PEPRs* (Zipfel et al., 2004; Huffaker et al., 2006; Zipfel et al., 2006; Denoux et al., 2008). Strong parallels between MAMP and DAMP perception in plants also exist. The defense responses that are triggered after stimulation by MAMPs flg22 or elf18 and DAMPs *At*Peps are very similar and well documented. In fact, both flg22 or elf18 and *At*Peps caused ROS production (Huffaker et al., 2006; Krol et al., 2010; Ma et al., 2012; Flury et al., 2013), ethylene synthesis (Krol et al., 2010; Bartels et al., 2013; Flury et al., 2013), seedling growth inhibition (Krol et al., 2010; Qi et al., 2010), membrane depolarization (Krol et al., 2010), increase of cytosolic Ca^{2+} concentration (Qi et al., 2010; Flury et al., 2013), activation of the MAPKs MPK3 and MPK6 (Bartels et al., 2013; Flury et al., 2013), and medium alkalinization (Yamaguchi et al., 2006; Huffaker et al., 2006; Yamaguchi et al., 2010; Flury et al., 2013), defense genes induction (Huffaker et al., 2006; Huffaker and Ryan, 2007; Qi et al., 2010; Yamaguchi et al., 2010; Ma et al., 2012; Flury et al., 2013) and protection against the virulent bacterium *P. syringae* (Ma et al., 2012). Accordingly, an interaction of the leucine-rich repeat receptor-like kinase BAK1 (BRI1-associated kinase 1) with PEPR1 and PEPR2 was recently found, either in a direct yeast two-hybrid approach (Postel et al., 2010) or *in vivo*, based on an immunopurification approach (Schulze et al., 2010). Furthermore, BAK1 and a second signal corresponding to PEPR1 and PEPR2 are phosphorylated *in vivo* in response to AtPep1 stimulation (Schulze et al., 2010). This indicates that BAK1, which is known to be required for the signal transduction of FLS2 (Chinchilla et al., 2007), mediates both PAMP and DAMP signaling, a finding that enlarges the overlap in DAMP and PAMP signaling. A reported significant decrease in amplitudes of MAMP/DAMP-triggered membrane depolarization in *bak1* mutants is also well in line with the signaling overlap and lend good substance to a general observation that the PAMP/DAMP signaling convergence begins already at the plasma membrane (Krol et al., 2010).

Recent studies focused primarily on plant responses triggered by the addition of the synthetically produced AtPep peptides, firmly established that treatment with these peptides enhances plant immunity via PEPRs. MAMP perception has an impact on the ROS production triggered by AtPeps. The perception of MAMPs, flg22 as well as elf18, enhanced

a subsequent AtPep1-triggered production of reactive oxygen species (ROS) (Flury et al., 2013). Likewise, in wild-type plants, all of the AtPep peptides stimulated ROS production after pretreatment with flg22 in a similar way as *At*Pep1 (Flury et al., 2013). Surprisingly, the pretreatment of leaf discs with flg22 did not enhanced the elf18-triggered ROS or *vice versa* (Flury et al., 2013). Intriguingly, other components of *At*Pep-triggered immunity like alkalinization of the surrounding medium, increase of cytosolic [Ca^{2+}], mitogen-activated protein kinase phosphorylation, ethylene production, and expression of early defense genes, as well as ROS-activated genes, remained unchanged (Flury et al., 2013). The MAMP-induced enhancement of *At*Pep-triggered ROS depends on functional RbohD and RbohF, is independent of the abundance of the two PEPRs or RbohD/RbohF, is not based on changes in the ROS detoxification machinery and is independent of mitogen-activated protein kinase and Ca^{2+} signaling pathways (Flury et al., 2013). In contrast to the effect of flg22, JA but not SA enhances all *At*Pep-elicited responses, most likely by regulating PEPRs expression (Flury et al., 2013).

In Figure 3 is represented the working model for Pep/PEPR-mediated basal immunity (Yamaguchi and Huffaker, 2011). It is assumed that Pep peptides are secreted to amplify defense responses initiated by MAMPs based upon the following observations: (1) Peps and MAMPs increase transcription of PROPEP genes (Zipfel et al., 2004; Huffaker et al., 2006; Zipfel et al., 2006; Huffaker and Ryan, 2007). (2) Peps and MAMPS induce similar defense responses (Zipfel et al., 2004; Huffaker et al., 2006; Zipfel et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010). (3) PEPR receptors are predicted cell surface LRR receptor kinases (Yamaguchi et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010) that are structurally similar to FLS2 and EFR, the LRR-RKs that bind the peptide MAMPs (Zipfel et al., 2004; Zipfel et al., 2006). (4) The PEPR, EFR and FLS2 receptors all complex with the same co-receptor, BRI1-associated kinase1 (BAK1), which is required for both MAMP and *At*Pep signaling (Heese et al., 2007; Postel et al., 2010; Schulze et al., 2010). (5) Overexpression of *AtPROPEPs* results in constitutive defense gene expression in the absence of infection or wounding and enhances disease resistance (Huffaker et al., 2006). Along with other signals, the Peps likely act to sustain and fine-tune plant responses to MAMP perception and/or biotic attack.

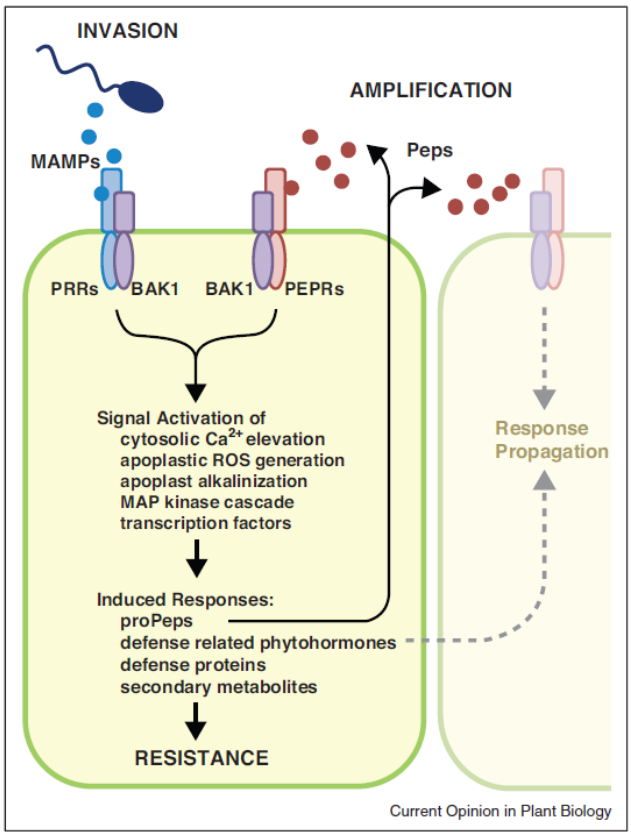

Figure 2. Amplification model of defense responses by plant elicitor peptides (Peps). Plant cells perceive microbe-associated molecular patterns (MAMPs) through pattern recognition receptors (PRRs) that function with BRI1-associated kinase (BAK1) to activate a signaling cascade involving components such as Ca^{2+}, reactive oxygen species (ROS) and mitogen activated protein kinases (MAPK). This cascade results in the production of defense proteins and secondary metabolites, leading to resistance. At the same time, precursor proteins of Peps (proPeps) and defenserelated phytohormones (jasmonic acid, salicylic acid, ethylene) are produced. After processing and release by unidentified mechanisms, the Pep peptides are perceived by Pep receptors (PEPRs) complexed with BAK1 to initiate a signaling cascade involving many of the same components utilized by MAMP signaling. Together the Peps and defense-related phytohormones amplify signaling and defense responses both spatially and temporally. Adapted from Yamaguchi and Huffaker, 2011.

However, the analysis of the defense responses induced by PAMP in the *pepr1*, *pepr2* and *pepr1 pepr2* loss of function mutants revealed some contrasting results. Some authors found that, unlike *At*Pep1-8, following flg22 or elf18 perception not only membrane depolarization, MPK3 and MPK6 activation, ethylene synthesis and seedling growth inhibition, but also ROS production and increase in cytosolic Ca^{2+} concentration were unaffected in *pepr1* and *pepr2* single mutants as well as in the *pepr1 pepr2* double mutant (Krol et al., 2010; Bartels et al., 2013; Tintor et al., 2013). Instead, others authors found that in leaves of *pepr1* as well as *pepr2* mutant plants, the flg22-induced cytosolic Ca^{2+} elevation was at least partially impaired compared to wild type (Qi et al., 2010; Ma et al., 2012). Moreover, a reciprocal sensitivity of AtPep3-dependent Ca^{2+} signaling to FLS2 presence was also found; absence of the flagellin receptor FLS2 partially impaired AtPep3-dependent cytosolic Ca^{2+} elevation (Qi et al., 2010). Some differences were identified between Pep/PEPR signaling and the Ca^{2+}-dependent immune signaling initiated by the flagellin peptide flg22 and its cognate receptor FLS2. FLS2 signaling may have a greater requirement for intracellular Ca^{2+} stores and inositol phosphate signaling, whereas Pep/PEPR signaling requires extracellular Ca^{2+}. In Arabidopsis protoplast (lacking an extracellular source of Ca^{2+} due to the lack of cell wall) WRKY33 was expressed in response to Pep3 only in presence of extracellular Ca^{2+} (Ma et al., 2012). The Pep3-triggered expression of WRKY33 was completely abolished in the

pepr1 pepr2 protoplasts co-expressing a mutant PEPR1 ($PEPR1^{m}$) construct, with null mutations within the GC catalytic domain of PEPR1, but not a WT PEPR1 construct, indicating that Ca^{2+}-dependent Pep/ PEPR1 immune signaling requires a functional GC domain of this receptor protein. Instead, flg22-dependent WRKY33 expression was maximal in the presence or absence of external Ca^{2+} (Ma et al., 2012). On the basis of experiments with *dnd1*-aeq plants, impaired in extracellular Ca^{2+} influx, and inositol polyphosphate 5-phosphatase (IP5-ptase)-aeq plants, impaired in phosphoinositide-specific phospholipase C (PI-PLC)–dependent generation of inositol triphosphate (IP3) and inositol hexakisphosphate (IP6), both important for signaling leading to vacuolar Ca^{2+} release, it was proposed that vacuolar Ca^{2+} release through PI-PLC signaling pathway may contribute to flg22- but not Pep-dependent cytosolic Ca^{2+} bursts, while CNGC function in Pep (and not in flg22) signaling (Ma et al., 2012). Impairment of IP3 and IP6 generation (in IP5-ptase-aeq plants) substantially inhibited the extent of cytosolic Ca^{2+} increase in response to flg22 but had no significant effect on the Ca^{2+} increase caused by Pep3. Conversely, null mutation of CNGC2 (in the *dnd1* mutant) had no effect on the extent of Ca^{2+} increase caused by flg22, but substantially impaired the increase in cytosolic Ca^{2+} elevation caused by Pep3. Besides Ca^{2+} spike, i) maximal flg22-dependent H_2O_2 generation required the presence of both PEPR receptors as well; ii) flg22-dependent WRKY33 expression was reduced (although to less of an extent) in *pepr1*, *pepr2*, and *pepr1 pepr2* plants and, in a corresponding fashion, Pep3-dependent *WRKY33* expression was reduced not only in the *pepr1*, *pepr2*, and *pepr1 pepr2* double mutant, but also in *fls2* plants (Ma et al., 2012). Similar trends were evidenced with regard to ligand-dependent expression of the pathogen defense gene *PDF1.2* in wild type, *pepr1*, and *fls2* plants. Moreover, both *pepr1 pepr2* and *fls2* mutants were affected in flg22- and pep3-induced protection against *P. syringae* (Ma et al., 2012)

V. 1. 5. Possible involvement of *At*Peps in OG signaling

AtPep1 and its homologues were the first endogenous peptide defence signals found in Arabidopsis, and the finding of orthologs throughout the plant kingdom suggests that this family of genes may have fundamental roles mainly related by authors to amplifying plant defences associated with innate immunity (Huffaker and Ryan, 2007).

Microarrays analyses showed that oligogalacturonides, like flg22, strongly induce the expression of *PROPEP2* and *PROPEP3* genes, and to a lesser extent *PROPEP1* (Denoux et al., 2008). Also PEPR1 is induced upon treatment with OGs as well as flg22. This data may let suppose a possible involvement of AtPEPs in amplifying OGs signaling.

In order to verify this, elicitor-triggered defense responses were analyzed in the *pepr1 pepr2* double KO mutant available in laboratory.

V. 2. RESULTS

V. 2. 1 *At*PEPR1 and *At*PEPR2 are not required for the ROS production, early defense genes induction and ethylene production triggered by OGs and flg22

A defect in fgl22-triggered ROS production has been described in the *pepr1 pepr2* mutant (Ma et al., 2012). In Arabidopsis seedlings, OGs are able to induce, over a period of six hours, a two-phase oxidative burst peaking early at 0.5 hours and late at 3 hours (Galletti et al., 2008). In order to establish if PEPRs are required also for the OG-induced early and late ROS production, H_2O_2 accumulation was quantified upon OG or flg22 treatments both in in leaf disks of Col-0 and *pepr1 pepr2* adult plants and in seedlings, after 0.5 and 3 hours after elicitation. Results show that OG- and, unexpectedly, flg22-triggered H_2O_2 production was not affected in the double mutant compared to wild type seedlings, even after 3 hours of treatment (Figure 1A). Coherently, the H_2O_2 production was not affected also in leaf discs of *pepr1 pepr2* adult plants compared to wild type (Figure 1B), indicating that PEPR1 and PEPR2 do not play a major role in the oxidative burst triggered by OGs and flg22.

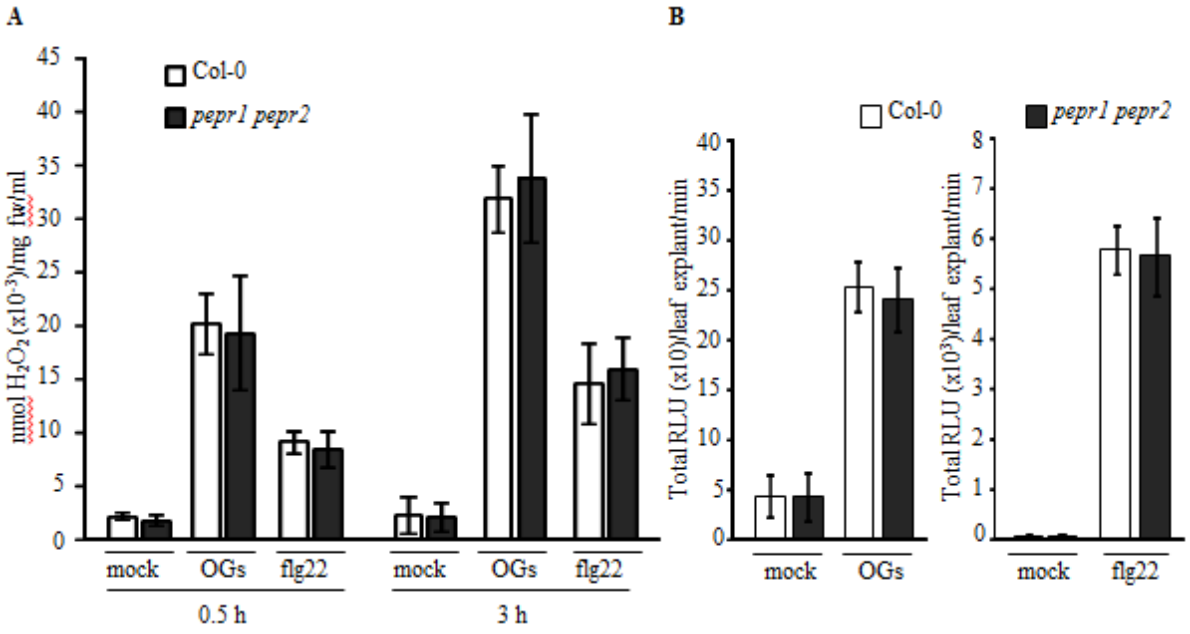

Figure 1. Accumulation of extracellular H_2O_2 in the Arabidopsis *pepr1 pepr2* mutant upon treatment with OGs or flg22. A) Col-0 and *pepr1 pepr2* 14-day-old seedlings were treated for 0.5 h and 3 h with mock (H_2O), OGs (100 µg/ml) or flg22 (1 µM). The H_2O_2 concentration in the incubation medium, expressed as nanomolar H_2O_2/mg fresh weight (fw) of seedlings/ml, was determined by a xylenol orange based assay. Results are average ±sd (n=4). Data are from one of two independent experiments that gave similar results. B) Total ROS production over a period of 40 min represented as relative light units (RLUs) in 4-wk-old Col-0 and *pepr1 pepr2* leaf discs after elicitation with mock (H_2O), 200 µg/ml OG or 1 µM flg22. Results are average ±sd (n=12). Data are from one of two independent experiments that gave similar results.

Next, searching for a possible involvement of PEPRs in the OG-triggered early defense genes induction, *pepr1 pepr2* mutant and Col-0 wild type seedlings were treated for 1 hour and 3 hour with OGs or mock (water) and transcripts levels of *WRKY33*, *CYP81F2* and *RET-OX* were analyzed by qRT-PCR. The *atpepr1 atpepr2* double KO seedlings

showed normal induction of early defense genes both after 1 h and 3 h of treatment with OGs, indicating that PEPR1 and PEPR2 are not required for the OG-induced early defense genes expression (Figure 2A).

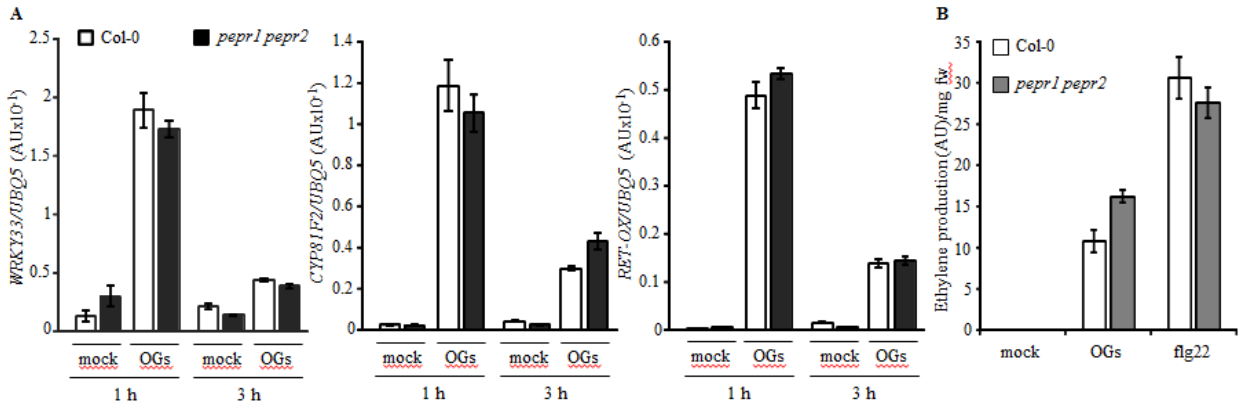

Figure 2. Early defense marker genes induction analysis and ethylene production upon treatments with OGs or flg22 in Col-0 and *atpepr1 atpepr2* double mutant line. A) Expression of defense gene markers *WRKY33*, *CYP81F2* and *RET-OX* was determined by quantitative RT-PCR in 10-day-old seedlings treated for 1 h and 3 h with water (mock) or OGs (100 µg/ml). Expression of *UBQ5* was used as a reference. Results are the mean of two independent experiments (±se). B) Ethylene production was measured in 12-day-old Col-0 and *pepr1 pepr2* seedlings treated with mock (water), OGs (50 µg/ml) or flg22 (1 µM) in vials and sealed for 4 h. Ethylene production expressed in arbitrary units (AU) was measured by gas chromatography. Results are average ±se (n=7).

V. 2. 2 AtPEPR1 and AtPEPR2 are differentially required for OG- and flg22-triggered expression of secondary response defense (late) genes

The role of PEPRs in the induction of late immune responses triggered by OGs and flg22, such as late defense genes induction and protection against *B. cinerea* was then analyzed. Previous works have described an involvement by PEPRs in the flg22-triggered induction of ethylene/jasmonic acid responsive genes (*PDF1.2, PLANT DEFENSIN 1.2*) and in the resistance to *B. cinerea* (Ma et al., 2012; Liu et al., 2013).

For analysis of late elicitor-induced defense gene expression, the genes *PDF1.2*, *PR-6-type*, a wound-inducible gene encoding a putative proteinase inhibitor that is not induced by any of the defense related hormones, and *PR-1* (*PATHOGENESIS-RELATED GENE 1*), a marker of salicylic acid responses, were chosen, all reaching maximal induction at time points between 6 and 12 hours in response to both OGs or flg22 (Ferrari et al., 2007; Denoux et al., 2008). Transcript levels were analyzed in *pepr1 pepr2* and wild type seedlings treated with OGs or flg22 for 6 and 12 hours. Three different expression behaviors were observed in the mutant compared to wild type: i) lower induced expression in response to flg22, but not to OGs (*PDF1.2*, Figure 3); ii) lower induced expression in response to both OGs and flg22 (*PR-6-type*, Figure 3); iii) lower induced expression in response to OGs, but not to flg22 (*PR-1*, Figure 3).

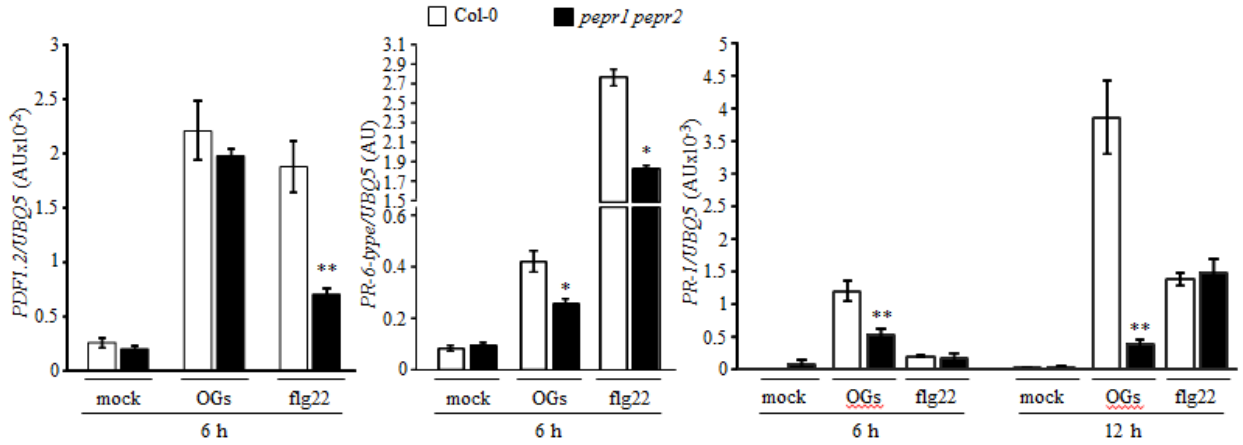

Figure 3. Late defense marker genes induction analysis and ethylene production upon treatments with OGs or flg22 in Col-0 and *atpepr1 atpepr2* double KO mutant line. Expression of defense gene markers *PDF1.2*, *PR-6-TYPE2* and *PR-1* was determined by quantitative RT-PCR in 10-day-old seedlings treated for 1 h and 3 h with OGs (100 µg/ml). Expression of *UBQ5* was used as a reference. Results are the mean of two independent experiments (±se).

V. 2. 3. OG- and flg22-triggered protection against *Botrytis cinerea* is affected by the loss of *At*PEPR1 and *At*PEPR2

Next, I examined whether, in the *pepr1 pepr2* mutant, the defence response defects affect the elicitor-induced protection response against *B. cinerea* (Ferrari et al., 2007; Galletti et al., 2011) The mutant has been described to be more susceptible than wild type to Botrytis infection (Liu et al., 2013).

Adult *pepr1 pepr2* and wild type plants were sprayed with OGs, flg22 or water, and excised leaves were drop-inoculated with *B. cinerea* conidia after 24 hours. In *pepr1 pepr2* water-pretreated leaves, the average of disease lesion diameter, measured 48 hours upon infections (hpi), was comparable to WT, indicating that, in contrast with literature data, basal susceptibility is not affected in this mutant. Unlike the wild type, *pepr1 pepr2* plants displayed neither OG- nor flg22-induced protection against Botrytis (Figure 4A). Also the analysis of the fungal growth on the infected leaves, quantifying the amount of *B. cinerea* tubulin 48 hpi by quantitative PCR, revealed that in water-sprayed leaves the amount of Botrytis tubulin transcript was comparable in wild type and mutant plants and that in both OGs- and flg22-sprayed leaves it was reduced by about 80% in Col-0 but not in *pepr1 pepr2* mutant (Figure 4B).

These results indicate an involvement of AtPeps also in the amplification of the OGs signal leading to the induction of SA-responsive genes (*PR1*) and protection to *B. cinerea*.

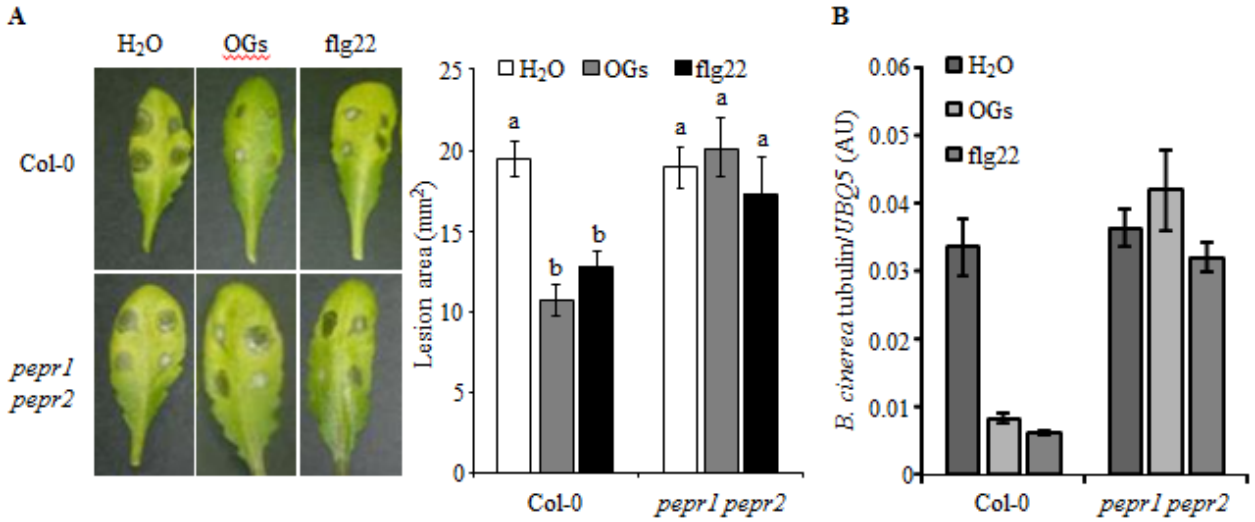

Figure 4. Elicitor-induced protection against *Botrytis cinerea* in Col-0 and *pepr1 pepr2* mutant. Four-week old plants were sprayed with OGs (200 µg/ml), flg22 (1 µM) or water; after 24 h, leaves were inoculated with B. *cinerea* spores ($5x10^5$ conidia/ml). A) Lesion areas were measured 48 h after inoculation (hpi). Results are average ±sd (n=12). Letters indicate P<0.01 by one-way ANOVA with Tukey's HSD test. Data are from one of two independent experiments that gave similar results. B) Botrytis tubulin expression was analyzed 48 hpi. Results are average of three technical replicates ±sd.

V. 2. 4. Elicitor-induced ethylene production is not affected by the loss of *At*PEPR1 and *At*PEPR2

Recently, two studies involving the *pepr1 pepr2* double mutant suggest an interaction of *At*Pep signaling with the defense hormone ethylene to maintain PTI responses (Tintor et al., 2013; Liu et al., 2013). To verify if the OG-triggered ethylene production is dependent on PEPR1 and PEPR2 seedlings of *pepr1 pepr2* mutant and Col-0 wild type were treated with OGs, flg22 (as a control) or water (mock) in sealed in vials. The ethylene amount, measured 4 h after treatment, was not affected in the mutant compared to wild type, neither after OG treatment nor after flg22 treatment (Figure 1B).

V. 3. DISCUSSION

Arabidopsis Pep peptides are considered amplifier of PAMP triggered immunity on the bases of the following findings: 1) Peps and PAMPs increase transcription of *PROPEP* genes (Zipfel et al., 2004; Huffaker et al., 2006; Zipfel et al., 2006; Huffaker and Ryan, 2007) and 2) Peps and PAMPs induce similar defense responses (Krol et al., 2010). Moreover, the loss of function of PEPR1 and PEPR2 seems to partially affect immune responses triggered by flg22, including Ca^{2+} spikes, ROS production, defense gene expression and protection to pathogens (Ma et al., 2012).

Microarray analyses showed that treatment with OGs induces, like flg22, three *PROPEP* genes (*PROPE1-3*) as well as *PEPR1* (Denoux et al., 2008). However, the flg22-triggered induction of these genes is stronger and more sustained. Moreover, the defense responses triggered by Peps and PAMPs are also very similar to those induced by OGs (Denoux et al., 2008; Krol et al., 2010). On the basis of these observations, the possible involvement of *At*Peps in amplification of the OGs signaling was taken into account.

Both early and late defense responses were analyzed in the *pepr1 pepr2* double mutant in response to OGs. I found that the *At*Peps are required for the full induction of some late defense-related genes but not for early responses, such as ROS production, and induction of *WRKY33*, *CYP81F2* and *RET-OX* expression. According to data obtained by Krol *et al.* (2010), but in contrast with results of Ma *et al.* (2012), I found that the H_2O_2 production triggered by flg22, used as control, was not affected in the *pepr1 pepr2* double mutant. Also the elf18-triggered ROS production is not affected in this mutant (Tintor et al., 2013). Also found that elicitor-induced ethylene synthesis is normal in double mutant seedlings.

Interestingly, the comparison between OG- and flagellin-triggered defense gene expression revealed that the PEPR/Pep system differentially regulate the responses induced by the two different elicitor. Indeed, my results indicate that the expression of *PDF1.2* is not affected in the *pepr1 pepr2* mutant in response to OG treatment, but it is affected in response to flg22, in agreement with literature data (Ma et al., 2012). Instead, *PR-1* showed an opposite behavior, i.e. a defective induction in response to OGs, but not to flg22. Finally, the expression of *PR-6-type*, a wound-inducible gene also induced by OGs and flg22/elf18, but little induced by the known defense related hormone, was affected in the *pepr1 pepr2* mutant in response to both elicitors.

In the *pepr1 pepr2* mutant the induction of *PR-1* is affected in response to another PAMP (elf18) and that the induction of *PR-1* triggered by elf18, but not by Pep2, is dependent on ethylene, since it is completely abolished in the *ein2* mutant (Tintor et al., 2013). It was proposed that the EFR-triggered signal might be sequentially relayed via ET signaling and then the PEPR pathway leading to *PR1* activation. The fact that *PR-1* can be induced by elf18 in an Et-dependent manner, and by Pep2 independently of ethylene, suggests that PEPR activation keeps this branch on and thus compensates in part for the PTI defects caused by dysfunctional ET signaling. This seems to be achieved in part by the PAMP-triggered activation of *PROPEP2* and *PROPEP3* in an ET-dependent or -independent manner, respectively (Tintor et al., 2013). However, whether the OG-triggered induction of *PR-1* is dependent on ethylene signaling and the *PROPEP* genes induction triggered by OGs differentially require ethylene signaling is not known. Collectively, these results indicate that loss of PEPR1 and PEPR2 leads to altered elicitor-triggered induction of defense response genes, with different effects depending on the gene, and suggest that the defense response gene up-regulation likely occurs through multiple pathways, some dependent and other independent from the two PEPRs.

My results also show that both OGs- and flg22-triggered protection against *B. cinerea* is affected in the *pepr1 pepr2* mutant. Flg22-triggered protection against Botrytis is dependent on ethylene, since the *ein2* mutant loses the flg22-induced PTI to this fungus. I obtained preliminary data that indicate that is might be the also for the protection against

Botrytis induced by OGs (data not shown). My data show that both OGs- and flg22-triggered production of ethylene is not affected in the *pepr1 pepr2* mutant, at least in seedlings. However, PEPR1 and PEPR2 are known to be required for the response to ethylene, since both seedling growth inhibition and induction of Et-responsive genes triggered by ACC are partially affected in the *pepr1 pepr2* mutant, and even more important the ACC-induced protection against *B. cinerea* is completely lost in the double mutant (Liu et al., 2013). Since the treatment with ACC induces resistance against Botrytis and both OGs and flg22 promote ethylene synthesis, the loss of protection induced by the two elicitors observed in *pepr1 pepr2* might be explained by the fact that the double mutant is defective in the response to ethylene. This observation suggests that AtPEPs could be part of an endogenous feedback loop involved in the amplification of the plant responses elicited by the perception of danger signals, including OGs. Indeed, Huffaker and colleagues reached the same conclusion proposing AtPep peptides as amplifiers of defence-related elicitors (2007).

PART VI – GENERAL CONCLUSIONS

Research over the last 25 years has led to an increasingly clear conceptual understanding of the molecular components of the plant immune system. In this thesis I have identified some steps of the OG signal transduction pathway. I demonstrated the involvement of elements, including CDPKs, SERKs and PEPRs and shown that there is overlap between PAMP and DAMP signaling cascades, at least for some responses. Overexpression of CDPK members has been shown to confer resistance to several pathogens both in monocot and dicot (Coca and Segundo, 2010)(Kobayashi et al., 2012)(Asano et al., 2012)(Dubiella et al., 2013). Likewise, Arabidopsis plants overexpressing *AtPROPEP1* or *AtPROPEP2* showed enhanced resistance to a root pathogen (Huffaker et al., 2006; Huffaker and Ryan, 2007).

An improved understanding of plant signaling pathways in response to a wide range of pathogens, such as fungi and bacteria, will give the potential for engineering plants for resistance against individual devastating diseases or multiple pathogens.

The firsts commercially available transgenic plant species were those with increased resistance towards viruses; recently, thanks to different practices involved in breeding for disease resistance, such as the identification and the editing within the host genome disease-resistance (*R*) genes, reduced pathogen growth and symptom development, also against fungi and bacteria. Another strategy is to transfer pattern recognition receptors (PRRs) that detect common microbial products into species that lack them (Dangl et al., 2013).

In contrast to PRRs and NLRs, another class of plant disease resistance genes has evolved to open a “trap door” that stops pathogen proliferation. Transcription activator–like (TAL) effectors are DNA-binding proteins delivered into plant cells, where they activate host gene expression to enhance pathogen virulence; in a neat evolutionary trick, however, both the rice and pepper lineages independently evolved TAL-effector binding sites in the promoters of genes whose products induce hypersensitive host cell death when up-regulated and thus inhibit pathogen proliferation (Dangl et al., 2013).

Our current challenge is to define and to stack multiple resistance specificities active against the daunting array of economically important pathogens, including *Phytophthora*, *Magnaporthe*, *Fusarium*, *Pseudomonas*, *Ralstonia*, *Xanthomonas*, and gemini and potyviruses. At the same time, we must maintain complex agronomic traits - such as yield, form, and flavor - and avoid yield penalties.

PART VII - MATERIALS AND METHODS

VII. 1. Plant Material

Arabidopsis (*Arabidopsis thaliana*) Columbia-0 (Col-0) wild-type seeds were purchased from Lehle Seeds. The *cpk5 cpk6 cpk11* triple KO line and was kindly provided by Jen Sheen (Department of Molecular Biology, Massachusetts General Hospital, Department of Genetics, Harvard Medical School, Boston, USA). The *bak1-5* and *bak1-5 bkk1-1* mutant lines were kindly provided by Cyril Zipfel (The Sainsbury Laboratory). Seeds of the T-DNA insertional mutants *At1g17750* (*pepr2*) (SALK_098161), *At1g73080* (*pepr1*) (SALK_059281), *serk4* (SALK_ 057955C) were obtained from Nottingham Arabidopsis Stock Centre (School of Biosciences, University of Nottingham, United Kingdom). The *pepr1 pepr2* double mutant was previously generated by crossing by Daniel Savatin.

VII. 2. Growth conditions and plant treatments

For seedling treatments, seeds were surface sterilized and germinated in multiwell plates (approximately 10 seeds/well) containing 2 mL per well of Murashige and Skoog medium (SIGMA ALDRICH; Murashige and Skoog, 1962) supplemented with 0.5% sucrose. Plates were incubated at 22°C with a 16-h light/8-h dark cycle and a light intensity of 120 µmol m^{-2} s^{-1}. After 9 days, the medium was adjusted to a final volume of 1 mL and treatments were performed after an additional day.

OG pools with an average DP of 9 to 16 (OGs) were kindly prepared by Gianni Salvi and Daniela Pontiggia (Università di Roma "Sapienza") as previously described (Bellincampi et al., 2000). Matrix-assisted laser desorption/ionization time-of-flight MS was used to verify the DP of OG preparations. The flg22 peptide was synthesized by Maria Eugenia Schininà (Università di Roma "La Sapienza).

VII. 3. Immunoblot assay

For MPK3, MPK4 and MPK6 phosphorylated form revelation, seedlings were frozen in liquid nitrogen and proteins were extracted with a buffer containing 50 mM Tris at pH 7.5, 200 mM NaCl, 1 mM EDTA, 10 mM NaF, 2 mM sodium orthovanadate, 1 mM sodium molybdate, 10% (v/v) glycerol, 0.1% Tween 20, 1 mM PMSF, 1 mM DTT, 1 x protease inhibitor cocktail P9599 (SIGMA ALDRICH).

Equal amounts of proteins (from 30 µg) were resolved on 7.5% polyacrylamide (30% Acrylamide/Bis Solution, 29:1, BIO-RAD) SDS-gel and transferred onto nitrocellulose membranes (Hybond™-C Amersham, UK) in 25Mm TRIS, 192 mM glycine, pH 8.3, 20% methanol at 4°C for 1 h.. The filter was stained with Ponceau Red to assess equal loading and then blocked with 5% Albumin from bovine serum (BSA, SIGMA ALDRICH) in Tris-Buffered Saline (TBS; BIO-RAD) for 1h prior to incubation with primary antibody against phospho-p44/p42 MAP kinase (Cell Signaling Technologies) in TBS containing 0.5% BSA over night. After three washes of 5 min in TBS, membrane was incubated with anti-rabbit secondary antibody horseradish peroxidase–conjugated (Amersham, UK) in TBS containing 0.5% BSA. Membrane was washed as described above prior to detection with ECL detection reagent (Amersham, UK). For MPK3 and MPK6 native form revelation, the membrane was incubated in a stripping buffer (2% SDS, 100 mM 2-mercaptoethanol, 50 mM Tris-HCl pH 6.8) for 30 min at 50°C in water bath. After extensive washes in TBS, the filter was blocked with 5% Albumin from bovine serum (BSA, SIGMA ALDRICH) in Tris-Buffered Saline (TBS; BIO-RAD) for 1h prior to incubation with primary antibody against AtMPK3 and AtMPK6 (SIGMA ALDRICH) in TBS containing 0.5% BSA for 1 h. After three washes of 5 min in TBS, membrane was incubated with anti-rabbit secondary

antibody horseradish peroxidase–conjugated (Amersham, UK) in TBS containing 0.5% BSA. Signal detection was performed as described above.

VII. 4. Measurement of ROS

H_2O_2 produced by leaf discs was measured by a luminol-based assay as previously described (Roux et al., 2011). Discs (0.125 cm^2) obtained from 4-week-old plants were washed for 2 h with water and left to recover in a 96-well titer plate (one disc/well). After about 12 h, water was replaced with a solution of luminol (Sigma-Aldrich; 30 µg mL^{-1}) and horseradish peroxidase (Sigma-Aldrich; 20 µg mL^{-1}) containing flg22 (200 nM). For elicitation by OGs, discs were vacuum infiltrated with the OG solution (200 µg mL^{-1}) or water, as a control, for 2 min before addition of the luminol/peroxidase solution. Plates were analyzed for 40 min using a GloMax 96 microplate luminometer with dual injectors (Promega) and a signal integration time of 1 s. Luminescence was expressed in Relative Light Units.
The H_2O_2 concentration in the incubation medium of treated seedlings was measured by the FOX1 method (Jiang et al., 1990), based on peroxide-mediated oxidation of Fe^{3+}, followed by the reaction of Fe^{3+} 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. 14-day-old seedlings (about 100–120 mg in 1 mL of medium) were treated with OGs (100 µg/ml) or flg22 (1 µM) for 30 minutes. To determine H_2O_2 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 Fe^{3+}-xylenol orange complex (A_{560}) was detected after 45 min of incubation. Standard curves of H_2O_2 were obtained for each independent experiment. Data were normalized and expressed as micromolar H_2O_2/g fresh weight of seedlings.

VII. 5. Gene expression analysis

Treated seedlings or leaves were frozen in liquid nitrogen, homogenized with MM301 Ball Mill (Retsch), and total RNA was extracted with Isol-RNA Lysis Reagent (5 Prime) according to the manufacturer's protocol. Two micrograms of RNA were treated with RQ1 DNase (PROMEGA) and first-strand cDNA was synthesized using ImProm-II reverse transcriptase (PROMEGA) according to the manufacturer's instructions. Real-time quantitative PCR analysis was performed using a CFX96 Real-Time System (BIO-RAD). One microliter of cDNA (corresponding to 50 ng of total RNA) was amplified in a 20-µL reaction mix containing 1 × Go Taq qPCR Master Mix (PROMEGA) and 0.5 µm of each primer. 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). Semi quantitative reverse transcription (RT)-PCR analysis was performed in a 30-µL reaction mix containing 1 µL of cDNA, 1× buffer (RBCBioscience), 3 mm $MgCl_2$, 100 µm of each dNTP, 0.5 µm of each specific primer, and 1 unit Taq DNA Polymerase. 25, 30, and 35 PCR cycles were performed for each primer pair to verify linearity of the amplification. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. Primer sequences are listed in Table 1.

VII. 6. Callose deposition

Leaves from 4-week-old plants were infiltrated with water, (200 mg mL^{-1} OGs) or flg22 (1 µM) using a needleless syringe. After 24 h, for each treatment, about eight leaves, from at least five independent plants, were cleared

and dehydrated with 100% ethanol. Leaves were fixed in an acetic acid:ethanol (1:3) solution for 2 h, sequentially incubated for 15 min in 75% ethanol, in 50% ethanol, and in 150 mM phosphate buffer, pH 8.0, and then stained for 1 h at 25°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 UV epifluorescence using an Axioskop 2 plus microscope (Zeiss). Images were taken with a ProgRes C10 3.3 MegaPixel digital color camera (Jenoptik). Callose quantification was performed using ImageJ software.

VII. 7. Chlorophyll leaching assay

Chlorophyll extraction and quantification was performed according to the protocol of (Sieber et al., 2000). Leaves were cut at the petiole, weighed and immersed in 15 ml of 80% ethanol. Chlorophyll was extracted in the dark at room temperature with gentle agitation. Aliquots were removed at 5, 10, 20, 50, 60, 90, 120, 1020, 1440 min after immersion. The chlorophyll content was determined by measuring absorbance at 664 and 647 nm and the micromolar concentration of total chlorophyll per gram of fresh weight of tissue was calculated from the following equation: $(7.93x(A_{664}\ nm) + 19.53x(A_{647}\ nm))\ g^{-1}$ fresh weight.

VII. 8. Botrytis cinerea growth and plant inoculation

Botrytis cinerea was grown on 20 g l^{-1} malt extract, 10 g l^{-1} proteose peptone n.3 (Difco, Detroit, USA), and 15 g l^{-1} agar for 7–10 days at +24°C with a 12-h photoperiod before collection of spores. Rosette leaves from 4-week-old soil-grown Arabidopsis plants were spryed with water or elicitor containing solutions (OGs or flg22) 24 h before being placed in Petri dishes containing 0.8% agar, with the petiole embedded in the medium. Inoculation was performed by placing 5 µl of a suspension of 5×10^5 conidiospores ml^{-1} in 24 g l^{-1}potato dextrose broth (PDB; Difco, Detroit, USA) on each side of the middle vein. The plates were incubated at +22°C with a 12-h photoperiod. High humidity was maintained by covering the plates with a clear plastic lid. Under these experimental conditions, most inoculations resulted in rapidly expanding water-soaked lesions of comparable diameter. Lesion size was determined by measuring the diameter or, in case of oval lesions, the major axis of the necrotic area by using ImageJ software.

VII. 9. Camalexin, salicylic acid and jasmonic acid determination

Camalexin was extracted as previously described (Pan et al., 2010a) from water- and OG-sprayed leaves of 4-week-old Col-0 and *cpk5 cpk6 cpk11* mutant plants (approximately 100 mg), collected 14 and 20 hours after infection with *B. cinerea* conidia (5×10^5 mL^{-1}). After homogenization in liquid N_2, samples were suspended in 1 ml Dichloromethane, left at 4°C for 30 minutes and centrifuged at 5000 x g for 10 minutes. Samples were dissolved in 1 ml methanol and analyzed by liquid chromatography coupled to mass spectrometry using an Ultimate 3000 HPLC system connected to an Orbitrap XL Discovery (Thermo Fisher Scientific) equipped with an ESI source operating in positive mode. Samples were separated by reversed-phase HPLC using an Acclaim 120 C18 column [3 µm, 200 Å, 2.1 x 150 mm (Thermo Fisher Scientific)] equipped with a guard column, and eluted, using as mobile phases, water containing 0.1 % formic acid (eluent A) and methanol containing 0.1 % formic acid (eluent B). A 45 min gradient, from 30% to 100% B, was performed. The effluent from the HPLC was directly electrosprayed into the mass spectrometer. The spray voltage was set at 4.5 kV with the heated capillary temperature set at 350°C. The Orbitrap MS instrument

operated in full-scan MS with resolution R = 30,000 at m/z 400. The quantification was obtained using the calibration curve method.

Jasmonic acid and salicylic acid were extracted according to the same protocol described above from: i) leaves from 4-week-old plants sprayed 24 h before with water, OGs (200 µg/ml) or flg22 (1 µM); ii) leaves from 4-week-old plants inoculated with *B. cinerea* conidia (5×10^5 mL^{-1}) for 14 and 20 hours; iii) 14-day-old seedlings treated with water, OGs (100 µg/ml) or flg22 (10 nM).

VII. 10. Ethylene determination

For ethylene measurements, Arabidopsis seedlings were grown in sterile conditions into 10 ml flasks containing 2ml of Murashige and Skoog medium for 10 days. After treatment with H_2O or elicitor solution (50 µg/mL OGs, or 1 µM flg22) flasks were sealed. Headspace samples (450 µL) were withdrawn from the vial 4 h after treatment and analyzed by GC-MS using an Agilent 6850A gas chromatograph coupled to a 5973N quadrupole mass selective detector (Agilent Technologies). Chromatographic separations were carried out on an HP Plot-Q fused-silica capillary column (30 m × 0.32 mm i.d.) coated with polystyrene-divinylbenzene (film thickness 0.20 µm) as stationary phase. Injection mode: splitless at a temperature of 220°C. The initial temperature of the oven was held at 50 °C for 8 min then ramped to 220 °C at a rate of 15 °C/min and held for 5 min. Helium was used as carrier gas at a constant flow of 1.0 mL/min. Mass spectra were collected both in full scan and in SIM mode monitoring the ions m/z m/z 26, m/z 27 and m/z 28 (ionization energy 70 eV; ion source 280°C; ion source vacuum 10^{-5} Torr). Rosette leaves from 4-week-old soil-grown Arabidopsis plants were cut from the petiole and placed in 10 ml flasks containing 1.5 mL 0.8% agar, with the petiole embedded in the medium. Inoculation was performed by placing 5 µl of a suspension of 5×10^5 conidiospores ml^{-1} in 24 g l^{-1}potato dextrose broth (PDB; Difco, Detroit, USA) on each side of the middle vein. The flasks were sealed and incubated at +22°C with a 12-h photoperiod in condition of high humidity. Headspace samples (450 µL) were withdrawn from the vial 20, 40 and 60 h after treatment and analyzed as described above.

Table I. Primers used in this work.

GENE	AGI CODE	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')
UBQ5	AT3G62250	GTTAAGCTCGCTGTTCTTCAGT	TCAAGCTTCAACTCCTTCTTTC
PHI1	AT1G35140	TTGGTTTAGACGGGATGGTG	ACTCCAGTACAAGCCGATCC
WRKY33	AT2G38470	GAAACAAATGGTGGGAATGG	TGTCGTGTGATGCTCTCTCC
FRK1	AT2G19190	TTAAACTCGACGATGCAACA	GATGGAAGTTTTCCCGTTTT
CYP81F2	AT5G57220	GTGAAAGCACTAGGCGAAGC	ATCCGTTCCAGCTAGCATCA
RET-OX	AT1G26380	AGGTTCTCGAACCCTAACAACA	GCACAGACGACACGTAAGAAAG
PAD3	AT3G26830	CCGGTGAATCTTGAGAGAGCC	GATCAGCTCGGTCATTCCCC
PGIP1	AT5G06860	TCTTGAACTTAGCAGGAGGAAC	GAGAGCTGGTTATGTGATAG
PDF1.1	AT1G75830	TAAACAATAGTCATGGCTAAGTCTGC	ACTTGGCCTCTCGCACAACT
PDF1.2	AT5G44420	CGCACCGGCAATGGTGG	ATCCATGTTTGGCTCCTTCG
ORA59	AT1G06160	TCGCGGCCGAGATAAGAGACTC	TCCGGAGAGATTCTTCAACGACATCC
PR-4	AT3G04720	TACGCGCCACCTACCATTTCTAT	TTGCTGCATTGGTCCACTATTCTC
ERF1	AT3G23240	GTCTTTGAGGATTTGGGAGAACAGT	CACCAAGTCCCACTATTTTCAGAA
ERF5	AT5G47230	TGAATCCGTATGCAAACCTG	ATCTTCAATGGCGGTTTACG
PR-1	AT2G14610	GTAGGTGCTCTTGTTCTTCCC	CACATAATTCCCACGAGGATC
PR-6-type	AT2G38870	GCTTACGGGAACAAATGGTG	GACGACGATACGGTTTCCAT
THI2.1	AT1G72260	TTCCAAGGGAAGGTGTATGC	ACATCCCTTGGCACATTGTT
VSP2	AT5G24770	AGTGACCGTTGGAAGTTGTGGAAGA	CTCAAGTTCGAACCATTAGGCTTC
CPK5	AT4G35310	TCGTTCCAAATTGACCTTGAC	GAGGAAACAGCGGAGAGAGAC

CPK6	AT2G17290	CTCGCAAACTAACGCTTACCTG	TTTTGGGATCTATAATGATCG
CPK11	AT1G35670	AAATGATGGTGTTTTTATTTA	AAACCAATTAGGCGATGAACC
β-TUBULIN	-	GTTACTTGACATGCTCTGCCATT	CACGGCTACAGAAAGTTAGTTTCTACAA

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