Immune Responses to Pathogen Infection in *Arabidopsis*

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ACADEMIC DISSERTATION

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To my family

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II Hu, Z., Keceli, A., Piisilä, M., Li, J., **Survila, M**., Heino, P., Brader, G., Palva, E. T. & Li, J. F-box protein AFB4 plays a crucial role in plant growth, development and innate immunity.2012 In : Cell Research. 22, p. 777-781 5 p.

III **Survila, M.***, Davidsson, P. R.*, Pennanen, V., Kariola, T., Broberg, M., Sipari, N., Heino, P. & Palva, E. T. Peroxidase-Generated Apoplastic ROS Impair Cuticle Integrity and Contribute to DAMP-Elicited Defenses. 23 Dec 2016 In : Frontiers in Plant Science. 7, 16 p., 1945

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I The author designed the experiments with JL, performed the experiments with NN and wrote the manuscript with JL and NN.

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III The author designed all the experiments with TP and PD, performed the majority of experiments and wrote the manuscript with PD, TK and TP.

ABBREVIATIONS

ABA	abscisic acid
ABFs	ABA responsive element binding factors
ABI	abscisic acid insensitive
Avr	avirulence
BHNs	broad host-range necrotophs
BLs	barssinosteroids
DAMPs	damage-associated molecular patterns
eATP	extracellular ATP
EFR	EF-TU receptor
EIN	ethylene insensitive
ER	endoplasmic reticulum
ER-QC	ER-quality control
ET	ethylene
ETI	effector-triggered immunity
FLS	flagellin sensing
GA	gibberellic acid
H_2O_2	hydrogen peroxide
HR	hypersensitive response
HSNs	host-specific necrotrophs
HSTs	host-specific toxins
ISR	induced systemic resistance
JA	jasmonic acid
LRR	leucine-rich repeat
MAMP/PAMP	microbe/pathogen-associated molecular pattern
MAP	mitogen-activated protein
MAPKs	mitogen-activated protein kinases
MeSA	methylated salicylic acid
MPK	MAP kinase
NADPH	nicotine adenine dinucleotide phosphate (reduced form)
NPR	nonexpresser of PR genes
OGs	oligogalacturonides
PAD	phytoalexin deficient
PAMP	pathogen-associated molecular pattern
PCWDE	plant cell wall-degrading enzyme
PCD	programmed cell death
PDF	plant defensin
PI	proteinase inhibitor
PR	pathogenesis-related proteins
PRR	plasma-membrane-localized pattern recognition receptor
PTI	PAMP-triggered immunity
PYR/PYL/RCAR	pyrabactin resistance/PY- like/regulatory component of ABA receptor
R	resistance
RKs	receptor kinases
RLPs	receptor-like proteins
RNS	reactive nitrogen species
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic acquired resistance
SID	SA induction deficient
Ub	ubiquitin

ABSTRACT

To survive, plants must recognize the presence of danger and establish effective defenses against invading pathogens. Most plants are resistant to the majority of plant pathogens (Jones and Dangl, 2006). This passive protection is provided primarily by the cell wall and waxy cuticular layer that limit the progress of most attackers (Dangl and Jones, 2001). If these barriers are overcome, the second line of defense is triggered upon detection of pathogen-associated molecular patterns or damage-associated molecular patterns (PAMPs/DAMPs) by pattern recognition receptors (PRRs) (Boller and Felix, 2009; Felix et al., 1999; Zipfel, 2014). The activation of PRRs induces multifaceted intracellular signaling pathways that ultimately initiate defense responses. Many molecular components by which plants perceive pathogens and the downstream signaling cascades have been characterized on a molecular level. However, the mechanisms by which plants protect themselves from phytopathogens (in particular necrotrophs) remain to be elucidated.

Three aspects of plant immunity to phytopathogens are addressed in this thesis: (I) the role of glucosidase II β-subunit *AtGCSIIβ* in EFR receptor-mediated defense signaling, (II) the role of F-box protein AFB4 in plant innate immunity against necrotrophic pathogens, and (III) the role of class III peroxidases in cuticle formation that governs very strong and local resistance against necrotrophic bacterial and fungal pathogens.

Plants exploit membrane-localized PRRs for specific and rapid detection of the potential pathogens. Many eukaryotic membrane-localized proteins undergo quality control during folding and maturation in the endoplasmic reticulum (ER), a process termed endoplasmic reticulum quality control (ER-QC) (Anelli and Sitia, 2008). The biogenesis of EFR, and to a lesser extent FLS2 receptors, is regulated by this mechanism (Nekrasov et al., 2009; Saijo et al., 2009). Study I demonstrated that the glucosidase II β-subunit *AtGCSIIβ* is pivotal for the function of the plant innate immunity receptor EFR. Loss-of-function in *AtGCSIIβ* results in elf18-insensitive phenotype, confirming the importance of *AtGCSIIβ* in biogenesis of the EFR receptor.

F-box proteins are important components in plant hormone responses. They target regulatory proteins to the ubiquitin (Ub) proteolytic machinery and mediate hormone signaling transduction. In study II, we demonstrated that auxin signaling F-box protein (*afb4-1*) mutant plants are enhanced in their resistance to bacterial and fungal necrotrophic pathogens. This was accompanied with altered sensitivity to methyl jasmonate (MeJA), indole-3-acetic acid (IAA), and abscisic acid (ABA) phytohormones, thus providing evidence that ABF4-mediated signaling is involved in balancing growth and defense responses via coordination of hormone-mediated signaling pathways.

The ability to maintain the barrier properties of the epidermis is largely due to the cell walls, which are covered with specialized lipids. This fine structure at the outermost region of the cell walls of

epidermal cells is called the cuticle, which has been the subject of many studies (Jeffree, 2006). Plants perceive and ultimately activate defense mechanisms in response to cuticular and cell wall structural components e.g. oligogalacturonides (OGs) released by the action of degradative enzymes secreted by pathogenic bacteria or fungi. Cuticle alterations induce a battery of reactions that often result in reactive oxygen species (ROS) production and resistance to necrotrophic pathogens. However, the source of ROS generated upon altered cuticle status and the acute downstream defense signaling pathways involved in such defense remains elusive. Study III provides evidence that ROS produced by class III apoplastic peroxidases suppress the expression of cuticle-biosynthetic genes, and together with ABA, regulate the formation of the cuticle envelope. However, resistance to necrotrophic pathogens in cuticle-depleted plants is a result of activated OG signaling components and function independently of salicylic acid (SA) and jasmonic acid (JA) signaling pathways.

This thesis demonstrates the use of *Arabidopsis* in studying the genetic basis of plant defense mechanisms. It provides novel insights on plant resistance to pathogens, and reveals how cuticular defects activate defense via OG signaling pathway.

1. INTRODUCTION

Plants and plant pathogens have co-evolved for millions of years. Plants acquired the ability to perform photosynthesis through symbiosis with photosynthetic bacteria. The ability of plants to convert the energy from sunlight into oxygen (essential for most organisms) allowed them to become primary producers of the terrestrial ecosystem. In addition to animals, there is a huge variety of organisms that take advantage of plants. These include nematodes, insects, and herbivores, as well as pathogenic microbes such as viruses, bacteria, and fungi. The outcome of successful pathogen infection can be seen as rots, water-soaked lesions, blights, wilts, powdery or downy mildews, or rust lesions on plant tissue leading to severe crop losses (Gross, 2014). Many valuable crops are highly susceptible to disease and would have difficulty surviving in nature without plant protective measures taken by humans. This is because modern agriculture depends mostly on few plant varieties such as rice, wheat, and maize. Genetically homogenous plant populations grown in close proximity enable rapid pathogen spread under favorable environmental conditions. Therefore, the study of plant disease resistance is very important to overcome development of disease in monoculture crops. Breeders continuously search for new resistance genes and resistance gene combinations to improve existing crop varieties (Jaggard et al., 2010).

The lifestyle of the pathogen influences the disease phenotype. Accordingly, three classes of pathogens are recognized on the basis of how they acquire nutrients from plant tissue. Necrotrophic pathogens use a brute-force infection strategy by producing cell wall-degrading enzymes (CWDEs) and toxins to induce cell necrosis. This strategy provides necrotrophic pathogens leaked nutrients from dead plant cells during tissue colonization. In contrast, biotrophic pathogens secrete limited amounts of CWDEs and lack the production of toxic compounds, thus allowing these pathogens to obtain nutrients from living host cells (Glazebrook, 2005; Mendgen and Hahn, 2002). A third group, hemibiotrophs, start with a biotrophic phase followed by a necrotrophic mode of nutrition.

The defense response in plants consists of a basal, low intensity response, and a response known as effector-triggered immunity (ETI) that is highly specific and intense. Basal defense is divided into pre-existing and inducible defenses. Pre-existing defense involves structural barriers such as cell walls, waxy cuticular layer, and bark. Most pathogens are not adapted to penetrate these barriers and usually exist harmlessly at low population densities. Such organisms are

referred to as non-host pathogens. Induction of defense responses occurs when PAMPs or DAMPs are detected by highly conserved PRRs. Pathogens that are able to penetrate pre-existing defenses will trigger PAMP- (PTI) or DAMP triggered immunity (DTI), respectively (Jones and Dangl, 2006). PAMPs include bacterial structures such as the protein flagellin (Felix et al., 1999) or elongation factor EF-Tu (Kunze et al., 2004). DAMPs include host biomolecules such as polypeptides and extracellular ATP (eATP) (Walker-Simmons et al., 1983) or structural components derived from extracellular matrix such as oligogalacturonides (OGs), which are released from plant cell walls by the action of bacterial and fungal cell wall degrading enzymes (CWDEs) (Boller and Felix, 2009; Galletti et al., 2011). Despite the recognition of PAMPs and DAMPs, and resulting induction of PTI, some pathogens are nevertheless successful and cause disease. These pathogens produce or secrete effector proteins encoded by *avr* (avirulence) genes. These proteins are capable of suppressing basal defense responses elicited by the PAMP recognition, resulting in effector-triggered susceptibility (ETS). On the other hand, plants have evolved R (resistance) proteins capable of recognizing the effector proteins. This recognition leads to the activation of a much stronger line of defense, known as effector-triggered immunity (ETI). Defense responses triggered by the R-effector interaction is more specific, faster, stronger, and more prolonged than PTI. These responses usually act systemically throughout the plant and are effective against a broad range of invaders (Durrant and Dong, 2004). The biological distinction between PTI and ETI is rather vague, since the responses are highly overlapping. Many of the same defense genes are up-regulated, and the cellular processes involved in plant defense are centrally regulated by major plant phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA). Classically, SA promotes resistance to biotrophs, whereas JA and ET act antagonistically to SA and promote resistance to necrotrophs (Bari and Jones, 2009; Dahl and Baldwin, 2007; Grant and Lamb, 2006; Howe, 2004; van Loon et al., 2006; Lorenzo and Solano, 2005).

In summary, humans depend almost exclusively on plants for food, and plants provide many important non-food products including wood, paper, dyes, textiles, medicines, cosmetics, and a wide range of industrial compounds. Understanding how plants defend themselves against pathogens and herbivores is essential to secure human food supply and develop highly disease-resistant and economically important crops.

1.1 PLANT-PATHOGEN INTERACTIONS

Plant-pathogen interactions, in particular those involving biotrophic pathogens, often consist of specific interactions between pathogen *avr* genes and the corresponding plant *R* genes.

Plant resistance is successful if compatible *avr* and *R* genes are present during plant-pathogen interaction. If either is absent, disease results (Flor, 1971). For the pathogen, the first step towards successful infection is to gain entry into the plant apoplast. Plant pathogens may secrete sticky polysaccharides that help them attach to the host surface. Some bacteria can also use microstructures called pili for attachment. Pathogens can gain entry to the plant apoplast by different means. Bacterial pathogens enter through wounds or natural openings like stomata or lenticels, while pathogenic fungi and oomycetes can penetrate host tissue by forming specialized organs called appressoria. Through the appressoria, the pathogen can secrete CWDEs, enabling penetration through the cuticle and the plant cell wall. Once inside the plant, the fungus forms specialized feeding organs called haustoria through which effectors can be introduced to suppress plant defenses. Viruses usually access the interior of plant cell using insect vectors. Viruses thus enter the plant through the wounds caused by insect feeding. Nematodes use brute physical force and literally dig into the host. Once inside, nematodes start feeding and introduce effectors through a structure called stylet (Glazebrook, 2005; Hématy et al., 2009; Hückelhoven, 2007).

Three broad groups of pathogens, necrotrophs, biotrophs, and hembiotrophs, are distinguished by their mode of pathogenicity and nutrient requirement (Glazebrook, 2005). Necrotrophs kill plant cells and acquire nutrients from the dead cells. Various fungal, bacterial, and oomycete pathogens belonging to this group attack with brute force: the production of toxins and CWDEs leads to extensive tissue maceration. Two types of necrotrophic pathogens exist: broad host-range necrotrophs (BHNs) and host-specific necrotrophs (HSNs). Examples of typical BHNs include the fungal pathogens *Botrytis cinerea*, *Alternaria brassicicola*, *Plectosphaerella cucumerina*, and *Sclerotinia sclerotiorum*, and the bacterial pathogen *Pectobacterium carotovorum*. These pathogens are capable of producing toxins that act on metabolic targets common to many plants. HSNs produce host-specific toxins (HSTs) that function only in susceptible cultivars lacking appropriate *R* genes. For example, the fungal pathogen *Cocbliobolus carbonum* produces HC-toxin and causes the Northern corn leaf spot (Mengiste, 2012a; Walton, 1996). In this sense, plant resistance response to this type of necrotrophs resembles the ETI, as it is conferred by single-gene encoded proteins that are able to detoxify HSTs.

On the other hand, biotrophic pathogens are obligate parasites, and propagate in living plant tissue without causing necrosis leading to cell death. Pathogens with a biotophic lifestyle include nematodes, viruses, and also some bacterial, fungal, and oomycete pathogens. They mostly penetrate host cell walls but not host cell membranes, and multiply between the cells without eliciting host defense. The level

of specialization required to establish an interaction between biotrophs and their hosts means that these types of pathogens tend to have a narrow host range (Glazebrook, 2005).

A third class of pathogens, called hemibiotrophs, have an initial biotrophic phase during which the pathogen actively suppresses the host immune system and multiplies in the host tissue. Later, the pathogen switches to a necrotrophic phase and induces cell necrosis, for example by massive secretion of toxins (Glazebrook, 2005). This class includes fungal, oomycete, and bacterial pathogens. For example, the oomycete pathogen *Phytophthora infestans* initially produces effectors that suppress plant defense responses, but at later phase produces necrosis-inducing effectors (Presti et al., 2015).

Upon pathogen recognition, all plants have the capacity to activate multilayered defenses. These include ROS production, phytohormones, and programmed cell death (PCD) that protect against disease. Since the diversity of organisms that interact with plants is enormous, our understanding of these interactions is still limited. In order to achieve broad-spectrum resistance in crop plants and to thoroughly understand immune recognition at the molecular level, identification of novel PAMP or DAMP recognition systems is necessary.

1.2 PATHOGEN RECOGNITION

There are surprising similarities in how animals and plants perceive pathogens. In animals, innate immunity is mediated by the Toll-like receptor (TLR) family that shares homology with plant transmembrane pattern recognition receptors (PRRs) (Ausubel, 2005; Jones and Dangl, 2006). In plants, two branches of recognition have been defined. There are the PRRs, which have the capacity to recognize a diverse range of pathogen /microbe-associated molecular patterns (PAMPs/MAMPs) resulting in PTI (Ausubel, 2005; Jones and Dangl, 2006; Macho and Zipfel, 2014; Zipfel et al., 2004, 2006). This type of defense is sufficient to resist non-pathogenic microbes, but not those capable of introducing effector proteins that suppress PTI. The second type of defense acts exclusively inside the cells using cytoplasmic receptors encoded by resistance (*R*) genes and has the capacity to recognize specific pathogen effectors resulting in effector-triggered immunity (ETI) (Jones and Dangl, 2006). Genetic studies of ETI have been tremendously influenced by Flor's gene-for-gene hypothesis, which posits that a single host resistance gene is matched by a single effector gene from a specific pathogen strain (Flor, 1971).

PRRs have the capacity to recognize biotrophic and necrotrophic pathogens by the structural patterns they bear, but necrotrophs may also be recognized as a result of the cellular damage they cause (Macho and Zipfel, 2014; Zipfel, 2014). Plant responses to biotrophic pathogens are better understood and usually involve the production of the defense

hormone SA and reactive oxygen species (ROS) (Mengiste, 2012b; Lai and Mengiste, 2013). Both further transmit the signal to induce late defense responses, such as cell wall fortifications, transcriptional activation of defense-related genes, synthesis of antimicrobial compounds (including phytoalexins), and production of callose. ROS can even act as an antimicrobial agent. Recognition of PAMPs and effectors triggers overlapping signaling responses in the plant and indicates differences in the speed, persistence, and robustness rather than the quality of response between PTI and ETI (Tsuda and Katagiri, 2010). Advances in understanding plant defense signaling include the recognition that the multitude of defense responses is mediated and amplified by an interacting set of phytohormones, i.e. jasmonic acid (JA), ethylene (ET), and SA that activate distinct sets of defense genes (Glazebrook, 2005; Reymond and Farmer, 1998).

1.2.1 Extracellular Recognition by pattern recognition receptors

Plants recognize a vast array of signals originating from microorganisms and the environment; recognition relies solely on each cell. In comparison to mammals, which use antigen-antibody interactions to recognize non-self, recognition in plants is based on a large number of extracellular surveillance-type receptors capable of detecting different types of pathogens and triggering defense signaling (Zipfel, 2014). Currently known plant PRRs are either surface-localized receptor kinases (RKs) or receptor-like proteins (RLPs) that recognize pathogen-derived PAMPs, but also the DAMPs that are present for recognition only after cell damage. The RK gene family contains approximately 610 members in the *Arabidopsis thaliana* genome, and many of these are responsive to biotic stresses (Lehti-Shiu et al., 2009). The RLP family has 57 members (Wang et al., 2008). In contrast to plants, animals possess 12 Toll-like receptors (TLRs) that fulfill equivalent roles to PRRs in plants (Gay and Gangloff, 2007). RKs have three common structures, a ligand-binding ectodomain, a single-pass transmembrane domain, and an intracellular kinase domain. RLPs share the same overall structure but lack an intracellular kinase domain. The PAMPs recognized by plants include proteins, carbohydrates, lipids, and small molecules such as ATP (Boller and Felix, 2009).

1.2.1.1 Recognition of bacteria

Recognition of bacterial PAMPs is best understood in the case of the *Arabidopsis* receptor kinase Flagellin Sensing 2 (FLS2), which binds bacterial flagellin directly and then assembles an active signaling complex (Gómez-Gómez and Boller, 2002). The recognition of bacterial flagellin by the LRR-RK FLS2 was the first plant PAMP/PRR pair to be characterized (Gómez-Gómez and Boller, 2002; Zipfel et al., 2004). Flagellin perception has also been described in animals, but FLS2 and mammalian TLR5 recognize

different flagellin domains. TLR5 binds to an epitope of flagellin formed by an N-terminal and a C-terminal part of the peptide chain (Smith et al., 2003). In plants, the receptor directly binds an epitope defined by a conserved stretch of 22 amino acids located close to the flagellin N terminus, referred to as flg22 (Chinchilla et al., 2006; Felix et al., 1999). Most higher plants are able to recognize flg22 (Boller and Felix, 2009), but species-specific differences of FLS2 revealed the ability of plants to recognize multiple epitopes of flagellin (Clarke et al., 2013; Takai et al., 2008). Comparative genome studies of field-isolated *Pseudomonas syringae* led to identification of a 28-amino acid epitope flgII-28 capable of inducing defense responses in *Solanum* and several other Solanaceae species. Interestingly, recognition of flgII-28 is FLS2-independent (Clarke et al., 2013). Since plants are unable to recognize flagellin inside the cell (Wei et al., 2013), PRR for flgII-28 derives most likely from RK or RLP.

Bacterial cold shock proteins and elongation factor Tu (EF-Tu) are another well-studied plant PAMP/PRR pair that activates defense responses similar to those triggered by recognition of flg22 (Zipfel, 2014; Zipfel et al., 2006). EF-Tu is directly recognized by the LRR-RK elongation factor Tu receptor (EFR). N-acetylated epitope elf18 (the first 18 amino acids of EF-Tu) binds to EFR. Interestingly, the ability to recognize elf18 is restricted within the plant kingdom to the family Brassicaceae (Boller and Felix, 2009; Kunze et al., 2004; Zipfel et al., 2006). Similarly to flg22, plants can also recognize EF-Tu through different epitopes besides elf18. For example, in *Oryza* the 50-amino acid epitope EFa50 obtained from the central region of EF-Tu was shown to induce immune responses through an unidentified PRR (Furukawa et al., 2013). Binding of flg22 and elf18 to FLS2 or EFR induces their association with co-receptor LRR-RK brassinosteroid insensitive 1-associated receptor kinase 1 (BAK1), leading to phosphorylation of both proteins and activation of downstream responses (Chinchilla et al., 2007; Roux et al., 2011; Schwessinger et al., 2011; Sun et al., 2013).

Plants can also recognize peptidoglycans (PGNs) derived from bacterial cell walls (Erbs and Newman, 2012; Gust et al., 2007). In *Arabidopsis*, two RLPs with lysine motif (LysM)-containing ectodomains, AtLYM1 and AtLYM3, were assigned to bind PGNs and to require LysMRK chitin elicitor receptor kinase 1 (CERK1) to induce immune responses (Willmann et al., 2011).

1.2.1.2 Recognition of fungi and oomycetes

Chitin is the major component of fungal cell walls and has been recognized as a classical PAMP for decades (Boller, 1995). LysM-RLP chitin oligomer-binding protein (CEBiP) was the first chitin-binding PRR identified in *Oryza* (Kaku et al., 2006). Recognition of chitin in *Oryza* requires homodimerization of the receptor and generation of a complex with OsCERK1. In *Arabidopsis*, AtCERK1 directly

binds to octamers of chitin leading to AtCERK1 homodimerization and sequential immune responses (Liu et al., 2012; Miya et al., 2007; Wan et al., 2008). Several other PAMP/PRR pairs have been implicated in plant–fungus interactions. LRR-RLP ethylene-inducing xylanase 2 (Eix2) is the PRR in *Solanum* for fungal xylanase (Ron and Avni, 2004), while fungal polygalacturonases (PGs) in *Arabidopsis* are recognized by RBGP1/RLP42 (Zhang et al., 2014). Heptaglucoside from the oomycete *Phytophthora infestans* is recognized by soluble betaglucan binding protein (GBP), but the transmembrane RK or RLP is still unknown. Many more PAMPs originating from oomycetes such as arachidonic acid (Bostock et al., 1982), major secreted elicitin INF1 of *P. infestans* (Tyler, 2002), and cellulose-binding elicitor lectin (CBEL) (Larroque et al., 2013) have also been identified, but thus far no PRRs have been identified for these.

1.2.1.3 Recognition of self-molecules

Plants can also sense endogenous molecules, referred to DAMPs, which can be recognized only after plant cell damage during pathogen attack or wounding triggered by herbivores (Boller and Felix, 2009; Galletti et al., 2009). In contrast to animals, only four well-characterized classes of DAMPs have been identified in plants to date (Table 1).

DAMP	Receptor	Co-receptor
Systemin	SR160 [a]	n.d.
Hydroxyproline-rich systemin	n.d.	n.d.
Plant elicitor peptides (Peps)	PEPR1/2 [b]	BAK1 [c] and BKK1 [d]
Oligogalacturonides (OGs)	WAK1 [e]	n.d.
Extracellular ATP (eATP)	DORN1 [f]	n.d.
*At*HMGB3 [g]	n.d.	BAK1 and BKK1

Table 1. Plant DAMPs. n.d. not determined; SR160: 160-kDa systemin cell-surface receptor; PEPR: PEP receptor; BAK1: BRI1-Associated receptor Kinase 1; BKK1: BAK1-LIKE Kinase 1; WAK1: Wall-Associated Kinase 1; DORN1: Does Not Respond to Nucleotides 1; AtHMGB3: Arabidopsis thaliana High Mobility Group Box 3 protein. Choi and Klessig, 2016.

The largest class are polypeptides/peptides isolated from *Salonum lycopercum*. These include three families of proteins universally referred to systemin – a term to describe polypeptide-induced defense signaling in response to physical damage (Pearce et al., 2001). Systemin was shown to induce the synthesis of wound-inducible proteinase inhibitor proteins (Pearce et al., 1991).

Another peptide-based DAMP/PRR pair was discovered in *Arabidopsis* (Huffaker et al., 2006). It involves plant elicitor peptides (Peps). In *Arabidopsis*, LRR-RKs PEPR1 and PEPR2 recognize Peps (Huffaker et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010; Yamaguchi-Shinozaki and Shinozaki, 2006). Peps induce a variety of innate immune response, including Ca^{2+} influx, induction of defense-associated genes (Yamada et al., 2016).

eATP is among the molecules that are released by cell damage and defines another class of plant DAMPs found in both plants and animals. *Arabidopsis* DORN1, a lectin receptor kinase, was shown to recognize

extracellular ATP (Choi et al., 2014). DORN1 is a member of a new purinoreceptor subfamily, P2K (P2 receptor kinase), which is plant specific and is required for ATP-induced cellular responses. Genetic analysis of loss-of-function mutants and overexpression lines demonstrated that DORN1 is involved in wound response (Choi et al., 2014). eATP treatment induces typical innate immune responses, however, it is not yet clear whether it contributes to resistance to pathogens.

A major category of plant DAMPs is the plant cell-wall fragments released by the action of CWDEs secreted by necrotrophic pathogens such as *P. carotovorum* or *B. cinerea*. Pectin is a central component in plant cell walls and forms the "glue" that keeps plant cells together. Consequently, many plant pathogens, including *P. carotovorum*, produce pectin-degrading enzymes as crucial virulence factors. However, the action of such enzymes releases oligomers of alpha-1,4-linked galacturonosyl residues (oligogalacturonides, OGs) from plant cell walls. OGs are subsequently recognized by the plant as DAMPs, leading to activation of innate immune responses. The wall-associated kinase 1 (WAK1) has been identified as a likely receptor for OGs in *Arabidopsis* (Brutus et al., 2010). Cuticle breakdown products can also act as potential signals that trigger plant defense. Treatment of *Arabidopsis* with cutin monomers was shown to induce the accumulation of defense-related genes, whereas cutinase-expressing plants displayed strongly enhanced immunity against the necrotrophic fungus *B. cinerea* (Chassot et al., 2007, 2008a).

Mechanical wounding of plant tissue either by herbivores or as a result of abiotic stress such as drought, cold, or UV irradiation also induces plant defenses. Thus far, little is known about the molecular recognition of herbivore-associated elicitors (HAEs). Cell wall fragments released from damaged cells might also be recognized by damage-associated mechanisms similar to recognition of DAMPs during microbial infection. Activation of signaling pathways during insect folivory shares high similarity to signaling pathways activated by PAMPs, further suggesting involvement of DAMP signaling in this type of recognition (Schuman and Baldwin, 2016).

1.2.1.4 PRR biogenesis and endoplasmic reticulum quality control

Recent studies have shown that endoplasmic reticulum quality-control mechanisms are crucial for PRR biogenesis. In eukaryotic cells, folding and maturation of the majority of membrane-localized proteins undergo quality control in the ER via a process termed as ER-QC. (Anelli and Sitia, 2008). A number of recent studies revealed that the EF-Tu receptor EFR is regulated by this mechanism (Häweker et al., 2010; Li et al., 2009; Lu et al., 2009; Nekrasov et al., 2009; Numers et al., 2010; Saijo et al., 2009). ER-QC relies mainly on Asn (N)-linked glycosylation of secreted proteins. Glycosylation is catalyzed by an

oligosaccharyltransferase complex (OST), which covalently attaches a complex polysaccharide containing three terminal glucose residues to the acceptor proteins. The glucose moieties are subsequently trimmed by glucosidase I (GI) and glucosidase II (GII) to produce mono-glucosylated glycans facilitating protein recognition and folding by the ER-resident chaperons, calnexin (CNX) and calreticulin (CRT). Properly folded proteins are transferred to their functional sites, whereas unfolded proteins are recognized by the UDP-glucose-glycoprotein glucosyl-transferase (UGGT). In this way, UGGT acts as a folding sensor, and the glycosylation process is closely related to protein maturation. Misfolded proteins are subsequently degraded (Hebert and Molinari, 2007; Pattison and Amtmann, 2009).

Another ER folding pathway is dependent on the binding immunoglobulin protein (BiP) chaperone. BiP binds to unfolded proteins using scaffolding with a set of proteins such as UGGT, calreticulin-3 precursor (CRT3), ER DnaJ 3 (ERdj3B), and ER lumen protein-retaining receptor B (ERD2b), which are required for EFR function and accumulation (Noh et al., 2003). Mutations within these genes determine plant susceptibility to pathogens, indicating that EFR is not the only immune protein controlled by ER-QC. Despite this, neither FLS2 nor CERK1 function is significantly affected in these mutants (Dodds and Rathjen, 2010).

1.2.2 Intracellular Effector Recognition

Pathogens produce small molecule effectors encoded by *avirulence (avr)* genes that can suppress PTI (Jones and Dangl, 2006; Zipfel, 2014). Successful pathogens manage to suppress PTI responses through the utilization of effectors, secreting them into the apoplast, or in the case of bacteria, directly into the plant cell using a type III secretion system (Chisholm et al., 2006; Jones and Dangl, 2006). Infection leads to disease development only if the pathogen manages to overcome ETI, a second layer of plant immunity. ETI depends on the recognition of effector proteins and is mediated by a class of intracellular receptor proteins that contain nucleotide-binding (NB) and leucine-rich repeat (LRR) domains. There are about 125 NB-LRR in the *Arabidopsis* genome. Many plant NB-LRR proteins also contain an N-terminal Toll-interleukin-like receptor (TIR) domain related to the intracellular signaling domain of animal Toll-like receptors (Gay and Gangloff, 2007). NB-LRR proteins directly or indirectly perceive highly variable effectors.

1.2.2.1 Direct and indirect recognition of effector proteins

Plant NB-LRR receptors are able to recognize pathogen-released effectors either by direct or indirect mechanisms (Caplan et al., 2008; Collier and Moffett, 2009; Zipfel, 2014). Three models have been postulated to describe these mechanisms. None of the ‘direct’, and indirect ‘guard/decoy’ and ‘bait-and-switch’ models

govern how recognition of effectors activates defense mechanisms and are limited to very specific examples. In direct recognition, effector proteins trigger immune responses resulting from physical association with the receptor leading to conformational changes. The fungal effectors Avrl567 and AvrM are the best studied examples of direct recognition (Catanzariti et al., 2010; Dodds et al., 2004). Nevertheless, in most of the studied cases indirect recognition has been observed. In the 'guard/decoy' model this type of recognition is mainly based on effector ability to modify the real binding partner of the R protein enabling the NB-LRR receptor to recognize it (Hoorn and Kamoun, 2008). In the 'bait-and-switch' model the interaction of an effector with its target protein is recognized by the R protein (Dodds and Rathjen, 2010).

A massive diversity in effector and receptor pairs suggests that novel recognition strategies are likely to be identified. The best-studied *Arabidopsis* R protein (resistance to *P. syringae* pv maculicola 1) RPM1-Interacting Protein 4 (RIN4) fits the guard model. Not only does RIN4 physically interact with the R proteins RPM1 and resistant to *P. syringae* 2 (RPS2), but it is also modified by three *Pseudomonas* effectors AvrRpm1, AvrB, and AvrRpt2 (Mackey et al., 2002, 2003).

1.2.2.2 NB-LRR activation of immune response

In general, NB-LRR is a conserved multidomain switch that translates pathogen signals into an immune response (Collier and Moffett, 2009). How effector recognition leads to NB-LRR activation is not yet fully understood. In the absence of an effector, NB-LRR proteins are retained in a restrained conformation. In most cases, NB-LRR proteins are self-inhibited by intramolecular interactions holding the protein in an inactive state until effector recognition releases the inhibition (Takken and Goverse, 2012). The NB domain appears to be essential for the function of all plant NB-LRR proteins and signal activation may involve an exchange of ATP and ADP in the binding site (Tameling et al., 2006). Additionally, TIR-NB-LRR proteins have similar signaling capacity as animal NB-containing leucine rich proteins such as NLRs and apoptotic factors apoptotic protease activating factor 1 (APAf1) and cell death protein 4 (CED4). Overexpression of TIR-NB-LRR proteins is sufficient to trigger HR and plant defense signaling in general (Swiderski et al., 2009). Recent observations suggest that NB-LRR proteins relocate to the nucleus where they interact with transcription factors to trigger changes in gene expression. However, no signaling partners of NB-LRR proteins have been identified in the nucleus thus far.

1.3 DEFENSE RESPONSES DOWNSTREAM OF PATTERN RECOGNITION RECEPTORS

Plants respond to pathogens with large-scale transcriptional changes. These early and late

defense responses include production of ROS, increased synthesis of phytohormones, up-regulation of pathogenesis-related (PR) genes, synthesis of antimicrobial compounds (including phytoalexins), production of the polysaccharide callose, HR and lastly, immunity (Boller and Felix, 2009; Jones and Dangl, 2006).

ROS play a central part in the activation of innate immunity signaling triggered by PAMP-PRR and DAMP-PRR interactions (Macho and Zipfel, 2014). The rapid accumulation of ROS after pathogen recognition is commonly referred to as oxidative burst (Bolwell et al., 2002; C J Baker and Orlandi, 1995; Mehdy, 1994) and is accompanied by changes in extracellular pH, ion fluxes, activation of mitogen-activated protein kinases (MAPKs) and Ca^{2+}-dependent protein kinases (CPKs and CDPKs) (Davies et al., 2006; Wojtaszek, 1997).

1.3.1 Short-term responses: minutes after pathogen recognition

Plant recognition of pathogen-derived PAMPs or effector proteins triggers several early defense responses, including ROS production, calcium flux, and MAPK activation. These early events mount late defense responses, including activation of defense-related genes, cell wall strengthening, induction of ethylene biosynthesis, and HR (Dixon, 2001; Greenberg and Yao, 2004; Ausubel, 2005; Glazebrook, 2005; Jones and Dangl, 2006; Boller and Felix, 2009; Coll et al., 2010; Reimer-Michalski and Conrath, 2016).

1.3.1.1 Oxidative burst

Among the responses downstream of the PAMP-PRR interaction, oxidative burst is one of the earliest, initiating only a few minutes after PAMP recognition (L'Haridon et al., 2011). Pharmacological studies suggest that the major sources of apoplastic oxidative burst are cell membrane localized NADPH oxidases and class III apoplastic peroxidases (Bolwell et al., 2002; Grant et al., 2000). Apoplastic oxidative burst is composed primarily of H_2O_2 and O_2^-. These oxidative species can be detected after pathogen recognition and are collectively termed as ROS. Formation of ROS in plants is generated in a biphasic pattern. A low- magnitude transient rise in ROS occurs several minutes after pathogen recognition and decreases within an hour. In plants, the first burst is usually followed by a sustained and stronger second burst that appears between 1.5 and 6 hours after a successful R-effector recognition event. Therefore, an acute HR response contributing to PCD and to SAR is a part of a successful ETI response (Dodds and Rathjen, 2010; Durrant and Dong, 2004; Stael et al., 2015).

The importance of oxidative stress in defeating invading pathogens has been shown both genetically and pharmacologically. For example, transgenic plants overexpressing apoplastic peroxidases are more resistant to bacterial and fungal pathogens (Chassot et al.,

2007). Apoplastic *PER33* and *PER34* peroxidases were also shown to play important roles in PTI in response to variety of PAMPs. T-DNA lines targeting *PER33* and *PER34* exhibited diminished oxidative burst after infiltration with PAMPs and increased susceptibility to local infections. Pharmacological inhibitor-based studies demonstrated that inhibition of ROS results in a reduced HR (Desikan et al., 1998; Levine et al., 1994). In general, ROS is critical for development of the HR response and to induce PCD. Once HR has been triggered, the plant tissues become highly resistant to a broad range of pathogens. This phenomenon is termed SAR, which provides resistance against secondary infections for an extended period of time (Gaffney et al., 1993).

In summary, it is rather clear that the primary apoplastic oxidative burst influences the further activation of generic plant immune responses associated with microbicidal actions.

1.3.1.2 Calcium flux

Increased Ca^{2+} concentration is one of the first detectable responses in plant-pathogen interactions closely linked to oxidative burst (Vadassery and Oelmüller, 2009). Two independent groups demonstrated that inhibition of calcium flux eliminates the oxidative burst (Blume et al., 2000; Grant et al., 2000). Ca^{2+} acts as a second messenger in numerous plant signaling pathways and even small changes in its concentration provide information for protein activation and signaling (Lecourieux et al., 2002). Downstream of PRR-PAMP activation, the activation of defense-related genes and accumulation of phytoalexins is mediated by Ca^{2+} fluxes at the plasma membrane. Ca^{2+} also plays a role in determining plasma membrane structure and function (Hepler, 2005). Ca^{2+} binds to phospholipids, stabilizes lipid bilayers and provides structural integrity as well as controlling plasma membrane permeability (Hepler, 2005). Calcium-dependent signaling responses are mediated by Ca^{2+} effectors in the nucleus, including calmodulin (CaM), CaM-binding protein, CDPKs, and CaM-regulated protein phosphatases (Bouché et al., 2005; Lee et al., 2004; Lévy et al., 2004). In addition, calcium-dependent processes are accompanied with post-translational modifications by reversible phosphorylation, including common signaling components such as MAPKs.

1.3.1.3 MAPK cascades in plant disease resistance

Plant MAPKs play important roles in plant defense against pathogen attack via signal transduction generated by PRRs or R proteins (Chisholm et al., 2006; Dodds and Rathjen, 2010; Rodriguez et al., 2010). Activation of PRRs triggers MAPKs within minutes after pathogen recognition, which leads to biosynthesis of stress hormones, stomatal closure, defense gene activation, phytoalexin biosynthesis, and HR cell death. Activation of MAPKs is carried out by upstream MAPK

kinases (MAPKK). MAPKK, in turn, are regulated by their upstream kinases, MAPKK kinases (MAPKKK). These three-kinase cascades, which function downstream of PRRs, generate signals into cellular responses (Chang and Karin, 2001; Widmann et al., 1999). Interestingly, ROS signaling is also mediated through the MAPK cascade. For example, H_2O_2 can specifically activate the MAPKKK ANP1, which then leads to an activation of the pathogen-inducible MAPKs MPK3 and MPK6 (Kovtun et al., 2000). Both of these MAPKs are regulated by NDPK2, another kinase that is involved in a feedback loop with ROS generation (Moon et al., 2003). The best-characterized plant PRRs include FLS2, EFR, and CERK1. All of these can trigger strong but transient activation of MAPKs in *Arabidopsis* (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006; Miya et al., 2007; Wan et al., 2008; Rasmussen et al., 2012; Liu and He, 2016; de Zelicourt et al., 2016). Very similar sets of genes are induced by elf18 and flg22, however, they do not show an additive effect in the activation of MPK3 and MPK6. *Arabidopsis* MPK3 and MPK6 are also activated by the fungal elicitor chitin and bacterial peptidoglycans (PGN) (Jones and Dangl, 2006; Zipfel et al., 2006; Miya et al., 2007; Macho and Zipfel, 2014). This data suggests that these MAPKs are crucial components in PAMP signaling. One major gap in our understanding of plant defense signaling is the linkage between PRR receptors and MAPK cascades, and identification of specific MAPK substrates.

1.3.2 Long-term responses: hours after pathogen recognition

Early local responses usually interact with late defense responses, ultimately leading to initiation of SAR, which is a result of enhanced resistance to pathogen challenge (Spoel and Dong, 2012; Vlot et al., 2008).

1.3.2.1 Hypersensitive response

The HR response in plants is highly localized cell death that may be triggered by pathogen attack (Govrin and Levine, 2000; Greenberg and Yao, 2004; Levine et al., 1994). HR is an effective host-regulated defense response and contributes to plant immunity by killing the infected host cell and thus, associated pathogens. In animals, PCD known as apoptosis shares many apparent parallels with those characterized in plants. Nevertheless, there are important differences between apoptosis and HR. In apoptosis, cytoplasmic condensation leads to the fragmentation of the cell into apoptotic bodies linked to proteolytic enzymes known as caspases. In plants, however, no gene sequence for a caspase has been found. Instead, pioneering work lea by Ikoko Hara-Nishimura has shown that vacuole-derived proteases are central for a mosaic-elicited HR in tobacco (Hatsugai et al., 2004). In plants, HR is a form of autophagy where cytoplasmic contents are packaged

within a membrane prior to degradation inside the vacuole or lysosome.

HR has different roles in plant responses to biotrophs and necrotrophs. HR increases the resistance to biotrophic pathogens but promotes susceptibility to necrotrophs (Mengiste, 2012b). However, it remains unclear whether this applies for to all plant-necrotroph interactions. Apparently, the HR response is induced and mediated by oxidative burst. ROS-induced HR-PCD also involves reactive nitrogen species (RNS), ER and Ca^{2+} in a coordinated regulation of HR (Bellin et al., 2012; Torres, 2010; Wang et al., 2013). Once HR has been triggered, the plant tissues become highly resistant to a broad range of pathogens. Activation of SAR provides resistance against secondary infections for an extended period of time (Gaffney et al., 1993).

1.3.2.2 Systemic acquired resistance

SAR in plants is the mechanism of induced defense that mounts long-lasting protection against a broad range of pathogens (Durrant and Dong, 2004; Grant and Lamb, 2006; Vlot et al., 2008). SAR requires the stress hormone SA and is associated with accumulation of PR proteins. Early grafting experiments demonstrated that SA itself is not the mobile signal for SAR. Results obtained in tobacco showed that despite the inability of *nahG*-expressing rootstocks to accumulate SA, the SAR signal was still produced and translocated into the scion (Vernooij et al., 1994). It is likely that systemic resistance may involve multiple signals. One of these may be methylated salicylic acid (MeSa). In plants where the lower leaves were treated with MeSA, SAR developed in the upper leaves. However, there is also evidence against MeSA being a systemic signal. For example, S-adenosy-lmethionine-dependent methyl-transferase (*bsmt1*) mutant plants unable to produce MeSA accumulate SA and induce SAR in distal leaves (Attaran et al., 2009; Liu et al., 2011a, 2011b; Park et al., 2007). There is also evidence for several small lipids possibly acting as mobile signals. Glycerol-3-phosphate and dehydroabietinal activate SAR, but the nature of these signals is still unclear (Chanda et al., 2011; Chaturvedi et al., 2012; Jung et al., 2009).

1.3.2.3 Cell wall fortification

The cell wall is the major boundary of defense against fungal and bacterial pathogens (Hückelhoven, 2007; Davidsson et al., 2013). The reinforcement of the cell wall is an important pathogen-induced defense response. Among the proteins induced during plant defense, class III peroxidases appears to be key enzymes by catalyzing the cross-linking of cell wall components such as polysaccharides, glycoproteins, lignin, and suberin (Almagro et al., 2009; Kärkönen and Kuchitsu, 2015).

Cell wall rigidity depends on lignin composed of phenolic compounds. Lignin has multiply roles in plant defense. It acts not only as physical barrier, the phenyl-propanoid pathway responsible for lignin biosynthesis

may also be recruited for defense purposes. For example, this pathway set up the synthesis of other phenolic compounds including phytoalexins, stilbenes, coumarins, and flavonoids implicated in plant defense (Dicko et al., 2005). Disruption of lignin biosynthesis pathway compromises resistance to pathogens. For example, the *Arabidopsis* caffeic acid O-methyltransferase 1 (*comt1)* mutant shows decreased levels of lignin when compared to wild-type controls, ultimately is more susceptible against *P. syringae* and *B. cinerea* (Goujon et al., 2003).

Cellulose deficient mutants were first discovered through screening for mutants with altered disease resistance. In *Arabidopsis*, cellulose synthase *cesa4*, *cesa7*, and *cesa8* mutants fail to develop disease symptoms against necrotrophic pathogens such as fungus *Plectosphaerella cucumerina* and the soil-borne bacterium *Ralstonia solanacearum* (Hernández-Blanco et al., 2007). Treatment with isoxaben, an inhibitor of cellulose biosynthesis, also demonstrated compromised resistance to necrotrophic pathogens (Hamann, 2012).

Hemi-celluloses are another group of cell wall polysaccharides that can negatively impact the accessibility of pathogen-derived enzymes to cellulose. Xylans are predominant hemi-celluloses in secondary plant cell walls. Some microbes secrete xylanases recognized as PAMPs. For example fungi *Trichoderma* produces ethylene-inducing xylanase (EIX). In *L. esculentum* EIX is recognized by RLPs LeEix1 and LeEix2 (Ron and Avni, 2004).

Variation in glycan and pectin composition has also been associated with pathogen resistance. Pectin strengthen the cellulose-hemicellulose network and is critical for tissue integrity and rigidity. Powdery mildew-resistant mutants, *pmr5* and *pmr6*, altered in pectin matrix showed enhanced resistance to the biotrophic pathogen *Erysiphe cichoracearum* (Vogel et al., 2002, 2004).

In summary, cell wall integrity is important in plant defense. Cell wall-associated plant defenses such as pathogen-triggered lignification, structural alterations to cell wall polysaccharides is therefore spatially a first line of defense and not a static barrier.

1.3.2.4 Callose deposition

Plant cells also respond to pathogen attack by synthesizing and depositing callose between the plasma membrane and the inner surface of plant cell wall adjacent to the invading pathogen (Ellinger and Voigt, 2014; Voigt, 2014). Callose deposits, called papillae, consist of β-1,3 glucan polysaccharide. Together with ROS and phytoalexins, papillae arrest pathogen penetration at the site of infection. Callose act as a barrier while ROS and phytoalexins are toxic to pathogens. Accumulation of ROS mediates callose deposition, since plants with defects in peroxidase-derived ROS generation exhibit impaired callose deposition (Daudi et al., 2012; Wrzaczek et al., 2013).

1.4 HORMONE CROSSTALK IN PLANT DISEASE AND DEFENSE

Resistance on the whole plant level depends on systemic signals mediated by plant hormones (Bari and Jones, 2009; Robert-Seilaniantz et al., 2011). Most studies on systemic defense signals in plant-pathogen interactions have focused on classical defense hormones, SA, JA, and ET, which are all central to plant immune responses. Gibberelic acid (GA), abscisic acid (ABA), auxin (IAA), brassinosteroids (BL), and cytokinins (CK) have recently emerged as important modulators of plant defenses against pathogens. Enhanced accumulation of different phytohormones is a common plant response to infection and mainly relies on positive and negative regulators, which modify hormonal crosstalk during disease and defense (Robert-Seilaniantz et al., 2011).

Classically, SA signaling triggers resistance towards biotrophic and hemibiotrophic pathogens, whereas JA and ET signaling trigger resistance against necrotrophic pathogens. These two signaling pathways usually function antagonistically. Accordingly, increased resistance to biotrophs often promotes enhanced susceptibility to necrotrophic pathogens, and vice versa (Glazebrook, 2005). The role of hormones in immune responses varies among plant species and depends on the lifestyle of the invading pathogen. For example, GA-induced degradation of DELLA protein growth repressors leads to elevation of ROS and SA, ultimately leading to attenuation of JA signaling and susceptibility to necrotrophic pathogens (Achard et al., 2008; Navarro et al., 2006). In contrast, both BLs and CKs promote resistance to pathogens due to enhanced SA signaling (Choi et al., 2010; Divi et al., 2010). Overall, pathogen-triggered activation of hormonal crosstalk establishes effective systemic immunity against a broad range of pathogens.

1.4.1 The Role of SA, JA and ET in modulating resistance and susceptibility to biotic stress

In response to pathogens, SA, JA, and ET activate distinct sets of genes involved in defense signaling (Glazebrook, 2005; Reymond and Farmer, 1998). By using an *Arabidopsis dde2*/*ein2*/*pad4*/*sid2* quadruple mutant, Tsuda et al. revealed complex interactions between SA, JA, and ET signaling (Tsuda et al., 2009). The immunity of the quadruple mutant was severely compromised against *Altenaria brassicicola* compared with the corresponding single mutants, suggesting that SA, JA, and ET signaling positively and synergistically contribute to immunity against *A. brassicicola.* During PTI, these three phytohormones seemingly amplify the response to maintain a sufficient level of pathogen resistance (Tsuda et al., 2009). In the ETI response, interactions between SA, JA, and ET result in an even more robust signal flux.

Less in known about responses to necrotrophic pathogens compared to biotrophs, since very few R genes conferring resistance to necrotrophs have been characterized so far. Classically, JA has been shown to play a central role in plant responses to necrotrophs. Accordingly, plants impaired in JA signaling are more sensitive to pathogens with a necrotrophic lifestyle (Mengiste, 2012a). JA responses are mostly mediated through the CORONATINE INSENSITIVE1 (COI1) receptor (Browse, 2009; Fonseca et al., 2009; Sheard et al., 2010). COI1 belongs to the F-box protein family and forms Skp1/Cullin1/F-box protein COI1 (SCF^{COI1}) complexes with *Arabidopsis* Cullin1and *Arabidopsis* Skp 1-like1 (ASK1) to recruit its substrate JA ZIM-domain proteins for ubiquitination and degradation. Loss of function in mutants of *coi1* results in insensitivity to JA and increased accumulation of SA. Ultimately, this leads to increased resistance to biotrophic bacterial pathogens and increased susceptibility to necrotrophic fungal pathogens (Thomma et al., 1998). The cross-talk between JA and SA has been supported by many experimental studies. For example, plant defensing *PDF1.2* is strongly induced by JA. However, when JA and SA are applied together, the expression levels of *PDF1.2* remain intact. Mutually antagonistic roles of JA and SA could be due to the fact that HR enhances necrotroph pathogenicity, whereas HR should be suppressed in the presence of necrotrophs. Interestingly, *B. cinerea* produces certain exopolysacharides, which (via activation of SA-dependent signaling) antagonize the JA pathway, leading to enhanced susceptibility in *Solanum* and *Arabidopsis* (Oirdi et al., 2011). Some pathogens like the hemi-biotroph *Pseudomonas* can take advantage of SA-JA signal cross-talk. Coronatine produced by *Pseudomonas* is an mimic of JA-Ile, the active jasmonate hormone (Geng et al., 2014). Bacteria capable of producing coronatine significantly enhance their pathogenicity by modulating plant defense signaling on their own benefit.

ET shares synergism with JA signaling and accordingly also has an important role in resistance to necrotrophic pathogens. Recognition of ET promotes EIN2-dependent expression of EIN3 transcription factor (Boutrot et al., 2010; Zhao and Guo, 2011). EIN3 is involved in the regulation of FLS2-BIK1 complex in early PTI responses, while EIN2 is required for flagellin-induced PTI to necrotrophic and biotrophic pathogens (Boutrot et al., 2010). JA-ET signaling pathway is also a central component of induced systemic resistance (ISR) defense response. Genetic studies indicate that the JA and ET pathways are both necessary for ISR, which does not involve the accumulation of defense proteins or an increase in the levels of JA or ET hormones. A current model suggests that the ISR includes elevated levels of inactive defense-associated transcription factors, ready for a rapid response when required (Groen et al., 2013; Pieterse et al., 2014).

1.4.2 The role of ABA in modulating resistance and susceptibility to biotic stress

ABA is known to have a central role in plant development, seed germination, and dormancy processes, as well as in abiotic stress responses (Nambara and Marion-Poll, 2005; Ton et al., 2009). More recently, it has become clear that ABA signaling also influences disease resistance. Depending on the lifestyle of the invading pathogen, ABA can have either a negative or positive role in influencing the outcome of the interaction. For example, elevated levels of ABA negatively affect defense against the soil-born fungus *Fusarium oxysporum* by having an antagonistic effect on the JA-ET signaling network (Anderson et al., 2004). Similarly, resistance to fungal and bacterial pathogens is enhanced in the ABA-deficient *Solanum* mutant *sitiens* associated with production of H_2O_2 and enhanced cuticle permeability (Asselbergh et al., 2007; Curvers et al., 2010). Drought-induced accumulation of ABA was shown to decrease resistance to *P. syringae* and *B. cinerea* in *Arabidopsis* plants (L'Haridon et al., 2011; Mohr and Cahill, 2003). These studies indicate that ABA accumulation during abiotic stress results in enhanced susceptibility both to necrotrophic and biotrophic pathogens. Accordingly, enhanced resistance to necrotrophic pathogens was also observed in *aba1* and *aba2* mutants deficient in ABA biosynthesis, and in an *abi4-1* mutant insensitive to ABA, further supporting the negative role of ABA in resistance to necrotrophic pathogens (Asselbergh et al., 2007; Curvers et al., 2010). On the other hand, *aba1*, *aba2*, and *abi4-1* mutants were more susceptible to biotrophic *Pythium irregulare* and *Altenaria solani* pathogens, highlighting the different roles of ABA in resistance to necrotrophic and biotrophic pathogens (Adie et al., 2007). Furthermore, in *Arabidopsis,* bodyguard *bdg* and long chain acyl-CoA synthetase 2, 3 *lacs2.3* cuticular mutants were previously shown to have increased cuticle permeability, increased accumulation of ROS, severe leaf deformations, increased accumulation of cuticular waxes, and enhanced resistance to *B. cinerea.* Exogenous application of ABA completely removed ROS and restored both the cuticle as well as plant susceptibility to *B. cinerea* (Asselbergh et al., 2007; Curvers et al., 2010; L'Haridon et al., 2011), further indicating the negative impact of ABA on ROS production and resistance to necrotrophic pathogens. Long known for its role in biotic stress, ABA can also promote plant defense. Its negative or positive role in disease resistance depends on the type of pathogen and evidently modulate immune responses through ROS generation, defense gene expression, cuticle permeability, and callose accumulation (Robert-Seilaniantz et al., 2011).

1.4.3 The role of F-box proteins in hormone Sensing

Plant genomes encode large numbers of F-box proteins. F-box genes can be categorized on the basis of the presence of recognizable domains. Out of ~700 F-box protein genes encoded in *Arabidopsis*, 67 F-box proteins contain Kelch repeats, 29 leucine-rich repeats (LRRs), and two F-box proteins contain tryptophan-aspartic acid (W-D) WD40 repeats that are found in humans and other organisms (Kuroda et al., 2002). Kelch repeats, LRRs, and WD40 repeats are implicated in protein-protein interactions. The rest of F-box proteins were originally categorized as F-box only (FBXO) proteins, but contrary to their name, these F-box proteins often have conserved homology domains that were either not recognized or are not present in a large number of F-box proteins. Many of these F-box proteins act as important receptors in plant hormone signaling pathways (Gagne et al., 2002). For example, the F-box protein transport inhibitor response 1 (TIR1) is an auxin receptor in *Arabidopsis* (Dharmasiri et al., 2005; Kepinski and Leyser, 2005), while the F-box protein GID2 is a GA receptor that directly interacts with a negative regulator SLR1, a DELLA protein (Ikeda et al., 2001; Itoh et al., 2003). DELLA proteins in *Arabidopsis* are major negative regulators of GA signaling. An interaction with the Skp1-Cullin-F-box (SCF) complex induces rapid degradation of DELLA proteins and promotes transcription of GA-responsive genes (Gomi et al., 2004). F-box proteins contain a conserved signature F-box domain of 35-60 amino acids at the amino-terminus, which is an important component in the ubiquitin (Ub) proteasome pathway (Kipreos and Pagano, 2000). Recent research in plant hormone signaling pathways has implicated the ubiquitin (Ub) proteasome pathway as central regulatory mechanism in signal transduction mediated by different hormones. F-box proteins bind to Skp1 or Skp1-like proteins and form an E3 ubiquitin ligase SCF protein complex (Zheng et al., 2002). The JA signaling pathway is central in modulating defense against necrotrophic pathogens. Most of the JA responses are mediated through the JA receptor, COI1 F-box protein (Browse, 2009; Sheard et al., 2010). Moreover, in the ET signaling pathway, two *Arabidopsis* F-box proteins, ethylene insensitive 3 (EIN3)-binding F-box protein 1 (EBF1) and EBF2, target the transcriptional activator EIN3 for degradation (Gagne et al., 2004; Guo and Ecker, 2003; Potuschak et al., 2003). This suggests that the Ub proteasome pathway negatively regulates ET signaling (de Torres Zabala et al., 2009; Tsuda et al., 2008). Taken together, F-box proteins in plants target regulatory proteins of hormone signaling pathways to the Ub complex for destruction, and these networks cross-talk with each other through these modified regulatory proteins.

1.5 THE ROLE OF CUTICLE IN PLANT PATHOGEN INTERACTIONS

The cuticle is an extracellular hydrophobic layer that covers the outer surface of epidermal cells. The cuticle provides plants with protection against water loss and environmental biotic and abiotic stresses (Yeats and Rose, 2013). The hydrophobic nature of the cuticle also prevents water from collecting on the leaf surface, which inhibits spore germination or adhesion of fungal and bacterial pathogens. Generally, the cuticle consists of cutin and epicuticular and intracuticular waxes. Cutin consists of esterified hydroxy and epoxy C16 and C18 fatty acids and glycerol (Heredia, 2003). The cuticular wax contains very long-chain fatty acids between 20 to 40 carbon atoms. Most of the genes involved in cuticle biosynthesis, transportation, and assembly have been characterized in *Arabidopsis* (Bourdenx et al., 2011; Lee and Suh, 2013; Li et al., 2007). Recently, a number of studies implicated the cuticle as a signal source in relation to leaf pathogen interactions (Reina-Pinto and Yephremov, 2009). The action of fungal and bacterial CWDEs releases cuticle breakdown products that can be recognized by plants as stress signals. A wide range of plants was tested with synthetic C18 family analogs that were effective in triggering defense against *Erysiphe graminis* in barley and *Magnaporthe grisea* in *Oryza*. This defense involved the production of ET and enhanced expression of defense-related genes (Schweizer et al., 1994, 1996). Interestingly, cutinase-induced resistance against *Rhizoctonia solani* was observed in bean, independent of the SA-mediated signaling pathway (Parker and Köller, 1998). This led to further investigations where cutinase-expressing plants (CUTE plants) generated with a partly absent cuticle were shown to exhibit immunity against the necrotrophic fungus *B. cinerea* independently of SA, JA, and ET signaling (Chassot et al., 2007). This intriguing association between increased cuticular permeability and increased immunity against a necrotrophic fungus led to a number of studies in *Arabidopsis* mutants impaired in cuticular biosynthesis. All tested cuticular mutants lacerate (*lcr*), hothead (*hth*), *bdg, lacs2/bre1,* symptoms to multiple avr genotypes 4 (*sma4*), and permeable cuticle 1 (*pec1*) and transgenic line CUTE displayed increased resistance to *B. cinerea* (Bessire et al., 2007; Chassot et al., 2007). In addition to resistance, many of these cuticular mutants spontaneously accumulated ROS (Benikhlef et al., 2013; L'Haridon et al., 2011). While the action of fungal cutinase also leads to the accumulation of ROS (L'Haridon et al., 2011), the site of this ROS production has remained elusive. Increased cuticular permeability was also observed in *aba2* and *aba3* mutants deficient in ABA biosynthesis. These plants also showed enhanced accumulation of ROS (L'Haridon et al., 2011), suggesting that the ABA signaling pathway is involved in the regulation of cuticle formation. Overall, the resistance of plants with increased

permeability could be explained by several yet unverified scenarios. Increased cuticular permeability can involve a faster perception of cell wall components upon the action of CWDEs. Additionally, cutin monomers might also be overproduced in cuticular mutants. Recognition of such monomers would trigger defense responses involving ROS production, antimicrobial proteins, and antifungal metabolites (Bessire et al., 2007; Chassot et al., 2007, 2008b).

2. AIMS OF THE PRESENT STUDY

This work aimed at gaining novel insights in the interaction between plants and bacterial and fungal phytopathogens. The model system employed a biotrophic bacterial pathogen *Pseudomonas syringae* and two necrotrophs, the soft rot bacterium *P. carotovorum* and the fungus *B. cinerea*. The effects of these pathogens were studied on the model plant *Arabidopsis*. Specifically, this included the identification of DAMP-induced components involved in plant innate immunity and functional studies of mutants isolated based on alterations in their responses to OGs and phytopathogens.

Another approach of this study was to analyze a non-redundant protein database of *Arabidopsis* with the C-terminal HDEL motif implicated in EFR-biogenesis and to determine whether any other ER-localized proteins are potentially involved in EFR biogenesis and signaling. Additionally, this study combined physiological, molecular, and genetic approaches to characterize the role of AFB4 in defense against necrotrophic pathogens.

The specific aims of this study were:

1. To characterize the role of glucosidase II β-subunit in EFR biogenesis and EFR-mediated defense signaling.

2. To characterize the role of AFB4-mediated signaling involved in biotic stress tolerance and plant development.

3. To characterize the role of class III apoplastic peroxidases in OG-triggered signaling and in plant immunity against necrotrophic fungal and bacterial pathogens.

3. MATERIALS AND METHODS

Materials, methods, and model organisms used in this study are presented in detail in the following publications (I-III).

Method	Publication
Plasmid construction	I-III
Generation and characterization of transgenic plants	III
Transformant selection	III
Determination of bacterial growth in plants, (CFU)	I, III
Plant growth retardation assays	I, III
Cuticle permeability and cell wall fortification assays	III
Infection of *Arabidopsis* with *P. carotovorum*	III
Infection of *Arabidopsis* with *B. cinerea*	III
Infection of *Arabidopsis* with *P. syringae*	I, III
Isolation of plant proteins	III
Hormonal assays	III
Peroxidase activity assays	III
Protein inhibitory assays	III
Quantitative ROS production analysis	I, III
Callose staining	I, III
Treatment with elicitors of defense responses	I-III
DNA/RNA extraction and purification	III
Genetic mapping with SSLP markers	III
DNA sequencing data analysis	III
PCR	I-III
RT-PCR	I-III
Quantitative RT-PCR	I-III

Organism/model	Publication
All plants and mutant lines were in the background of *Arabidopsis,* ecotype Columbia (Col-0), and ecotype C24	
Arabidopsis thaliana	I-III
P. carotovorum spp. *carotovorum* SCC1	III
B. cinerea	III
Pseudomonas syringae pv. *Tomato* DC3000	I, III

4. RESULTS AND DISCUSSION

4.1 REQUIREMENT OF GLUCOSIDASE II B-SUBUNIT (ATGCSIIB) IN EFR-MEDIATED DEFENSE SIGNALING (I)

Plants sense attacking pathogens through recognition of PAMPs/DAMPs by PRRs located on the cell surface (Jones and Dangl, 2006; Zipfel, 2008). For example, *Arabidopsis* detects a variety of PAMPs/DAMPs including bacterial flagellin and EF-Tu, or their peptide epitopes flg22 and elf18, through the PRRs FLS2 and EFR, respectively (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006). Both FLS2 and EFR are transmembrane glycoproteins that undergo maturation within the secretory pathway before reaching their final destination at the plasma membrane. In endoplasmic reticulum (ER), a long chain glycan (Glc3Man9GlcNAc2) is initially attached to an asparagine residue of the polypeptide. This glycan is then stepwise trimmed by removing two terminal glucose residues by glucosidases I and II, respectively. This trimming is monitored by different chaperones in a process called ER-quality control (ER-QC). The chaperones mediate proper folding of the proteins only if the two glucose units are removed, after which the third glucose is also removed and the proteins are transported to the Golgi apparatus for further processing. (Anelli and Sitia, 2008). Unfolded proteins are retained in the ER until they are properly folded or ultimately degraded by ER-associated degradation (ERAD) in the cytosol (Vitale and Boston, 2008). Study I elucidated the role of the glucosidase II β-subunit required for proper accumulation of the EFR in the plasma membrane.

4.1.1 ATGCSIIβ mutants are compromised in EFR but not FLS2 signaling

Previous studies have established that EFR biogenesis requires a subset of ER-QC components including CNX, CRT3, UGGT, SDF2, ERdj3B, and BiP (Crofts et al., 1998; Denecke et al., 1995). A loss of any of these leads to a complete loss of EFR accumulation (Li et al., 2009; Saijo et al., 2009). All of these proteins harbor a C-terminal ER-retention signal, either HDEL or KDEL (Lewis and Pelham, 1992; Semenza et al., 1990). The retention of soluble proteins in the ER depends on the ER-lumen protein-retaining receptor ERD2 that binds to the C-terminal sorting signal of ER-escaped proteins and retrieves them back to the ER. To determine whether any other ER-localized soluble proteins are putatively involved in EFR biogenesis, we performed a BLASTP analysis with HDEL C-terminal motif. BLASTP analysis identified 15 proteins sharing the HDEL motif in *Arabidopsis*. Interestingly, one of these, a calmodulin binding protein, shared high homology with the human Glucosidase II β subunit, and was chosen for further analysis and named ATGCSIIβ. To investigate whether ATGCSIIβ plays a role in biogenesis of

functional EFR, we obtained *atgcsIIβ* insertion lines and compared their growth to that of wild type *Arabidopsis* in response to continuous elicitation with elf18 and flg22. Interestingly, *atgcsIIβ* alleles showed strongly reduced or abolished seedling growth inhibition after elf18 treatment (see article I, Fig. 5A, B). However, the fresh weight of seedlings treated with flg22 was the same as in wild-type treated controls (Figure 1).

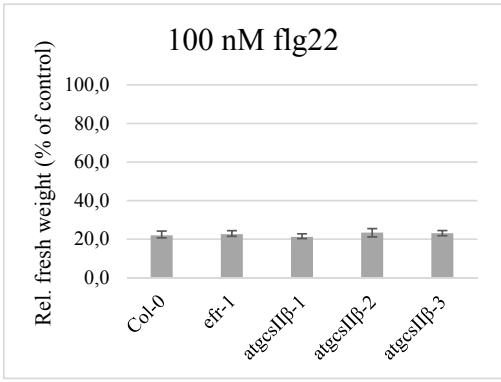

Figure 1. *ATGCSIIβ* is not required for flg22 responses. Seedling growth of Col-0 and *atgcsIIβ* mutants after treatment with 100 nM flg22. Treated and control seedlings were grown for 7 days in the presence of flg22, (n=8, ±SD).

These results indicate that ATGCSIIβ is required for EFR-mediated seedling growth inhibition, but is not required for FLS2 function.

We next compared *atgcsIIβ* alleles for elf18-trigerred oxidative burst, activation of MAPK kinases, callose deposition, and expression of defense-related marker genes. The *atgcsIIβ* mutants were insensitive or their response to elf18 was strongly decreased as measured by oxidative burst, MAPK activation, and defense gene expression (see article I, Fig. 6A-E). Taken together, this suggests that *ATGCSIIβ* is required for EFR function. This data also indicates that maturation of EFR requires ER quality control components that are dispensable for FLS2 function. Since EFR is only found in Brassicaceae, while FLS2 has been identified in several dicotyledonous and monocotyledonous plants, one speculation could be that EFR has evolved more recently than FLS2 and is less capable of folding properly in the absence of these components.

4.1.2 Loss-of-function in ATGCSIIβ confers enhanced disease susceptibility to bacteria

Previous studies have demonstrated that the loss-of-function mutant *efr-1* does not have an increased susceptibility to the virulent bacterial strain *P. syringae pv. tomato* DC3000 (Zipfel et al., 2004, 2006), indicating redundancy in recognition of PAMP signals during pathogen attack. Insensitivity to elf18 suggested that the *atgcsIIβ* mutant might share the pathogen phenotype with *efr-1*. Therefore, we examined whether *atgcsIIβ* shows enhanced susceptibility to infection by *P. syringae pv. tomato* DC3000. The *atgcsIIβ* mutants were found to be clearly more susceptible to the DC3000 strain. Taken together, the enhanced susceptibility phenotype of *atgcsIIβ* along with the intact susceptibility phenotype of *efr-1* suggest that in addition to EFR, the *ATGCSIIβ*

mutation is compromising the function of other PRRs as well.

4.2 REQUIREMENT OF AFB4 IN PLANT GROWTH AND INNATE IMMUNITY (II)

Ubiquitin-mediated proteolysis controls many cellular processes, particularly those that must proceed unidirectionally, such as cell cycle and circadian rhythm (Patton et al., 1998; del Pozo and Estelle, 2000). The largest class of ubiquitin ligases in plants are the Skp1-Cullin-F-box protein (SCF) complexes, where the F-box proteins are the target-recruiting subunits. In *Arabidopsis*, the plant phytohormone auxin is recognized by the F-box protein TIR1. In addition to TIR1, *Arabidopsis* encodes 5 highly similar F-box proteins. Three of these, the auxin-signaling F-box proteins 1-3 (AFB1-3), have been characterized and found to have similar functions as TIR1 (Dharmasiri et al., 2005). AFB4, in turn, was shown to have a negative regulatory function on auxin signaling in seedlings (Greenham et al., 2011). Study II attempted to address the function of the F-box protein AFB4 in *Arabidopsis* using a complete loss-of-function *abf4-1* mutant line.

4.2.1 Loss-of-function in AFB4 confers pleiotropic developmental phenotypes

To assess the role of AFB4 in *Arabidopsis*, we utilized the *afb4-1* mutant line to study several auxin-dependent growth processes, including petiole and hypocotyl elongation, and lateral root formation. The *afb4-1* mutant was found to have shorter petioles and hypocotyls than the corresponding wild-type seedlings, and less lateral roots (see article II, Fig. 1D, J, Q). This suggested that ABF4 has a role in auxin signaling. Most striking was the observation that loss-of-function in AFB4 strongly affected seedling size. The *afb4-1* developed small distorted rosette leaves and produced a smaller rosette overall (see article II, Fig. 1O). To verify that the phenotypes observed in the *afb4-1* line resulted from the absence of the functional AFB4, complementation studies were carried out with constructs harboring *ABF4* cDNA and GUS-tagged versions of the cDNA, driven by the constitutive CaMV35S promoter. As seen in article II (Fig. 1N, O) the expression of the ABF4 cDNA rescued wild-type growth in *abf4-1*. Interestingly, heterozygous plants displayed a phenotype intermediate between homozygous *afb4-1* and wild-type plants, as seen by rosette diameter (Figure 2).

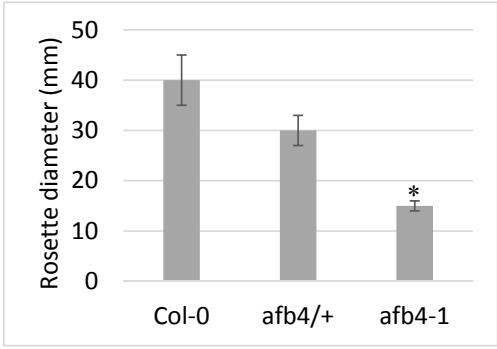

Figure 2. Heterozygous *afb4* plants exhibit an intermediate phenotype. 20-day-old soil grown Col-0, *afb4*/+ and *afb4-1* plants were measured for their

developed rosettes. (n=12, ±SD). *p < 0.05, Student's *t*-test.

RT-PCR analysis of *AFB4* in wild-type and heterozygote lines did not show clearly reduced expression in a heterozygous mutant (see article II, Fig. S4D). Weaker growth defects are likely due to a dose-dependent effect. Measurement of silique length also indicated that *afb4-1* had 50 % smaller siliques than the corresponding wild-type (Figure 3).

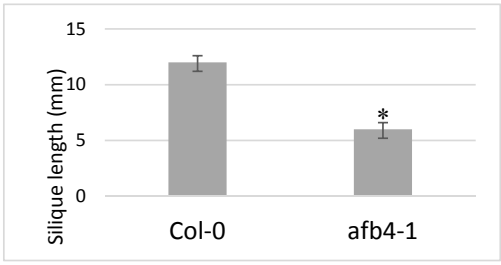

Figure 3. Appearance of siliques in *afb4-1* mutant plants. Silique length was measured in 7 to 8-weeks-old Col-0 and *afb4-1* plants. (n=12, ±SD). *p < 0.05, Student's *t*-test.

The smaller siliques contained slightly smaller seeds. Col-0 and *afb4-1* seeds had an average size of 13 mm^2 and 11 mm^2, respectively. Importantly, seed viability in *afb4-1* was not reduced suggesting a normal embryo development. To explore if abnormal seed development had any effects on embryo development, embryos at different developmental stages were examined at wild-type and mutant plants (see article II, Fig. S2). When the embryos were cleared and examined under microscopy, no defects were observed.

In summary, our data demonstrate that AFB4 is important for various aspects in plant development and affects multiply traits during life cycle of plant.

4.2.2 The *abf4-1* mutant shows enhanced resistance to necrotrophic bacterial and fungal pathogens

Crosstalk between growth and immunity signaling is essential for plants to finely balance resource allocation (Lozano-Durán and Zipfel, 2015). An effective PTI serves to fight off most pathogens, however, PTI activation is costly in terms of cellular resources and often results in growth retardation. Although the auxin-mediated processes in plant-pathogen interactions are rather complicated, most studies implicate auxin in promotion of disease symptoms in many plants (Nafisi et al., 2015). Suppression of auxin signaling was shown to increase resistance against both hemibiotrophic and biotrophic pathogens (Navarro et al., 2006; Robert-Seilaniantz et al., 2011). The negative effect of auxin in SA-mediated defense against biotrophic pathogens has been demonstrated by several independent studies (Chen et al., 2007; Wang et al., 2007). However, the auxin mutant *aux1* (defective in auxin influx) is unable to develop systemic resistance against *B. cinerea* (Korolev et al., 2007). The auxin signaling mutants *axr1*, *axr2*, and *axr6* are more susceptible to *B. cinerea* than wild-type plants (Korolev et al., 2007; Llorente et al., 2008). The role of auxin has also long been

known in plant cell wall loosening, while cell wall loosening is implicated in resistance against *B. cinerea* (Abuqamar et al., 2013).

The trade-off between growth and immunity prompted us to examine the role of AFB4 in development of disease resistance against necrotrophic bacterial and fungal pathogens. Col-0 and *afb4-1* plants were infected with *P. carotovorum* and *B. cinerea,* followed by scoring of disease symptom development. After three days of infection, *afb4-1* plants exhibited strong resistance against both *P. carotovorum* (see article II, Fig. 1K) and *B. cinerea* (Figure 4) compared to the wild-type controls.

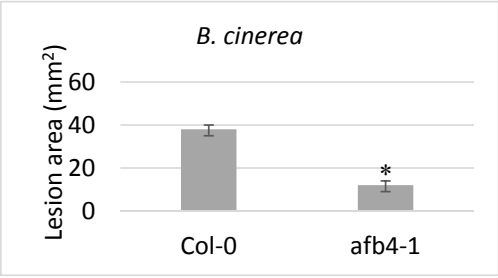

Figure 4. Resistance of *afb4-1* mutant to *B. cinerea*. 21-day-old Col-0 and *afb4-1* plants were subsequently infected with *B. cinerea*. The lesion area was measured 3 days post-inoculation (n=8, ±SD). *p < 0.05, Student's *t*-test.

Taken together, our data indicate that lost-of-function mutation in *AFB4* confers resistance to necrotrophic bacterial and fungal pathogens further supporting the notion that auxin is involved in plant-pathogen interactions.

4.3 CLASS III PEROXIDASES MODULATE DEFENSE SIGNALING AND AFFECT DISEASE RESISTANCE (III)

Oligogalacturonides (OGs) are cell-wall breakdown products released from plant cell walls upon infection by necrotrophic pathogens, wounding, or insect chewing (Nürnberger et al., 2004; Palva et al., 1993). Recognition of OGs elicit defense responses, including accumulation of ROS and pathogenesis-related proteins that protect the plants against pathogen infection (Ferrari et al., 2007). In 2010, Brutus and co-workers demonstrated that OGs are recognized by cell wall-associated kinases (Brutus et al., 2010). OGs are currently considered true damage-associated molecular patterns (DAMPs), able to activate plant innate immunity. Early defense responses triggered by OG elicitors released by the action of CWDEs produced by necrotrophic pathogens are pivotal in determining plant resistance to necrotrophs. To identify OG-induced processes required for disease resistance, we developed a high-throughput screen for altered OG-response by utilizing the fact that OG treatment strongly inhibits seedling growth. A population of 62000 T-DNA activation-tagged *Arabidopsis* lines were treated with OGs and their growth phenotypes were assessed (Weigel et al., 2000). This was followed by screening of associated necrotrophic pathogen susceptibility and resistance phenotypes. The screen yielded 46 activation-tagged mutant

lines with altered growth and pathogen phenotypes in response to OGs. One of these 46 lines exhibited a strong hypersensitive response to OGs (DP 2-20), complete immunity to *B. cinerea*, and strong resistance to *P. ca*. This line was named *ohy1* (OG hypersensitive 1) and chosen for further characterization. The phenotype in *ohy1* was subsequently shown to be mediated by overexpression of the *PER57* gene, which encodes an apoplastic peroxidase. Study III elucidated the role of *PER57* and some other members of the class III (CIII) peroxidase gene family in plant defense response to OG elicitors and induced resistance to necrotrophic pathogens.

4.3.1 Overexpression of PER57 enhances ROS accumulation, OG signaling and resistance to necrotrophic pathogens

All living organisms contain peroxidases that catalyze oxidative reactions using H_2O_2 as the electron acceptor. Higher plants harbor at least four types of peroxidases; these include glutathione peroxidases, catalases, ascorbate peroxidases (class I), and secreted peroxidases (class III) (Shigeto and Tsutsumi, 2016). In contrast to the first three types of peroxidases, class III (CIII) peroxidases are involved in a broad range of physiological processes throughout the plant life cycle. For example, the activities of CIII peroxidases are essential in cell wall metabolism (Passardi et al., 2004), wound healing (Allison and Schultz, 2004), hypersensitive response (Bindschedler et al., 2006), and defense against pathogens (Cosio and Dunand, 2009). In *Arabidopsis*, five CIII peroxidases, *PER21, PER33, PER34, PER62*, and *PER71*, have been shown to play a role in plant defense. Interestingly, all of these CIII peroxidases are responsive to OG elicitors and wounding (Davidsson et al., 2013). The overexpression of *PER21, PER62*, *and PER71* peroxidases has been shown to confer immunity to *B. cinerea* (Chassot et al., 2007), while knockdown of *PER33* and *PER34* transcripts increased susceptibility to both fungal and bacterial pathogens (Daudi et al., 2012). The *ohy1* mutant line identified in our screen was carrying the T-DNA insertion in the extragenic DNA region between the At5g17820 and At5g17830 genes. The CaMV 35S enhancers present in the T-DNA were found adjacent to the At5g17820 (*PER57*) and accordingly, the expression of the *PER57* gene and accumulation of ROS was clearly enhanced in the mutant (see article III, Fig. 1E, G). To verify that the phenotypes observed in *ohy-1* were indeed caused by the activation of the *PER57* gene, we used the CaMV35S promoter to drive the expression of *PER57* in transgenic plants. The phenotypes of the generated transgenic plants were identical to those observed in *ohy-1*, demonstrating the role of PER57 activation.

To study the role of PER57 in the plant response to OG elicitors, the expression of OG-signaling marker genes were analyzed in *Arabidopsis* plants overexpressing the gene

and in wild-type controls. The expression of OG marker genes in OG-treated *PER57* overexpressed plants were strongly up-regulated when compared to their wild-type controls (see article III, Fig. 2A). Enhanced expression of OG-marker genes has earlier been shown to correlate with increased resistance to nectrotrophic pathogens (Ferrari et al., 2007). Accordingly, disease resistance to necrotrophic pathogens was also determined. As expected, the plants overexpressing *PER57* exhibited markedly enhanced resistance to both *B. cinerea* and *P. carotovorum* (see article III, Fig. 2C-F). Furthermore, to test if the plants overexpressing *PER57* also demonstrated enhanced defenses against pathogens with different lifestyles, we infected these and wild-type plants with the bacterial hemibiotroph *Pseudomonas*. Surprisingly, in contrast with the observed resistance to necrotrophic pathogens, the overexpression of this CIII peroxidase clearly increased the susceptibility of the plants to *Pseudomonas* (see article III, Fig. 2G, H). This indicates that overexpression of *PER57* in the *ohy1* line confers increased resistance to necrotrophic pathogens, but enhanced susceptibility to biotrophic pathogens. Additionally, our observations indicate that elevation of CIII apoplastic peroxidase-derived ROS lead to cuticle permeation.

4.3.2 CIII peroxidase-generated ROS negatively modulate the formation of the cuticle

CIII peroxidases catalyze cross-linkage formation in between cell wall polymers, such as lignin and suberin leading to increased rigidity. To elucidate the role of *PER57* in cell wall adjustment, we performed a fortification assay using CWDEs extracted from *P. carotovorum*. Unexpectedly, the cell walls of plants overexpressing *PER57* were much less fortified than those of the wild-type plants (see Fig. 3B). There is no evidence for the involvement of peroxidase-generated ROS in cuticle thickness. In general, decreased fortification of plant cell walls could be coupled with increased permeability of the leaf cuticle. Interestingly, a number of studies (Bessire et al., 2007; Chassot et al., 2007, 2008b; L'Haridon et al., 2011; Voisin et al., 2009) reported that mutants impaired in the biosynthesis of the cuticle are more resistant to *B. cinerea* and more susceptible to *Pseudomonas*. In addition, cuticular permeability was associated with the increased accumulation of ROS and ROS-induced immunity (Asselbergh et al., 2007; L'Haridon et al., 2011). However, the source of altered cuticle-induced ROS has remained elusive. Disease resistance phenotype of plants overexpressing *PER57* appeared to be very similar to that reported for *bdg* and *lacs2.3* mutants, which are both impaired in cuticle biosynthesis. To test if PER57-derived ROS had any effect on cuticle formation of the

transgenic plants, adaxial sides of *ohy1* leaves were tested with a hydrophilic toluidine blue (TB) dye exclusion assay, in which TB only permeates defective cuticles to stain the cell walls (Tanaka et al., 2004). As expected, no staining was observed in the leaves of wild-type plants while dark blue staining was clearly visible on the leaves of *ohy1* already 1 min after the application of TB, indicating strongly increased permeability of the leaf cuticle (see article III, Fig. 3A). Since CIII peroxidases exist as large multigene families and act redundantly, we generated six transgenic lines overexpressing different CIII peroxidases to address the issue of specificity on resistance and cuticle permeability; *PER10, PER28, PER34* responsive to OGs and *PER44*, *PER53*, *PER64* involved in other aspects of the plant life cycle. Overexpression of all these apoplastic peroxidases resulted in increased permeability of the leaf cuticle (see article III, Fig. 3C) and strongly enhanced resistance to *B. cinerea* (see article III, Fig. 3E). Importantly, we showed that the effect of *PER57* on the resistance is not specific and could be achieved by overexpression of the other CIII peroxidases as well. To determine how peroxidase-generated ROS promote alterations of the leaf cuticle, we examined the expression of the major cutin-biosynthetic genes *MYB96*, *BDG*, and *LACS2.3* in *ohy1* plants. Both *BDG* and *LACS2.3,* encoding structural cutin components, and *MYB96*, a positive regulator of cutin formation, were strongly down-regulated in *ohy1*, suggesting that the loss of cuticle integrity is influenced by altered cutin biosynthesis (see article III, Fig. 4A).

4.3.3 NADPH oxidase RBOHD-derived ROS do not appear to have a role in regulation of cuticle formation

Genome-wide and pharmacological studies have implicated a family of 10 *Arabidopsis RBOH* genes that share homology to the mammalian gp91^{phox} NADPH oxidase (Sagi and Fluhr, 2001, 2006). Plasma membrane-localized *RBOHD* and *RBOHF* are involved in PAMP-induced ROS (Nühse et al., 2007; Torres et al., 2002; Zhang et al., 2007). Despite their role in ROS generation in response to pathogen attack, neither the single *AtrbohD* mutant nor the double mutant *AtrbohD/AtrbohF* showed compromised resistance to either bacterial or fungal pathogens (Torres et al., 2002, 2005). Galletti (Galletti et al., 2008) demonstrated that the *AtrbohD* mutant was compromised in callose deposition following elicitation with OGs, however, neither reduced OG-elicited defense gene induction nor compromised resistance to fungal pathogens was detected. A recent study has shown that RBOHF is not expressed in response to flg22 or chitin, suggesting that RBOHF has no obvious function in PTI responses (Morales et al., 2016). Unlike RBOHF, RBOHD is activated upon PAMP recognition and is critical for the PAMP-induced ROS and PAMP-triggered stomatal closure (Kadota et al., 2014). The *rbohD* mutant cannot close stomates in response to

flg22 or elf18, indicating its importance in PTI responses (Marino et al., 2012; Morales et al., 2016). In addition to the role of RBOHD in PTI, we wanted to further examine the role of RBOHD-derived ROS in cuticle formation. Cuticle permeability and cuticle-biosynthetic gene expression were examined in plants overexpressing RBOHD (Torres et al., 2005). Despite increased accumulation of ROS, cuticular permeability and expression of cuticle biosynthetic genes *BDG* and *LACS2.3* were intact in RBOHD overexpressing plants (Figure 5).

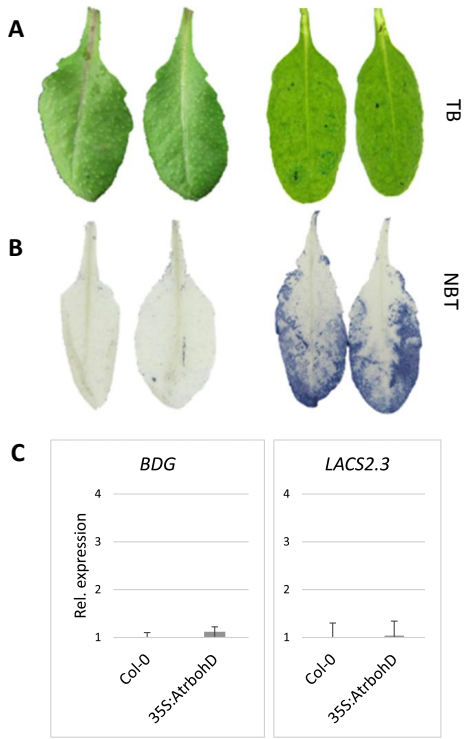

Figure 5. Cuticle permeability and expression of cuticle biosynthetic genes is not impaired in plants overexpressing *RBOHD*. (A) Toluidine blue (TB) staining was used to indicate cuticle permeability in 28-day-old Col-0 and *35S:AtrbohD* plants (n=8). (B) Superoxide formation was detected using NBT staining in 21-day-old Col-0 and *35S:AtrbohD* plants (n=8). (C) Quantitative RT-PCR analysis was performed to evaluate the levels of, *BDG* and *LACS2.3* transcripts in 28-day-old Col-0 and *35S:AtrbohD* rosette leaves. Transcript levels were plotted relative to the expression level in the Col-0 line at 0-hpt. The *EF1α* and *UBQ10* reference genes were used as internal controls.

Interestingly, plants overexpressing RBOHD were slightly less susceptible to *B. cinerea* (Figure 6) when compared to corresponding wild types.

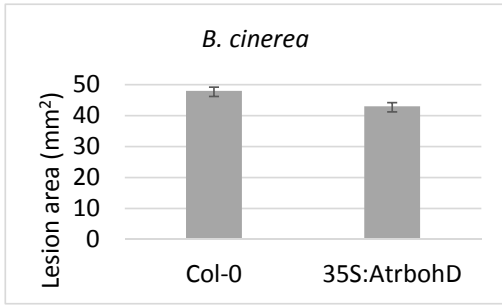

Figure 6. Response of *35S:AtrbohD* overexpressor to *B. cinerea* infection. 5 µl droplets of a suspension of *B. cinerea* spores (2 x 10^5 spores/mL) were placed on the leaves of 28-day-old plants. Images were taken three days post inoculation. The lesion area was measured in Fiji (n=12, ±SD). The experiment was repeated twice with similar results.

Unlike peroxidase overexpressors, full immunity to *B. cinerea* has never been observed in RBOHD-overexpressing plants. Our data indicate that *Arabidopsis* plants gain full immunity against *B. cinerea* only if they manifest defective cuticles, whereas enhanced accumulation of ROS without cuticle alterations (for example as in RBOHD

overexpressors) did not protect plants from *B. cinerea* infection.

4.3.4 Cuticular defects activate defense priming via OG-signaling pathway independently of SA and JA signaling

To elucidate the role of SA and JA signaling in the observed resistance of *PER57*-overexpressing plants to necrotrophic pathogens, we monitored the expression of marker genes for SA and JA signaling. The expression of these genes remained unaltered in response to OG elicitors (see article III, Fig. S1), suggesting that the OG signaling pathway is the major contributor to defense mediated by CIII peroxidase-generated ROS. Since the expression of OG marker genes PAD3, PGIP1, and PER4 was much stronger and faster in OG-treated *ohy1* plants than the levels observed in the wild-type controls, we suspected defense responses might be primed in *PER57*-overexpressing plants. To test this, we examined the expression of OG-marker genes in response to the bacterial PAMP flg22. Flg22-induced expression of the tested genes was even stronger. One could speculate that the primed defense response observed in *ohy1* plants could simply reflect an increase in the amount of elicitor capable of diffusing across the more permeable cuticle. Therefore, we further tested the priming of defense in leaves infiltrated with the OG and flg22 elicitors and determined the deposition of callose, which has emerged as a popular model system to quantify the activity of plant immunity. Increased callose deposition was observed in leaves infiltrated with either one of the elicitors used (see article III, Fig. 2B), suggesting that defense responses are indeed primed in plants with a permeable cuticle and this could be mediated through the OG-signaling pathway.

4.3.5 The antagonism between ABA and peroxidase-derived ROS plays a key role in controlling permeability of the cuticle

ABA is a major phytohormone that regulates a broad range of plant adaptive responses. Early studies showed that pretreatment of potato plants with ABA increased susceptibility to *Phytophtora infestans* and *Cladosporium cucumerinum* (D. M. Henfling, 1980). To elucidate the role of ABA in the observed resistance of plants overexpressing *PER57*, ROS accumulation and disease resistance to *B. cinerea* were also examined in ABA-deficient *aba2*, and ABA-insensitive *pyr/pyl* 112458 sextuple mutant plants treated with ABA. In the presence of ABA, all plants were equally susceptible to *B. cinerea,* except the ABA-insensitive *pyr/pyl* 112458 sextuple mutant (see article III, Fig. 5D). In addition, ABA signaling mutants displayed increased accumulation of ROS. To elucidate the source of ROS in ABA signaling mutant and *ohy1* plants, we performed peroxidase activity assays. All of the tested plants exhibited increased peroxidase activities as compared to the wild-type plants (see article III, Fig. 5C),

pointing to CIII peroxidases as the source of ROS. A separate set of these plants was treated with diphenylene iodonium (DPI), an inhibitor of NADPH oxidase-dependent oxidative burst (see article III, Fig. 5B). However, the application of DPI had no prominent effect on ROS formation in any of the tested lines, indicating that ROS was indeed produced by the peroxidases (see article III, Fig. 5B). Since the ABA signaling mutants displayed excessive levels of ROS, the cuticular permeability was determined in these plants. Increased cuticular permeability and strong down-regulation of *BDG* and *LACS2.3* were observed in ABA signaling mutants (see article III, Fig. 5B). In the presence of ABA, the expression of cutin biosynthetic genes (see article III, Fig. 5A) and cuticular permeability were restored to wild-type levels, indicating significance of the ABA signaling pathway in the regulation of excessive ROS formation. Restored expression of cutin biosynthetic genes was observed after ABA-induced removal of ROS. This type of antagonistic regulation between ROS and ABA demonstrates why plants exposed to pathogens in natural conditions do not continuously produce ROS, since pathogen-induced accumulation of ABA attenuates ROS generation. Induction of defense responses usually negatively correlates with plant fitness. Apparently, continuous ROS production would result in growth retardation and reproduction defects. To prevent mass loss and fertility defects, plants should develop redundant mechanisms that are important for adaptation.

5. CONCLUSIONS AND FUTURE PROSPECTS

The studies presented in this thesis provide novel data on how plants resist bacterial and fungal pathogens and how such resistance is modulated by the ABA signaling pathway. The data provided by Study I highlight the importance of *ATGCSIIβ* in EFR-mediated defense signaling. Mutations in ER-QC components have been reported to specifically suppress protein secretion. Compromised defense responses in *atgcsIIβ* mutant plants suggest ER-QC physiological requirement in the biogenesis of the plant innate immunity receptor EFR. The enhanced disease susceptibility phenotype of *atgcsIIβ* and insensitivity to flg22 suggest that *ATGCSIIβ* may have a very specific role in EFR-mediated plant innate immunity. Therefore, other PRRs involved in bacterial and fungal recognition might involve as yet uncharacterized ER-QC components that should be identified in the future.

Study II characterized the role of AFB4 in plant development and disease resistance. This study demonstrated signal crosstalk between defense and hormonal pathways. First, loss-of-function in *AFB4* triggered growth retardation and HR response against *P. carotovorum.* Second, the *afb4* mutant responded differently to plant phytohormones, indicating an important role for ABF4 in hormonal signaling pathways in *Arabidopsis*. To establish a causal link between growth and disease resistance, we performed a complementation experiment. 84 transgenic lines overexpressing *AFB4* were generated. Two lines displayed completely restored growth and disease susceptibility. Despite the role of AFB4 in defense signaling and hormone-mediated signaling pathways, the mechanism by which AFB4-impaired plants resist necrotrophic bacterial pathogens needs to be determined at the molecular level.

Oxidative burst has long been associated with the response to pathogen attack. The source of defense-related ROS and its role in cell wall remodeling were thoroughly investigated. However, role of ROS in cuticle permeability and cuticular permeability-derived resistance to necrotrophic pathogens was uncharacterized until now. Disease resistance described in study III apparently depends on increased cuticular permeability. Upregulation of class III peroxidases, but not NADPH oxidases, triggers strong repression of cuticle biosynthetic genes. Plants with a permeable cuticle, in turn, switch to a primed state of defense responses mediated via the OG signaling pathway. Our data indicates that elevated levels of ROS without cuticle alterations cannot protect plants from necrotrophic fungal and bacterial pathogens. Plants resist necrotrophic fungal pathogens only if they produce defective cuticle. Study III also demonstrated that enhanced accumulation of ROS in response to pathogens is tightly regulated by pathogen-triggered accumulation of ABA. ABA negatively controls the formation of ROS. However, ABA-triggered removal of ROS restores both the expression of cuticle biosynthetic genes and cuticular integrity.

The results of this thesis serve to further establish the roles of PTI components in plant immunity. This in turn facilitates our understanding of the early genetic determinants involved in disease

resistance against phytopathogens. Knowledge on how plants recognize and respond to necrotrophs has been behind our understanding of plant responses to biotrophs. Recent research focused on mechanisms of interactions between hormonal pathways, natural variation, and quantitative genetics. However, it is still unclear how changes in hormonal levels during infection are transmitted into specific immune responses. For example, *B. cinerea* infection results in increased accumulation of SA, JA, ABA, and ET, and the expression of marker genes associated with these pathways. However, the contributions of these hormones to resistance are clearly different. The data presented in this thesis should facilitate the development of crop plants resistant to necrotrophic fungal and bacterial pathogens.

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