Analysis on the BAK1-interacting RLKs BIR2 and BIR3, two proteins that differentially regulate BAK1-dependent pathways

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List of abbreviations

°C	degree Celsius
µ	micro (10^{-6})
35S	Promotor of cauliflower mosaic virus
A. brassicicola	*Alternaria brassicicola*
A. thaliana	*Arabidopsis thaliana*
aa	Amino acid
ACD	Accelerated cell death
Ade	Adenine
ARF-GEF	ADP ribosylation factor - guanine nucleotide exchange factor
ATG	Autophagy
*At*GRXC2	*Arabidopsis thaliana* Glutaredoxin C2
*At*MC	*Arabidopsis thaliana* metacaspase
*At*MIN7	*Arabidopsis thaliana* HopM interactor 7
*At*Pep1	Arabidopsis thaliana peptide 1
avr	Avirulence
avrB	Avirulence protein B from *P. syringae* pv. *glycinea*
avrRPM1	Avirulence protein 1 from *P. syringae* pv. *maculicola*
avrRpt2	Avirulence protein 2 from *P. syringae* pv. *tomato*
BAK1	BRI1-associated kinase
BES1	BRI1–EMS-suppressor 1
BIK1	Botrytis-induced kinase 1
BIN2	Brassinosteroid insensitive 2
BIR	BAK1-interacting RLK
BKI1	BRI1 kinase inhibitor 1
BKK1	BAK1-like 1
BL	Brassinolide
BON1	Bonzai 1
BR	Brassinosteroid
BRI1	Brassinosteroid-insensitive 1
BRZ	Brassinazole
BSK1	BR signaling kinase 1
BSA	Bovine serum albumin
BSU1	*bri1* suppressor 1
BZR1	Brassinazole resistant 1
CBB	Coomassie brilliant blue
CC	Coiled-coil
cDNA	complementary DNA
CDPK	Calcium dependent protein kinase
CERK1	Chitin elicitor receptor kinase 1
cfu	Colony forming units
CoIP	Coimmunoprecipitation
Col-0	Columbia-0
CPD	Constitutive photomorphism and dwarfism
CPR22	Constitutive expresser of PR 22
CPK28	Calcium dependent protein kinase 28
CSM	Complete synthetic medium
DAMP	Danger-associated molecular pattern
DNA	Desoxyribonucleic acid

DND1	Defense no death 1
dNTPs	Deoxyribonucleotide triphosphate
dpi	Days post inoculation
DWF4	Dwarf 4
EDS	Enhanced disease susceptibility
EFR	EF-TU receptor
e.g.	for example
eMAX	Enigmatic MAMP activity from *Xanthomonas*
ET	Ethylene
ETI	Effector-triggered immunity
EtOH	Ethanol
ETS	Effector-triggered susceptibility
F	Filial generation
FLS2	Flagellin-sensing 2
FLU1	Fluorescent 1
FRK1	Flagellin responsive kinase 1
Fwd	Forward
g	Gram
g	Gravitational acceleration
GA	Gibberellic acid
h	Hour
HBI1	Homolog of BEE2 interacting with IBH 1
His	Histidine
HopM1	HRP (hypersensitive response and pathogenicity) outer protein M1
Hpa	*Hyaloperonospora arabidopsidis*
HR	Hypersensitive response
HRP	Horseradish peroxidase
IOS1	Oomycete susceptibility 1
IP	Immunoprecipitation
JA	Jasmonic acid
KAPP	Kinase-associated protein phosphatase
KAS2	Kashmir 2
Kond	Kondara
l	Liter
LB	Lysogeny broth
Ler	*Landsberg erecta*
LES22	Lesion 22
Leu	Leucine
LIN2	Lesion initiation 2
LLS1	Lethal leaf spot 1
LMM	Lesion mimic mutant
LORE	Lipooligosaccharide-specific reduced elicitation
LRR	Leucine-rich repeat
Lsd1	Lesions simulating disease resistance response 1
LYK5	LysM-containing receptor-like kinase 5
LYM	Lysine motif
LysM	Lysine motif
M	Molar
m	Milli (10^{-3})
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MEKK	MAPK / ERK (extracellular signal-regulated kinase) kinase kinase

Met	Methionine
min	Minute
MKK	Mitogen activated protein (MAP) kinase kinase
MKS1	MAP kinase substrate 1
MS	Murashige and Skoog
n	nano (10^{-9})
N. benthamiana	*Nicotiana benthamiana*
NB-LRR	Nucleotide-binding leucine-rich repeat
NDR1	Non-race specific disease resistance 1
NLP	Ethylene-inducing peptide 1 (Nep1)-like protein
NOI	Nitrogen oxide intermediates
OD	Optical density
PAD3	Phytoalexin deficient 3
PAMP	Pathogen-associated molecular pattern
PAT1	Protein interacting with APP tail-1
PBL	AvrPphB Susceptible1 (PBS1)-like proteins
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDF1.2	Plant defensin 1.2
PEPR1	Pep1 receptor 1
PP2A	Protein phosphatase 2A
PR	Pathogenesis related
PRR	Pattern recognition receptor
PSKR	PSK receptor
PSKα	Phytosulfokine
PSY1	Plant peptide containing sulfated tyrosine 1
PSY1R	PSY1 receptor
PTI	PAMP or pattern triggered immunity
Pto	*Pseudomonas syringae pv. tomato*
PUB12	Plant U-Box 12
R-gene	Resistance gene
R-protein	Resistance protein
RBOHD	Respiratory burst oxidase homolog D
ReMAX	Receptor of eMAX
rev	Reverse
RIN4	RPM1-interacting protein 4
RLCK	Receptor-like cytoplasmic kinase
RLK	Receptor-like kinase
RLP	Receptor-like protein
RLU	Relative light units
RNA	Ribonucleic acid
ROS	Reactive-oxygen species
RPM1	Resistance to *Pseudomonas syringae pv. maculicola* protein 1
RPP1	Recognition of *Peronospora parasitica* 1
RPS2	Resistance to *Pseudomonas syringae* protein 2
RT	Reverse transcription
s	Second
SA	Salicylic acid
SAG101	Senescence associated gene 101
SAR	Systemic acquired resistance
ScFE1	*Sclerotinia* culture filtrate elicitor1
SERK	Somatic embryogenesis receptor kinase

SID2	Salicylic acid induction deficient 2
SNC1	Suppressor of NPR1-1 constitutive 1
SOBIR1	Suppressor of *bir1*
SOC	Super optimal broth (SOB) with catabolite repression
SRF3	Strubbelig receptor kinase 3
SUMM2	Suppressor of *mkk1mkk2* 2
T-DNA	Transfer DNA
Ta	Annealing temperature
TIR	Toll-Interleukin 1
Tm	Melting temperature
TMV	Tobacco mosaic virus
Trp	Tryptophan
Ura	Uracil
v/v	Volume per volume
VPE	Vacuolar-processing enzyme-like
w/v	Weight per volume
WB	Western blot
WRKY	Tryptophane, arginine, lysine, tyrosine containing transcription factor
WT	Wild-type
YPD	Yeast extract peptone dextrose

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1. Introduction

Plants are constantly exposed to a variety of different pathogens. Nevertheless, most plants in nature look healthy which argues for an effective immune system. Plants do not have mobile immune cells or an adaptive immune system as animals do; instead, each cell is able to establish an innate immune response. The first barriers for pathogens are preformed defense mechanisms like the plant cell wall, the cuticle or phytoanticipins, preformed toxic components (Eigenbrode et al., 1991; Osbourn, 1996; Malinovsky et al., 2014a). The second barriers are inducible defense mechanisms such as the production of reactive oxygen species, cell wall fortification or the production of phytoalexins (O'Brien et al., 2012; Ellinger and Voigt, 2014; Poloni and Schirawski, 2014). Defense mechanisms have to be adapted to the pathogens life style, which can be biotrophic or necrotrophic. Biotrophic pathogens colonize living plant tissue and feed on plant metabolites. Necrotrophic pathogens kill the plant and feed on the dead plant tissue. Hemibiotrophic pathogens have a biotrophic life style at the beginning of their life cycle and are able to switch to a necrotrophic life style at the end of their life cycle (Glazebrook, 2005). As the induction of defense mechanisms is energy consuming they should only be activated after pathogen recognition and need to be tightly controlled.

1.1. The two layered plant immune system

The first step for the induction of an immune response is the recognition of possible pathogens. Plant pathogens can be feeding insects, bacteria, viruses, fungi or oomycetes. Microbial pathogens can be recognized by microbe associated molecular patterns (MAMPs or PAMPs for pathogen associated molecular patterns), which are conserved structures present in a large group of microbes (Medzhitov and Janeway, 1997). Examples are the bacterial flagellin, the elongation factor TU, the fungal cell wall component chitin or nlp20, a peptide pattern found in ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) from different microbes (Felix et al., 1993; Felix et al., 1999; Kunze et al., 2004; Böhm et al., 2014b). These structures are indispensable for the life of microbes, therefore well conserved and cannot be easily mutated (Boller and Felix, 2009). MAMPs are recognized via membrane bound pattern recognition receptors (PRRs). PRRs belong to the class of receptor-like kinases (RLKs) or receptor-like proteins (RLPs) with extracellular domains composed of e.g. leucine rich repeats (LRR) or lysine motif (LysM) domains (Böhm et al., 2014a). Recognition of MAMPs by PRRs leads to the induction of a certain level of defense that is effective against most non adapted pathogens and is summarized as PAMP triggered immunity (PTI) (Jones and Dangl, 2006).

Typical responses are calcium influx, alkalization of the apoplast, production of reactive oxygen species (ROS), activation of mitogen-activated protein kinase (MAPK) cascades or transcriptional activation of pathogenesis related (PR) genes such as chitinases or glucanases (Zipfel, 2009). However, by recognition of MAMPs plants cannot distinguish between pathogenic and non-pathogenic microbes and cannot determine the pathogen´s life style. Plants cannot only recognize non-selves but also structures that originate from the plant during infection. Many plant pathogens use lytic enzymes to destroy the plant cell wall and invade the cells. The generated cell wall fragments can be recognized as danger signals or damage-associated molecular patterns (DAMPs) (Matzinger, 2002).

During an evolutionary arms race, pathogens developed mechanisms to suppress the plant immune response. They secrete effector proteins via a type III secretion system directly into the plant cell. These effectors evolved to interrupt the signaling cascade downstream of MAMP perception and therefore shut off the plant defense. The microbes are able to colonize the now susceptible plant and are called virulent. An example is the *Pseudomonas syringae* effector HRP outer protein M1 (HopM1) that targets the *A. thaliana* protein HopM interactor 7 (*At*MIN7), an ADP ribosylation factor - guanine nucleotide exchange factor (ARF-GEF) protein involved in vesicle transport and extracellular secretion (Nomura et al., 2006).

Effectors are specific for a species or a small group of adapted pathogens and are therefore a good target for recognition of specific pathogens by the host. Indeed, plants evolved perception systems, the resistance (R)-proteins, to specifically recognize pathogens via their effectors and induce specific immune responses that are summarized as effector triggered immunity (ETI) (Jones and Dangl, 2006). Here the effectors that were destined by microbes to suppress ETI become avirulence (avr) factors. ETI responses resemble MTI responses but are stronger and accelerated and often lead to the induction of a hypersensitive response (HR), a locally restricted cell death response that aims to stop the proliferation of biotrophic pathogens (Morel and Dangl, 1997). HR responses would be beneficial for necrotrophic pathogens and are therefore not part of PTI. ETI was already described decades ago as the gene for gene hypothesis which states that the interaction of an *avr*-gene product with a cognate *R*-gene product in the host plant leads to an incompatible interaction due to induction of host defense responses (Flor, 1971). During that time this hypothesis was understood as a direct interaction between the effector and the R-protein, where the effector is the ligand and the R-protein is the receptor. Only a few cases for such a direct interaction have been described so far, for example the recognition of a tobacco mosaic virus (TMV) helicase by the tobacco R-protein N (Ueda et al., 2006). Most effectors are sensed indirectly, what explains how the small number of plant R-proteins can recognize the broad diversity of

pathogen effectors. The Arabidopsis genome contains about 125 NB-LRR proteins but each strain of the plant pathogen species *Pseudomonas syringae* already contains 10 to 40 effectors (Baltrus et al., 2011). An alternative recognition concept besides direct interactions is the guard hypothesis, which states that R-proteins monitor or "guard" the integrity of the host cellular target of the effector (Jones and Dangl, 2006). The third model describes that plants evolved mimics of real effector targets, that have no function in MTI, but solely as effector targets and are called decoys (van der Hoorn and Kamoun, 2008).

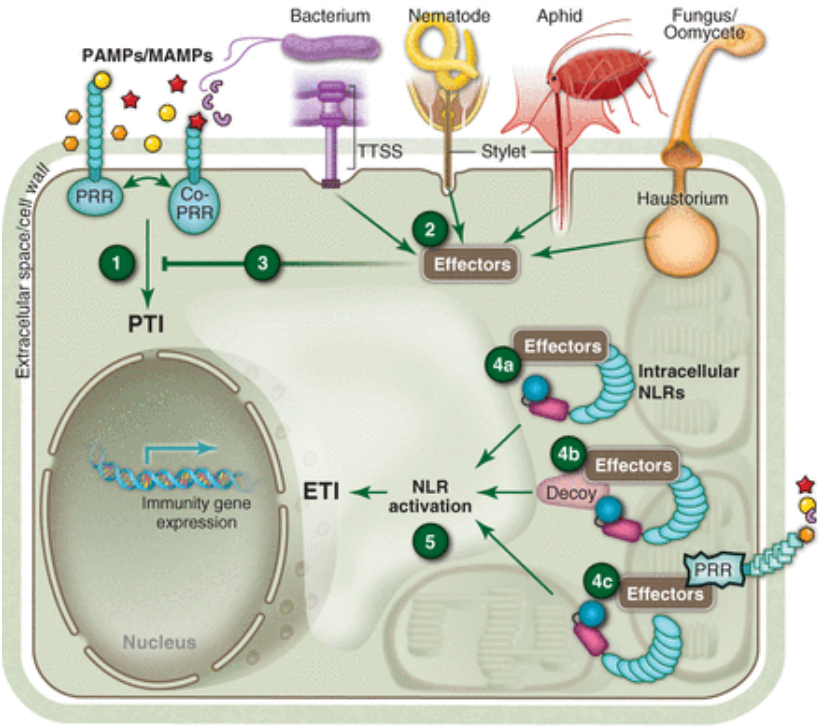

Figure 1-1: Simplified representation of the plant immune system (Dangl et al., 2013).
Recognition of MAMPs from pathogens with different life styles leads to the initiation of MTI (1). Pathogens deliver effectors into the plant cell (2) to suppress MTI (3). R-proteins can sense effectors in three distinct ways: direct effector R-protein interaction (4a), sensing alterations of a decoy (4b), or sensing alterations of a virulence target (4c). Effector recognition leads to ETI (5). For details see text.

The relatively small number of R-proteins compared to effectors can also be explained by the fact that effectors from different pathogens target common plant proteins and thus effectors converge onto plant cellular hubs, which are highly interconnected proteins (Mukhtar et al., 2011). For example, two different *Pseudomonas syringae* effector avrRPM1 and avrB target RIN4, a regulator of basal defense, and phosphorylate it (Mackey et al., 2002). Phosphorylation of RIN4 is recognized by the R-protein RPM1 and ETI is induced. But the pathogen strikes back: another type III effector, avrRpt2, evolved that also targets RIN4 and leads to proteolytic cleavage of RIN4, blocking RPM1 function (Kim et al., 2005). The plant

counter strikes and evolved the R-protein RPS2 which detects cleaved RIN4 (Axtell and Staskawicz, 2003; Mackey et al., 2003). In this way the evolutionary arms race continues, natural selection leads to pathogens with new effector proteins to suppress ETI and plants with additional R-proteins that recognize the newly evolved effectors.

Plant R-proteins structurally belong to the class of nucleotide-binding leucine-rich repeat proteins (NB-LRRs) with either N-terminal coiled-coil (CC) or Toll/Interleukin1-Receptor (TIR) domains. These two groups of NB-LRR proteins use different downstream signaling components (Aarts et al., 1998). CC-type NB-LRRs signal via the GPI-anchored Non-race specific disease resistance 1 (NDR1) protein (Century et al., 1997), TIR-type NB-LRRs require the Enhanced disease susceptibility 1/ Phytoalexin deficient 4/ Senescence associated gene 101 (EDS1/PAD4/SAG101) complex (Wiermer et al., 2005). Both signaling pathways lead to the accumulation of the plant defense hormone salicylic acid (SA). In general, different phytohormones play a key role in coordinating defense responses against different types of pathogens (Pieterse et al., 2009). Biotrophic pathogens are typically sensitive to immune responses that are regulated by salicylic acid whereas necrotrophic pathogens are sensitive to immune responses induced by jasmonic acid (JA) and ethylene (ET) (Penninckx et al., 1998; Loake and Grant, 2007). The wound response after insect feeding is regulated by JA alone (McConn et al., 1997). As the different lifestyles of pathogens require different defense strategies, coordination is needed. This is achieved through a negative crosstalk between the SA and JA signaling pathways (Spoel et al., 2003). Phytohormones are also involved in systemic defense responses. Upon induction of defense at the site of infection, a systemic defense response is triggered in distal parts of the plant to protect the so far undamaged tissue against the subsequent pathogen invasion (systemic acquired resistance, SAR) (Durrant and Dong, 2004).

1.2. Receptor-like kinases and MAMP signaling

As described above, the first layer of plant immunity is the recognition of MAMPs by PRRs. One of the best studied examples is the recognition of flg22, a 22 amino acid peptide from the bacterial flagellum, by the receptor Flagellin sensing 2 (FLS2) (Gomez-Gomez and Boller, 2000). FLS2 is a leucine-rich-repeat receptor-like kinase (LRR-RLK) consisting of 24 extracellular leucine-rich repeats, a single transmembrane domain and an intracellular kinase domain. Flg22 binding to FLS2 leads to rapid heterodimerization of FLS2 with the co-receptor BRI1-associated kinase 1 (BAK1) (Chinchilla et al., 2007; Heese et al., 2007). BAK1 is also a LRR-RLK, but with only 5 extracellular leucine rich repeats and was initially identified as co-receptor of the

brassinosteroid receptor Brassinosteroid-insensitive 1 (BRI1) and named BRI1-associated kinase 1 (Li et al., 2002; Nam and Li, 2002). BAK1 is important for flg22 signaling, as *bak1* knockout mutants show strongly reduced flg22 responses (Chinchilla et al., 2007). BAK1 belongs to a family of five somatic embryogenesis receptor kinase (SERK) proteins; these have partially redundant function and lead to residual MAMP responses in *bak1-4* mutants (Heese et al., 2007). Recently the co-crystal structure of BAK1 and FLS2 extracellular domains has shown that flg22 first binds to FLS2, creating a new interaction interface for BAK1. Flg22 serves as a "glue" between the two molecules and BAK1 binds to the ligand, making BAK1 a real co-receptor (Sun et al., 2013). Binding of flg22 leads to transphosphorylation events between the two kinase domains within seconds (Schulze et al., 2010). This activation leads to phosphorylation and activation of the receptor like cytoplasmic kinase (RLCK) Botrytis-induced kinase 1 (BIK1) which in turn transphosphorylates FLS2 and BAK1 and leads to the dissociation from both RLKs (Lu et al., 2010). BIK1 transduces the signal by activating the membrane bound NADPH oxidase Respiratory burst oxidase homolog D (RBOHD), which leads to rapid generation of ROS (Kadota et al., 2014; Li et al., 2014). Independently from BIK1, mitogen-activated protein kinase (MAPK) cascades are activated, but the direct link between receptors and MAPKs is missing (Asai et al., 2002). MAPK cascades are composed of a MAPKKK or MEKK, a MAPKK or MKK and a MAPK which are consecutively activated. Two distinct MAPKs cascades are activated upon flg22 treatment. One is composed of a so far unknown MEKK, MKK4/5 and MPK3/6 and the other one is composed of MEKK1, MKK1/2 and MPK4 (Rodriguez et al., 2010). At the same time a calcium influx leads to the activation of calcium dependent protein kinases (CDPKs) (Boudsocq and Sheen, 2013). Therefore, flg22 signaling cannot be seen as a linear cascade. Activation of the MAP kinases MPK3 and MPK6 could be directly linked to activation of WRKY type transcription factors, here WRKY22 and WRKY29 (Asai et al., 2002). MPK4 phosphorylates MAP kinase substrate 1 (MKS1), which leads to release of MKS1-WRKY33 complexes and induction of PAD3, the enzyme responsible for the production of the antimicrobial compound phytoalexin (Qiu et al., 2008). Flg22 signaling generally leads to strong gene expression reprogramming and among the induced genes RLKs are overrepresented leading to a positive feedback loop (Zipfel et al., 2004). Other flg22 responses like ethylene biosynthesis or callose deposition cannot be brought in line with the known signaling cascade. All these responses together are thought to lead to pathogen defense, as *fls2* mutants are more susceptible to *Pto* DC3000 bacteria (Zipfel et al., 2004).

The rapid induction of these defense responses is necessary to fend off pathogens but tight control of FLS2 and negative regulation is as important to avoid fitness costs and damage from constitutive defense responses. Negative regulators are for example the kinase-

associated protein phosphatase Kinase-associated protein phosphatase (KAPP) that interacts with the inactive FLS2 kinase domain and prevents unwanted phosphorylation events (Gomez-Gomez et al., 2001). After flg22 binding BAK1 leads to recruitment of the E3 ligases Plant U-Box 12 and 13 (PUB12 and PUB13) that poly-ubiquitinate FLS2 and lead to its degradation (Lu et al., 2011). Moreover FLS2 undergoes ligand induced endocytosis into late endosomal compartments for degradation (Robatzek et al., 2006). In addition to the function of removing the activated receptor, endocytosed FLS2 could also have a signaling function (Robatzek, 2007). FLS2 additionally undergoes a constitutive cycling between the plasma membrane and early endosomes independent of BAK1 (Beck et al., 2012). More and more components of flg22-mediated signaling have been identified, like the calcium dependent protein kinase CPK28 that negatively regulates flg22 signaling by regulating the turnover of the rate limiting factor BIK1 (Monaghan et al., 2014). The protein phosphatase PP2A negatively regulates immune signaling by controlling the phosphorylation status of the co-receptor BAK1 (Segonzac et al., 2014).

In addition to flg22-FLS2, several other ligand receptor pairs have been identified. The leucine rich repeat-receptor kinase EF-Tu receptor (EFR) recognizes the bacterial elongation factor Tu or the minimal peptide elf18 (Zipfel et al., 2006). Similar to FLS2, EFR also heterodimerizes with BAK1 or other SERKs but has less BAK1 specificity, as *bak1-4* mutants still show elf18 responses (Roux et al., 2011). The FLS2 and EFR signaling cascades seem to converge at an early step, as both lead to almost the same gene induction pattern (Zipfel et al., 2006). The fungal cell wall component chitin was believed to be recognized by the LysM receptor Chitin elicitor receptor kinase 1 (CERK1) for a long time (Miya et al., 2007; Petutschnig et al., 2010). However, it was recently proposed that CERK1 only functions as co-receptor and that the LysM-RLK LysM-containing receptor-like kinase 5 (LYK5) is the major chitin binding protein as it shows a much higher affinity to chitin (Cao et al., 2014). The bacterial cell wall component peptidoglycan is also recognized by LysM receptors, in this case the LysM –RLPs LYM1 and LYM3 (Willmann et al., 2011). Here, CERK1 also acts as a co-receptor. Another structural type of receptors are RLKs with extracellular lectin-like domains. The bulb-type lectin S-domain-1 receptor–like kinase Lipooligosaccharide-specific reduced elicitation (LORE) detects mainly the lipid A moiety of lipopolysaccharides (Ranf et al., 2015). In recent years, more and more RLPs have been identified as PRRs. For example RLP30 recognizes a proteinaceous MAMP called ScFE1 from the necrotrophic fungus *Sclerotinia sclerotiorum* (Zhang et al., 2013*)*. RLP1/ReMAX recognizes the not yet characterized MAMP eMAX from *Xanthomonas* spp. (Jehle et al., 2013). All RLPs described so far form a ligand independent complex with the small RLK Suppressor of BIR1 (SOBIR1) forming a structural and functional

equivalent of RLKs (Gust and Felix, 2014). Like most RLKs, RLP-SOBIR1 complexes are thought to heterodimerise with BAK1 or SERK family proteins after activation, as e.g. ScFE1 signaling is BAK1 dependent (Zhang et al., 2013). It is not yet understood how signaling specificity between different BAK1 dependent pathways is achieved or if this is actually needed. One exception is the chitin response pathway, as this is not BAK1 dependent. Several BAK1 interacting RLKs phosphorylate the intracellular kinase BIK1; CERK1 is also able to phosphorylate BIK1 but prefers PBS1-like protein 27 (PBL27) as a target. This could distinguish BAK1 dependent from BAK1 independent signaling pathways (Shinya et al., 2014). Despite the already described PRRs, it is likely that there are more MAMPs and corresponding receptors in Arabidopsis and even more in other plants. The Arabidopsis genome contains 221 LRR-RLK genes of which 49 are transcriptionally upregulated after different pathogen or MAMP treatments and are therefore likely to be involved in immune responses (Postel et al., 2010). One pathogen is usually detected by several MAMPs. Knockout of one MAMP receptor like FLS2 only leads to a certain amount of increased growth of *Pto* DC3000 bacteria whereas the *fls2 efr* double mutant or the *bak1-5* mutant shows stronger impairment in bacterial resistance (Schwessinger et al., 2011).

Other receptor like kinases that regulate immune responses sense endogenous signals. The LRR-RLKs Pep1 receptor 1 and 2 (PEPR1 and PEPR2) sense a 23-amino acid peptide *AT*Pep1 and also use BAK1 as a co-receptor (Yamaguchi et al., 2006; Krol et al., 2010; Postel et al., 2010). *At*Pep1 is processed from the *PROPEP1* gene, which is induced after wounding, methyljasmonate, ethylene or flg22 (Huffaker et al., 2006). This shows that *At*Pep1 serves as an endogenous signal for stress or wounding and acts as DAMP for neighboring cells (Liu et al., 2013; Tintor et al., 2013). The tyrosine sulfated peptides Phytosulfokine α (PSKα) and Plant peptide containing sulfated tyrosine 1 (PSY1) were originally described as growth promoting factors (Matsubayashi and Sakagami, 1996). They are recognized by PSK receptors 1 and 2 (PSKR1 and PSKR2) and the PSY1 receptor (PSY1R), respectively, which are also LRR-RLKs. The PSKR1 receptor is transcriptionally upregulated after *Pseudomonas* infection; receptor mutants show higher resistance to biotrophic pathogens as well as higher SA levels. However, they are more susceptible to necrotrophic pathogens (Igarashi et al., 2012; Mosher et al., 2013). Thus, these receptors could balance defense responses against biotrophic and necrotrophic pathogens by regulating SA JA homeostasis.

1.3. Brassinosteroid signaling

Brassinosteroids (BRs) are a class of plant hormones that regulate many developmental and physiological processes, for example cell elongation, vascular differentiation, root growth or abiotic and biotic stress (Fridman and Savaldi-Goldstein, 2013). The BR signaling pathway is one of the best studied signaling pathways and has been elucidated from BR recognition at the plasma membrane over several steps to gene induction in the nucleus. The main BR receptor Brassinosteroid-insensitive 1 (BRI1) was identified in different forward genetic screens for BR insensitive mutants, which have differentially strong dwarf phenotypes depending on the exact mutation (Clouse et al., 1996; Kauschmann et al., 1996). Cloning of the *BRI1* gene showed that it is a LRR-RLK with 24 extracellular leucine-rich repeats, an island domain between LRR 20 and 21, a transmembrane domain and a kinase domain (Li and Chory, 1997). Mutation analysis revealed that the island domain and LRR 21 are sufficient to bind BRs (Kinoshita et al., 2005), the crystal structure of the extracellular domain recently showed that BRs bind to the island domain and LRRs 21 to 25 (Hothorn et al., 2011; She et al., 2011). BL binding leads to a local structural rearrangement at the two loops linking the island domain with the flanking LRRs. The loops become ordered and form a protein-protein interaction platform (Hothorn et al., 2011). The protein BAK1 is a co-receptor for several ligand-binding receptors but was first identified as BRI1 associated kinase 1 (Li et al., 2002; Nam and Li, 2002). BR binding to BRI1 recruits the co-receptor BAK1 to BRI1 forming heterodimers that are necessary for the activation of the BRI1 kinase. Several auto- and transphosphorylation residues at serines, threonines and also tyrosines were identified. A sequential phosphorylation model is proposed, in which activated BRI1 transphosphorylates BAK1 and BAK1 transphosphorylates BRI1 to fully activate its kinase activity (Wang et al., 2008; Jiang et al., 2013). Microscopic studies have recently shown that large populations of BAK1 and BRI1 already colocalize in live root cells and that 7% of the BRI1 pool even constitutively interact with BAK1. The pre-assembled BRI1-BAK1 complexes probably get activated by BR binding and kinase activation of BRI1 (Bücherl et al., 2013). In the BR pathway different SERK proteins, which are homologs of BAK1, are functionally redundant as *bak1* knockouts only show a weak BR phenotype. The phenotype can be significantly enhanced in the *bak1 serk1* knockout (Albrecht et al., 2008), but only *serk1 bak1-4 bkk1-1* triple knockouts lead to a *bri1* mimicking phenotype, showing that these three SERKs have partially redundant functions and that SERKs are necessary for BR signaling (Gou et al., 2012).

In the absence of BR, BRI1 kinase inhibitor 1 (BKI1) additionally binds to BRI1 and seems to prevent interaction with BAK1 (Wang and Chory, 2006). This negative regulation

could be necessary if BAK1 is already in close vicinity to BRI1 in preformed receptor complexes. Moreover proteins of the BR signaling kinase (BSK) family of RLCKs interact with BRI1 and are kept inactive (Tang et al., 2008). Consequently the phosphatase *bri1* suppressor 1 (BSU1), which interacts with BSK, is also inactive and does not inhibit Brassinosteroid insensitive 2 (BIN2) phosphorylation (Mora-Garcia et al., 2004; Kim et al., 2009). The kinase BIN2 is active and able to phosphorylate the transcription factors Brassinazole resistant 1 (BZR1) and BRI1–EMS-suppressor 1 (BES1/BZR2) (Wang et al., 2002; Yin et al., 2002). Phosphorylation of BZR1 and BZR2 inhibits their DNA binding activity and leads to their proteasomal degradation (Yin et al., 2002; Vert and Chory, 2006). Moreover they are retained in the cytoplasm by binding of 14-3-3 proteins (Gampala et al., 2007).

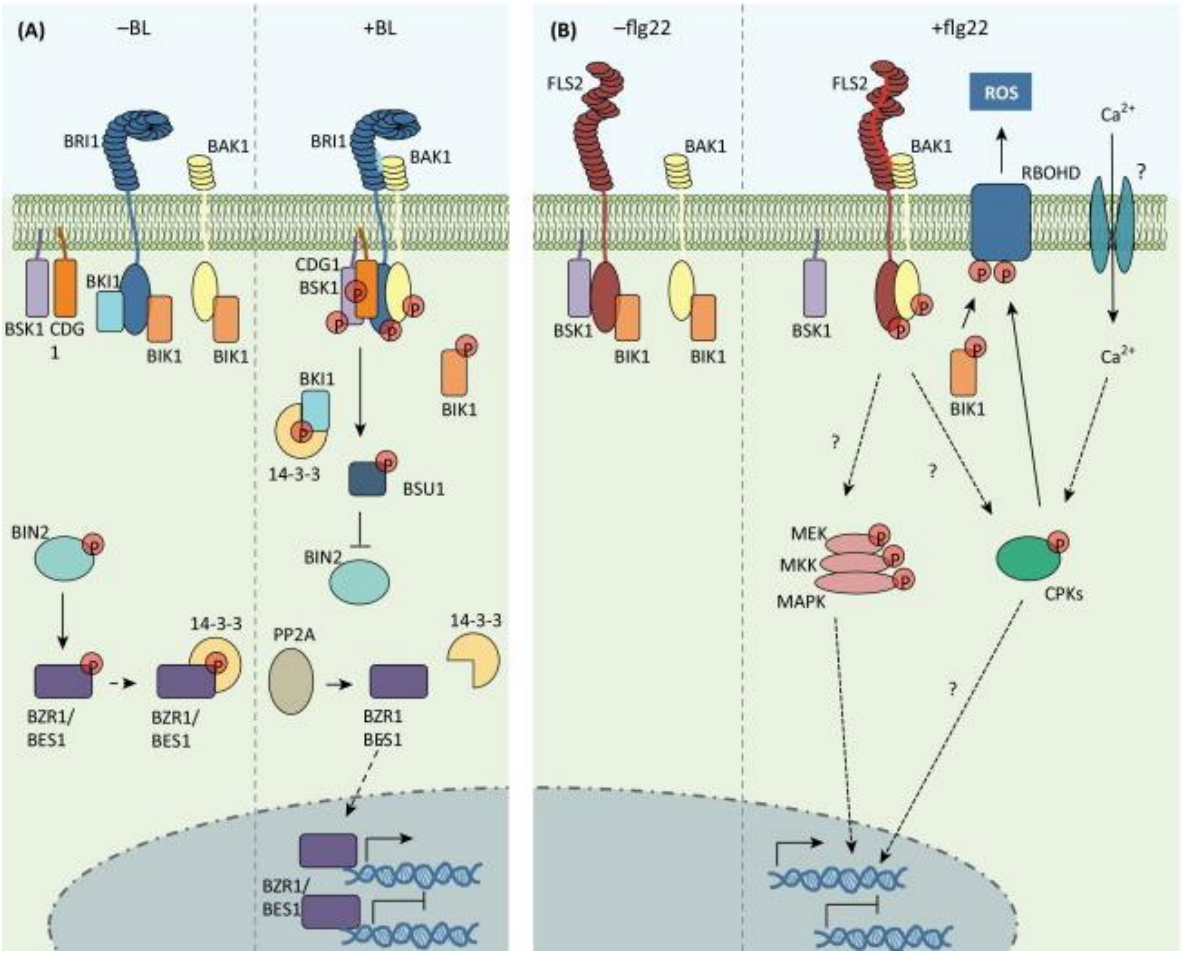

Figure 1-2: Schematic representations of the BL (A) and flg22 (B) signaling pathway (Lozano-Duran and Zipfel, 2015). For details see text.

In the presence of BRs, BKI1 gets phosphorylated by BRI1 and is released from the membrane (Wang and Chory, 2006). Likewise, members of the RLCK family BSKs get phosphorylated and released (Tang et al., 2008). This leads to interaction of BSKs with BSU1; now BSU1 is active and dephosphorylates BIN2 (Mora-Garcia et al., 2004). But BIN2 also directly interacts with BSKs and phosphorylates them, suggesting a positive feedback loop in the so far linear signal transduction pathway (Sreeramulu et al., 2013). BIN2 is no longer phosphorylating the transcription factors BZR1 and BZR2 which leads to their stabilization and nuclear localization. BZR1 and BZR2 directly regulate BR responsive genes (He et al., 2005; Yin et al., 2005). These are, for example, BL biosynthesis genes like Dwarf 4 (DWF4) or Constitutive photomorphism and dwarfism (CPD) which leads to a positive feedback regulation. Recently, an Arabidopsis-specific longer isoform of BES1 has been identified, BES1-L (Jiang et al., 2015). It has stronger activity than the short BES1-S form and an additional nuclear localization signal which also promotes nuclear localization of BES1-S and BZR1 via dimerization.

1.3.1. Trade-off between BR- and immunity signaling

In an uninfected situation plant defense responses are under negative control and developmental processes are favored (Huot et al., 2014). However, at the moment of pathogen infection, activation of defense responses has to be prioritized. This becomes visible as a growth inhibition of MAMP treated seedlings. The trade-off between growth and immunity might be achieved by a crosstalk between the corresponding signaling pathways. As BAK1 is the co-receptor for BL and different MAMPs it seems likely to be an interaction point. Indeed, overexpression of BRI1 or the BR biosynthesis enzyme DWF4 in Arabidopsis leads to reduced flg22 responses. The effect of BRI1 overexpression can be antagonized by simultaneously overexpressing BAK1-HA. Moreover, treatment with chitin, which signals independent of BAK1, leads to normal responses. This shows that the negative effect of BR on MAMP signaling is BAK1 dependent. Treatment with the BR biosynthesis inhibitor BRZ also leads to lower flg22 responses showing that BR homeostasis is critical for the induction of proper immune responses. However, flg22 signaling cannot block BR responses showing that the crosstalk is unidirectional (Belkhadir et al., 2012). This unidirectional crosstalk was also shown in another study, where pretreatment with BR led to reduced responses after flg22 or elf18 treatment (Albrecht et al., 2012). However, here it is stated that the crosstalk is independent of BAK1. In co-immunoprecipitation experiments with BAK1 and FLS2 the amount of BAK1-FLS2 association was not affected by treatment with BR. This shows that BAK1 is not titrated by active BRI1 and not rate limiting between the pathways.

These findings suggest that the crosstalk occurs downstream of the receptor level and indeed the transcriptional activator BZR has been recently described as interaction point (Lozano-Duran et al., 2013). BZR1 overexpression leads to impaired flg22- or chitin-induced ROS production, seedling growth inhibition or marker gene expression but interestingly does not alter BAK1-FLS2 complex formation or MAPK activation. BZR1 interacts with the WRKY transcription factor WRKY40 which transcriptionally inhibits MTI responses so that BZR1 and WRKY transcription factors co-regulate immune responses. However, BZR1 also targets WRKY transcription factors like WRKY11, 15 or 18 which also act as negative regulators of MAMP responses, and could therefore control a second wave of transcription. BZR can also be activated by DELLA proteins. This shows the complex hormone signaling network integrating another hormone, namely gibberellic acid (GA) into the model (Bai et al., 2012). GA is known to be involved in developmental processes like the stimulation of seed germination or transition from juvenile to adult leaf stage (Gupta and Chakrabarty, 2013). However, GA also influences the SA/JA homeostasis and thus influence defense responses (Navarro et al., 2008). A second BR induced transcription factor, Homolog of BEE2 interacting with IBH 1 (HBI1), was identified as suppressor of MTI (Fan et al., 2014; Malinovsky et al., 2014b). Both studies were able to show that MAMP signaling leads to a repression of HBI transcription, indicating a negative effect of MAMPs on BR-signaling. Two additional signaling components shared by MAMP and BR pathways are the RLCKs BIK1 and BSK1 that associate with BRI1 and FLS2 in the absence of the ligand (Lin et al., 2013; Shi et al., 2013). BIK1 acts as a positive regulator of MAMP signaling and negative regulator of BR signaling (Lin et al., 2013). In summary, different studies have identified different proteins as executioners of crosstalk between MAMP and BR signaling pathways. Especially in regard to BAK1 contradictory results have been published so that further studies are needed to show the importance of single proteins in the interaction network between BR and MAMP signaling pathways.

1.4. Autoimmunity and cell death

During the development of multicellular organisms not only cell division and growth is important but also cell death. Programmed cell death (PCD) is a genetically regulated sequence of events that leads to the controlled destruction of a cell (Lockshin and Williams, 1964). In plants, PCD is involved in developmental processes like the formation of trachea elements but also in defense responses against abiotic and biotic stress (Bruggeman et al., 2015). In animals, three cell death pathways can be distinguished and the molecular mechanisms are already well characterized (Lockshin and Zakeri, 2004). The first one is apoptosis, which is accompanied by

shrinkage of the cell, nuclear condensation and fragmentation and the breakup of the cell into apoptotic bodies. On the molecular level, perception of pro-death signals lead to disruption of the mitochondria outer membrane potential and subsequent release of cytochrome C from mitochondria. This leads to activation of caspases (a class of cysteine proteases) which degrade the cell (Adrain and Martin, 2001). The second pathway is autophagy, a “self-eating” process where cytoplasmic material is isolated in vesicles, known as autophagosomes, and then degraded through lysosomes or recycled. Therefore, autophagy is a process also needed to recycle nutrients during starvation. The third pathway is necrosis which is activated after overwhelming cellular stress, when the cell is unable to induce apoptotic pathways. It is accompanied by swelling of the cell because of a lack of osmoregulation (Lockshin and Zakeri, 2004). However, there is also interplay between these three types of PCD.

In plants, the molecular mechanisms of cell death are not that well described and the classification of cell death pathways is difficult. Nevertheless, homologies to the animal system led to the use of the same terms or terms like apoptotic like cell death (Reape et al., 2008). Moreover morphological criteria where used to describe plant cell death pathways that were named as autolytic and non-autolytic (van Doorn, 2011). Autolytic PCD is characterized by the formation of large lytic vacuoles, tonoplast rupture and rapid clearance of the cytoplasm. Autolytic PCD is comparable to animal autophagy. Non-autolytic PCD lacks rapid clearance of the cytoplasm and matches necrosis in animals. Autolytic PCD occurs mainly during plant development and non-autolytic PCD during stress responses. Despite these plant-specific terms, the terms autophagy and necrosis are widely used in the plant field. A core set of autophagy components is conserved in eukaryotes; in Arabidopsis, several *ATG* genes have been described as well with functions in development and stress responses (Hofius et al., 2011). Interestingly, autophagy can have pro-death and pro-survival function during immune responses. The autophagy component Beclin1 restricts HR to the infection side of TMV in *N. benthamiana*. Beclin1-silenced *N. benthamiana* plants show HR lesions adjacent to the infection side and also in distal leaves indicating that autophagy restricts cell death in uninfected tissue (Liu et al., 2005). Arabidopsis *atg5*, *atg10* and *atg18* mutants show enhanced resistance to the hemibiotrophic pathogen *Pto* DC3000 and enhanced susceptibility to infections with necrotrophic pathogens, indicating a pro-survival role of ATG proteins as well (Lenz et al., 2011). In contrast to this, another study described that *atg7* mutants show enhanced growth of *Pto* DC3000 and less cell death after *Hyaloperonospora arabidopsidis* (*Hpa)* infection, demonstrating a pro-death role of this *ATG* gene (Hofius et al., 2009).

Van Doorn stated that there is no apoptotic cell death in plants, however, similarities between plant cell death and animal apoptosis can be observed (van Doorn, 2011). For

example, the potential of the outer mitochondrial membrane is changed which leads to the release of cytochrome c from mitochondria in cucumber (Balk et al., 1999). Plant genomes do not contain caspases which are key enzymes in animal apoptosis. However, caspase-like activity has been described (Danon et al., 2004) and the search for caspase homologues let to the identification of metacaspases (Uren et al., 2000). Different metacaspases such as *At*MC1, *At*MC2 and *At*MC2d have now been studied and are indeed involved in plant cell death control (Coll et al., 2010; Watanabe and Lam, 2011). Interestingly, *At*MC1 has a pro-death function, but *At*MC2 is genetically a negative regulator of *At*MC1, as *At*MC2 over-expression mimics *atmc1* mutant phenotypes (Coll et al., 2010). Crossing of *atmc1* with two autophagy mutants, *atg5* and *atg18a*, led to double mutants with an additive effect, showing that metacaspases act in a cell death pathway parallel to autophagy (Coll et al., 2014). Other plant PCD executioners beside metacaspases could be vacuolar processing enzymes (VPE) which have caspase-1 like activity and VPE deficiency prevents virus induced HR in tobacco (Hatsugai et al., 2004).

A prominent cell death response in plants is the hypersensitive response, which can be induced during immune responses against biotrophic pathogens. HR seems to be a unique type of cell death (Coll et al., 2011). HR leads to cytoplasmic shrinkage, chromatin condensation, mitochondrial swelling, and plant specific reactions like vacuolization and chloroplast disruption. The chloroplast is generally very important for the HR reaction as it is the source of ROS, nitrogen oxide intermediates (NOI), SA and JA (Coll et al., 2011). Moreover, light is required for HR reactions and some bacterial effectors have chloroplast localization signals, highlighting the importance of chloroplasts in cell death control (Jelenska et al., 2007). During immune responses, negative regulation of cell death is as important. Necrotrophic pathogens induce cell death via phytotoxins and cell wall degrading enzymes to feed on the dead plant material (Choquer et al., 2007; Davidsson et al., 2013). *Botrytis cinerea* is even able to trigger HR to facilitate host colonization and thus uses a host defense mechanism for its own pathogenicity (Govrin and Levine, 2000).

Deregulation of cell death leads to autoimmunity, a constitutive immune response of plants against their own tissue. Mutants with deregulated cell death can be used to screen for the involved molecular components. These mutants are called lesion mimic mutants (LMMs) because they show either constitutive or unregulated cell death that resembles HR like lesions after pathogen infection (Bruggeman et al., 2015). LMMs are divided into initiation mutants that show HR-like lesions without pathogen infection and propagation mutants that show cell death spreading once it was initiated by pathogens (Lorrain et al., 2003). The identification of LMMs in forward genetic screens leads to the identification of proteins that are inhibitors of

cell death. Later on, second site mutagenesis in these LMMs in order to find suppressors of cell death led to the identification of positive regulators. Different LMMs revealed the novel cellular functions or processes during plant PCD. These are Ca2+ ion influx (*defense no death 1*, (*dnd1)*), ROS formation and sensing (*constitutive expresser of PR 22 (cpr22)* and *lesions simulating disease resistance response 1 (lsd1)* respectively), sphingolipid metabolism (*accelerated cell death 5 (acd5)* and *acd11*) and porphyrin/chlorophyll biosynthesis and catabolism (*acd1*, *acd2*, *lesion initiation 2* (*lin2)*, *lethal leaf spot 1* (*lls1*), *lesion 22* (*les22*) and *fluorescent 1* (*flu1)*) (Moeder and Yoshioka, 2008). Many LMMs are linked to R-proteins; mutation of the guardee mimics the pathogen effector and leads to ETI induction by the guard. Another mutant that shows autoimmunity, but is no classical LMM, is *mpk4* (Petersen et al., 2000). *mpk4* mutants do not exhibit necrotic lesions, have very high SA levels and block JA. MPK4 can be genetically seen as a negative regulator, however, it activates defense responses upon flg22 perception via WRKY33 and PAD3 (Droillard et al., 2004; Andreasson et al., 2005; Qiu et al., 2008). MPK4 is an effector target and the *mpk4* mutant phenotype can be suppressed by mutation of the R-protein SUMM2, showing that MPK4 is a guardee and its absence induces ETI (Zhang et al., 2012). It was recently discovered that MPK4 interacts with PAT1, a component of the mRNA decapping machinery. PAT1 accumulates after flg22 treatment, so that mRNA decay could be another mechanism by which MPK4 rapidly initiates defense responses.

The small RLK BIR1 was identified to be induced after infection with *Psm* ES4326 and *bir1* mutants interestingly have a seedling lethal phenotype when grown at 22°C (Gao et al., 2009). *bir1* mutants show intrinsic cell death, constitutive activation of defense responses, resistance against *Hpa* and high SA levels. BIR1 constitutively interacts with BAK1. The *bir1* mutant phenotype can be partially suppressed by *eds1* and *pad4* and to a lower extent by *ndr1*, showing that cell death activation in *bir1* is likely via R-protein pathways. During the same study another small RLK was identified whose mutation suppresses the *bir1* mutant phenotype, Suppressor of *bir1* (SOBIR1). *bir1 sobir1* double mutants are larger compared to *bir1* single mutants but do not reach wildtype size. Overexpression of SOBIR1 also leads to constitutive activation of defense responses, showing that it is a positive regulator of cell death. Later studies showed that different mutations in components of the ER quality control can suppress cell death in *bir1*. SOBIR1 protein amounts are reduced in these mutants showing that ER quality control is important for SOBIR1 biogenesis (Sun et al., 2014). Another mutation in the ER quality control machinery, *stt3a*, also suppresses the autoimmunity phenotype of *bir1* but does not alter SOBIR1 protein amounts (Zhang et al., 2015). This indicates that additional signaling components leading to cell death in *bir1* are regulated by ER quality

control. In different studies it could be shown that SOBIR1 interacts with several RLPs to form PRR complexes. SOBIR1 is necessary for recognition of the MAMP ScFE1 from the necrotrophic fungus *Sclerotinia sclerotiorum* and *sobir1* mutants are more susceptible to *S. sclerotiorum* infections (Zhang et al., 2013). Recognition of ScFe1 is not only dependent on SOBIR1 but also on BAK1. Interestingly, BAK1 also plays a role in cell death control. *bak1* mutants show spreading cell death after pathogen infection and double mutants of BAK1 and the closest homologue BKK1 show spreading cell death and are seedling lethal (He et al., 2007; Kemmerling et al., 2007).

Another cell death mutant showing lethality at 22°C is *bonzai1* (*bon1*). BON1 is a phospholipid binding protein which locates to the plasma membrane. The mutant phenotype can be rescued by growth at 28°C, where BON1 is less expressed (Hua et al., 2001). The *bon1* phenotype can be rescued by mutation of the R-protein Suppressor of NPR1-1 constitutive 1 (SNC1) (Yang and Hua, 2004). BON1 interacts with BAK1 and BIR1 which are themselves described to be involved in cell death regulation (Wang et al., 2011). BON1 and BIR1 have a synergistic effect because the double mutant shows a more severe phenotype compared to the single mutants. In the same study it was shown that the *bir1* phenotype can also be attenuated by *SNC1* knockout. This connection between BAK1 and the R-protein SNC1 provides a link between MTI and ETI.

Another phenomenon where spontaneous cell death occurs is hybrid necrosis. To maintain species or sub-species, gene flow barriers need to be sustained. Species hybrids are often less fertile than the parental strains or even embryonic lethal showing a mechanism for hybrid incompatibility (Lafon-Placette and Kohler, 2015). Many plants show a common type of hybrid incompatibility which resembles the phenotype after pathogen attack or autoimmunity mutants and is therefore called hybrid necrosis (Bomblies and Weigel, 2007). After crossing of different *Arabidopsis thaliana* strains, approximately 2 % of the F1 progeny showed hybrid necrosis (Bomblies et al., 2007). It is caused by synergistic interactions of genes that have functionally diverged among the respective parents. One gene family in Arabidopsis that shows this high genetic variation and was shown to be involved in hybrid incompatibility is the NB-LRR gene family. One example is the incompatibility between a strain from Poland, *Landsberg erecta* (*Ler*), and strains from central Asia, *Kashmir 2* and *Kondara* (*Kas2* and *Kond*). The polymorphic TIR-NB-LRR cluster RPP1 in *Ler* is incompatible to the RLK Strubbelig receptor family 3 (SRF3) in *Kas* and *Kond* causing temperature dependent autoimmunity (Alcazar et al., 2010).

1.5. The multifunctional co-receptor BAK1

In the previous chapters it became clear that BAK1 is a multifunctional co-receptor that acts in different signaling pathways. It was first named SERK3 as a member of the SERK protein family (Hecht et al., 2001). BAK1 was identified as a co-receptor of the BL receptor BRI1 and named as BRI1 associated kinase 1 (Li et al., 2002; Nam and Li, 2002). However, BAK1 also plays a role in plant immunity as it interacts with several MAMP receptors like FLS2 and EFR, the DAMP receptor PEPR1 and PEPR2 and the phytosulfokine receptor PSKR1 (Yamaguchi et al., 2006; Chinchilla et al., 2007; Heese et al., 2007; Krol et al., 2010; Postel et al., 2010; Roux et al., 2011; Ladwig et al., 2015). It is also assumed that BAK1 forms ligand induced complexes with RLPs and SOBIR1, e.g. with RLP30 that recognizes the MAMP ScFE1 from *Sclerotinia sclerotiorum.* An RLP-BAK1 interaction that is experimentally validated is the interaction with the RLP44, however, BAK1 and RLP44 interact constitutively. RLP44 is involved in cell wall remodeling by activation of BR signaling (Wolf et al., 2014). Additionally, BAK1 plays a role in cell death control as *bak1* mutants show spreading cell death after pathogen infection and are more susceptible to necrotrophic pathogens. The cell death phenotype of *bak1* is BL independent, as exogenous application of BL does not restore immunity to infection by necrotrophic fungi (Kemmerling et al., 2007). The cell death regulatory role of BAK1 becomes more obvious in double mutants with the closest homologue BAK1 like 1 (BKK1 or SERK4). b*ak1 bkk1* double mutants show constitutive activation of immune responses, display spontaneous cell death and are seedling lethal (He et al., 2007). The SERK family consists of 5 members that have partially redundant functions in somatic embryogenesis (SERK1 and SERK2), tapetum formation (SERK2), BR-signaling (SERK1, SERK3 and SERK4), MAMP-signaling (SERK3 and SERK4) and cell death (SERK3 and SERK4) (Li, 2010). Their functions overlap, but each provides a specific subset of signaling roles. Specific residues in the extracellular domain that can be assigned to one specific signaling function are conserved even if that SERK does not function in that pathway. For example SERK2 does not function in the brassinosteroid pathway and does not interact with BRI1 but is conserved in its BRI1 interaction domain (Aan den Toorn et al., 2015). SERKs are ancient genes that have been recruited as co-receptors to newly evolved signaling pathways.

The fact that BAK1 is a shared signaling component obviously leads to the question how signaling specificity is achieved. It was shown that signaling is indeed specific, because activation of BAK1 by BR treatment cannot induce typical immune responses like ROS production or MAPK activation and vice versa (Albrecht et al., 2012). Here it could also be shown that BAK1 is not rate limiting between the pathways which fits to the model of

preformed receptor complexes. This means that receptors and the co-receptor BAK1 are already in close vicinity in distinct membrane compartments before ligand-binding, as it was shown for BRI1 and BAK1 (Bücherl et al., 2013). This would mean that distinct sub-pools of BAK1 exist that are already preassembled with a certain ligand-binding receptor and that e.g. a BAK1 molecule assembled with BRI1 cannot be activated by flg22 treatment. Another model for signaling specificity would be phospho-coding, differential phosphorylation of BAK1 by different ligand-binding receptors. One hint for this is the *BAK1-5* allele, a point mutation of C408 in the kinase domain that leads to lower phosphorylation activity of BAK1 and only affects the MAMP signaling pathway but not BR responses or cell death (Schwessinger et al., 2011). Another study aimed to identify BAK1 phosphorylation patterns associated with other RLKs (FLS2, EFR, BRI1 and BIK1) and performed different in vitro assays to quantify BAK1 phosphorylation (Wang et al., 2014). It could indeed be shown that different RLKs lead to different phosphorylation patterns in BAK1. These different BAK1 phosphopatterns then lead to different phosphorylation of RLK targets, however, this data still has to be confirmed in planta. Another shared signaling component between MAMP and BL signaling is the RLCK BIK1. In the flg22 signaling cascade the co-receptor BAK1 phosphorylates BIK1 which leads to its activation and dissociation from the receptor complex. In contrast, BR perception leads to phosphorylation of BIK1 directly by the receptor BRI1, also leading to activation and dissociation of BIK1 (Lin et al., 2013). This data also points into the direction of signaling specificity via differential phosphorylation patterns, but the exact phosphorylation events need to be elucidated.

Recently, a study used BAK1 overexpressing plants to further study the role of BAK1 in immunity and cell death (Dominguez-Ferreras et al., 2015). BAK1 overexpressors showed a stunted phenotype with leaf necrosis, 75% of the lines died prematurely, and the others produced less seeds. In the same study it could be shown that inducible overexpression of BAK1 leads to cell death, visualized by trypan blue staining in cotyledons, similar to that after elf18 treatment observed in this study. Overexpression of BAK1 also lead to induction of MAPKs and ethylene production without MAMP treatment and the plants are more resistant to *Pto* DC3000 infections. Interestingly, the BAK1 overexpression phenotype can be rescued by *sobir1*. The molecular mechanism is not further elucidated but as SOBIR1 is important for autoimmunity cell death, it is discussed that the extra BAK1 could be detected by R-proteins. These findings show that BAK1 and SOBIR1 not only act together as co-receptors for RLPs but also in cell death control. The fact that BAK1 overexpression leads to cell death was already observed before (Belkhadir et al., 2012). Here the phenotype could be rescued by

simultaneous overexpression of BRI1. This indicates that balanced receptor levels are crucial for repression of cell death.

1.6. The BIR family of receptor-like kinases

BAK1 is a central regulator of different immunity pathways and additionally controls cell death responses, but it is not yet clear how BAK1 itself is regulated. Therefore, a former PhD student Sara Mazzotta purified in vivo BAK1 complexes and identified two new interactors named as BAK1 interacting RLK 2 and 3 (BIR2 and BIR3) (Halter et al., 2014). BIR2 and 3 belong to a small family of RLKs that consists of four members and builds the subgroup Xa of Arabidopsis RLKs. Another family member, BIR1, was already described and plays a role in cell death control (Gao et al., 2009). *bir1* mutants show very strong cell death, high SA levels, resistance to biotrophic bacteria and are seedling lethal when grown at 22°C. Crossing with *pad4, eds1* or *ndr1* can partially rescue the cell death phenotype so that the authors concluded that knocking out BIR1 results in activation of multiple R-protein mediated resistance pathways. Analysis of BIR2 and 3 showed that both proteins have a differential expression pattern after pathogen infection. Similar to BAK1, BIR2 is upregulated after infection with different *Pseudomonas syringae* strains whereas BIR3 is downregulated. BIR2 and 3 are structurally receptor like kinases but do not show kinase activity. The BIR2 crystal structure has shown that it has an occluded ATP binding pocket, is not able to bind ATP and execute phosphorylation activity (Blaum et al., 2014). However, BIR2 gets phosphorylated by BAK1 and the BAK1 phosphorylation activity is necessary for BAK1 BIR2 interaction. BIR3 gets only weakly phosphorylated by BAK1 in in vitro kinase assays (Mazzotta, 2012). Functional analysis of BIR2 mutants has shown that BIR2 differentially affects BAK1 regulated pathways. In contrast to *bak1* mutants, *bir2* mutants show hyperresponsiveness to MAMPs, they are not affected in BL signaling and show stronger cell death after pathogen infection. *bir3* mutants do not show defects in cell death or BL sensitivity but BIR3 overexpressors have a very strong stunted growth phenotype and are BL insensitive. Moreover, 35S-BIR3 plants show stronger symptom development after *A. brassicicola* infection and less ROS production after flg22 treatment (Halter, 2014).

1.7. Aim of the thesis

BIR family proteins were identified to interact with BAK1 and to regulate different BAK1 dependent processes. *bir2* mutants are hyperresponsive to MAMPs and show stronger cell death after pathogen infection. BIR3 overexpressors are BL insensitive and at the same time

show stronger cell death and lower MAMP responses. However, the full functional potential and the molecular mechanisms how BIR proteins regulate BAK1 are not yet understood. Moreover, the relationship of the different BIR proteins, whether they are redundant or specify different BAK1 dependent processes, needs to be clarified. The aim of this thesis is to elucidate which BAK1 dependent processes are regulated by BIR2 and BIR3 and how the different BIR proteins exert these functions.

2. Materials and Methods

2.1. Materials

2.1.1. Plant genotypes

Genotype	Mutation	Reference/Source
Col-0	wildtype	
bak1-4	SALK_116202, T-DNA insertion in *BAK1* (At4g33430)	(Kemmerling et al., 2007)
bak1-3	SALK_034523, T-DNA insertion in *BAK1* (At4g33430	(Kemmerling et al., 2007)
bir2-1	GABI N 733599, T-DNA insertion in *BIR2* (At3g28450)	(Halter et al., 2014)
amiR-*BIR2*	artificial micro RNA against *BIR2*	(Halter et al., 2014)
bir3-1	Salk_132078, T-DNA insertion in *BIR3* (At1g27190)	(Halter, 2014)
bir3-2	Salk_116632, T-DNA insertion in *BIR3* (At1g27190)	(Halter, 2014)
35S-BIR3	pB2GW7-BIR3 transformed in Col-0	(Halter, 2014)
35S-BIR3-YFP	pB7YWG2-BIR3 transformed in Col-0	(Halter, 2014)
35S-BIR2-YFP	pB7YWG2-BIR2 transformed in Col-0	(Halter et al., 2014)
bak1-3 bir2-1	Crossing of *bak1-3* with *bir2-1*	this work
bak1-4 amiR-*BIR2*	Crossing of *bak1-4* with amiR *BIR2*	this work
bir2-1 bir3-2	Crossing of *bak2-1* with *bir3-2*	this work
bak1-3 bir3-1	Crossing of *bak1-3* with *bir3-1*	this work
bak1-3 bir3-2	Crossing of *bak1-3* with *bir3-2*	this work
bak1-4 bir3-2	Crossing of *bak1-4* with *bir3-2*	this work
bir1-1	WiscDsLox393-396D17, T-DNA insertion in *BIR1* (At5g48380)	(Gao et al., 2009)
bak1-4 bir1-1	Crossing of *bak1-4* with *bir1-1*	this work
bir2-1 35S-BIR3	Crossing of *bir2-1* with 35S-BIR3	this work
bir1-1 bir3-1	Crossing of *bir1-1* with *bir3-1*	this work
bir1-1 35S-BIR3	Crossing of *bir1-1* with 35S-BIR3	this work
bir1-1 amiR-*BIR2*	Crossing of *bir1-1* with amiR-*BIR2*	this work
bak1-5	point mutation in *BAK1* C408Y	(Schwessinger et al., 2011)
bkk1-1	SALK_057955, T-DNA insertion in BKK1 (At2g13790)	(He et al., 2007)
nahG	expression of the bacterial NahG gene, a salicylate hydroxylase	(Lawton et al., 1995)
pad4	point mutation in PAD4 (At3g52430)	(Glazebrook et al., 1996)

snc1-11	SALK_047058, T-DNA insertion in SNC1 (At4g16890)	(Kim et al., 2010)
bir3-2 bkk1-1	Crossing of *bir3-2* with *bkk1-1*	this work
bir3-2 bak1-5	Crossing of *bir3-2* with *bak1-5*	this work
BIR3-GFP bak1-4 bir3-	Expression of BIR3-GFP construct under endogenous promotor in *bak1-4 bir3-2* background	Sacco de Vries
BAK1-GFP bak1-4 bir3 2	Expression of BAK1-GFP construct under endogenous promotor in *bak1-4 bir3-2* background	Sacco de Vries

2.1.2. Bacterial strains

Strain	Genotype
Escherichia coli DH5α	F-(Φ80lacZΔM15) Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1)
Agrobacterium tumefaciens GV3101	T-DNA- vir+ rifr, pMP90 genr

2.1.3. Media and Antibiotics

Components of the different media used in this study are given in the following table.

Medium	Components
LB	10 g/l Bacto-Trypton, 5 g/l Bacto-Yeast extract, 5 g/l NaCl, to solidify add 15 g/l Agar
King's B	20 g/l Glycerol, 40 g/l Proteose Pepton 3, 0.1 % K_2HPO_4, 0.1 % $MgS0_4$, to solidify add 15 g/l Agar
½ MS	2.2 g/l MS-salts (Duchefa), 1% sucrose when indicated, set pH 5.7 with KOH, to solidify add 8 g/l Select-Agar
SOC	2.0 g/l Trypton, 0.5 g/l Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose, set pH 7 with NaOH
YPD	20 g/l peptone, 20 g/l glucose, 10 g/l yeast extract, set pH to 6-6.3, to solidify add 15 g/l oxoid agar
CSM	6.9 g/l YNB without amino acids (Formedium), synthetic complete amino acid drop out according to manufacturer's instructions (Formedium), 20 g/l glucose, set pH to 6-6.3, to solidify add 1.5 % oxoid agar

After autoclaving media were cooled to 60°C and supplemented with antibiotics in the following concentrations.

Antibiotic	Stock	final concentration
Kanamycin	50 mg/ml in H_2O	50 µg/ml
Rifampicin	12.5 mg/ml in methanol	50 µg/ml
Cycloheximide	12.5 mg/ml in H_2O	50 µg/ml
Spectinomycin	50 mg/ml in H_2O	100 µg/ml
Gentamycin	10 mg/ml in H_2O	25 µg/ml
Carbenicillin	50 mg/ml in H_2O	50 µg/ml

2.1.4. Antibodies

1. Antibody	Source	Use	Reference/Provider
α-GFP	goat	1:10000	Acris
α-HA	mouse	1:2000	Sigma-Aldrich
α-Myc	rabbit	1:5000	Sigma-Aldrich
α-BAK1	rabbit	1:5000	Agrisera
α-FLS2	rabbit	1:2500	Custom made
α-BIR2	guinea-pig	1:2000	(Halter et al., 2014)

2. Antibody	Feature	Use	Reference/Provider
α-goat	HRP conjugated	1:10000	Sigma-Aldrich
α-mouse	HRP conjugated	1:10000	Sigma-Aldrich
α-rabbit	HRP conjugated	1:50000	Sigma-Aldrich
α-guinea-pig	HRP conjugated	1:5000	Santa Cruz

2.1.5. Primers

Primers were synthesized by Eurofins MWG Operon. Primer stocks were kept at a 100 µM concentration diluted in nuclease-free water. The sequences of primers used in this study are listed in the following table.

Name	sequence 5'→ 3'	Characteristics
a-N616202	TTATTGTTTGGCCGATCTTGG	genotyping *bak1-4,* fwd
b-N616202	ACATCATCATCATTCGCGAGG	genotyping *bak1-4*, rev
a-N534523	GGTGCTTCAAAGTTGGGATGC	genotyping *bak1-3*, fwd
N534523	CTATTTGGCGACACTACTTTCTGAC	genotyping *bak1-3*, rev
a-WISCP25D5	TTAAATAGGAAGTCGCTAACCATGGGAG	genotyping *bir2-1*, fwd

PGL3 rev	AGA AAC CAA GAA GCG GTG CT	genotyping *bir2-1*, rev
At1g27190R	GGTAGGAGATACAAGACAAACACGAGCAC	genotyping *bir3-1*, fwd
At1g27190F	TATTACCAGCCAACCAACCTTTCATACAA	genotyping *bir3-1*, rev
At1g27190R3	ACAGACAAAGGCTTTTGCCCTGTAACCA	genotyping *bir3-2*, fwd
At1g27190F3	CTCGCCGGTGAGATTCCTGAGTCTCTTA	genotyping *bir3-2*, rev
a-CS854273	GCAACTTGGACGGCTCAAAACATTCTCT	genotyping *bir1-1*, fwd
b-CS854273	CGTGATGACTGTATTCTCCTCAACCAAA	genotyping *bir1-1*, rev
Gabi-Kat-LB	CCCATTTGGACGTGAATGTAGACAC	GABI LB primer
Lba1	TGGTTCACGTAGTGGGCCATCG	Salk LB primer
p745	AACGTCCGCAATGTGTTATTAAGTTGTC	WiscDSLox LB primer
snc1-11 fwd	TCTGTTGCTTTAACCTTTGCTCC	genotyping *snc1-11*, fwd
snc1-11 rev	TGGTGATTCCGATTTTCTTCCAC	genotyping *snc1-11*, rev
pad4-1-PflmI-F	ATGAGTCGCATAAGACTAGCCAAG	genotyping *pad4-1*, fwd
pad4-1-PflmI-R	CCATTTCTTTCCTAAATGAAAATCA	genotyping *pad4-1*, rev
NahG-F	GGCTTGCGCATCGGTATCGTCGGC	genotyping nahG, fwd
NahG-R	GCCATGGGCCCGATAGGCTTCTCG	genotyping nahG, rev
BAK1-dCAPs-F	AAGAGGGCTTGCGTATTTAGATGATCACT	genotyping *bak1-5*, fwd
BAK1-dCAPs-R	GAGGCCAGCAAGATCAAAAG	genotyping *bak1-5*, rev
a2-N557955	TTGCTACCCCGCATTTAGTCA	genotyping of *bkk1-1*, fwd
b2-N557955	TGGTTCACGTAGTGGGCCATCG	genotyping of *bkk1-1*, rev
ef1a-100-f	GAGGCAGACTGTTGCAGTCG	EF1α qPCR, fwd
ef1a-100-r	TCACTTCGCACCCTTCTTGA	EF1α qPCR, rev
FRK1F	AGCGGTCAGATTTCAACAGT	FRK1 qPCR, fwd
FRK1R	AAGACTATAAACATCACTCT	FRK1 qPCR, rev
PR1F	GTGGGTTAGCGAGAAGGCTA	PR1 qPCR, fwd
PR1R	ACTTTGGCACATCCGAGTCT	PR1 qPCR, rev
PDF1.2F	AGGGGTTTGCGGAAACAGTAA	PDF1.2 qPCR, fwd
PDF1.2R	CGTAACAGATACACTTGTGTGC	PDF1.2 qPCR, rev
EF1 a s	TCACATCAACATTGTGGTCATTGG	EF1α RT-PCR, fwd
EF1a as	TTGATCTGGTCAAGAGCCTCAAG	EF1α RT-PCR, rev
BAK1-q-fwd	GACCTTGGGAATGCAAATCTATC	BAK1 RT-PCR, fwd
BAK1-q-rev	AAAACTGATTGGAGTGAAAAGTGAAA	BAK1 RT-PCR-rev

2.1.6. Plasmids

Plasmid	Features	reference
pB7YWG2-BIR3	Expression of 35S-BIR3-YFP in planta	Thierry Halter
pGWB14-BRI1	Expression of 35S-BRI1-3xHA in planta	This work
pGWB14-BKK	Expression of 35S-BKK1-3xHA in planta	Thierry Halter
pGWB17-FLS2	Expression of 35S-FLS2-4xMyc in planta	Thierry Halter
BAK1-pMetYC	Met repressible expression of BAK1 in yeast with C-terminal Cub-ProteinA- LexA-	Christopher Grefen

	VP16	
BRI1-pNubA22	Constitutive expression of BRI1 C-terminal NubA-3xHA in yeast	Christopher Grefen
pZMU-Dest	Expression vector for Constitutive expression with C-terminal myc in yeast	Christopher Grefen
pZMU-BIR3	Constitutive expression of BIR3 with C-terminal myc in yeast	This work

2.1.7. Chemicals

Chemicals and reagents were purchased from Carl Roth, Sigma-Aldrich, Fluka, Merck, Duchefa or Applichem. Enzyme used for nucleic acids studies were obtained from Thermo Scientific.

2.2. Methods

2.2.1. Plant methods

2.2.1.1. Plant growth conditions

A. thaliana plants were grown on soil for 6 weeks in a growth chamber under short day conditions (8 hours light, 16 hours dark, 22 °C, 110 $mEm^{-2}s^{-1}$, 60 % relative humidity). For seed production plants were grown under long day conditions (16 hours light, 8 hours dark). *N. benthamiana* plants were grown in the greenhouse under long day conditions for 3 weeks.

For CoIP experiments with *A. thaliana* material of sterile grown seedlings was used. Therefore, sterilized seeds were sown on ½ MS plates and grown for 7 days under long day conditions. Afterwards they were transferred into liquid ½ MS medium with 1 % sucrose. They were grown again for 7 days under long day conditions. One week later they were removed from the MS medium and incubated for 30 min in 0.1 M NaCl, 0.1 % BSA to distress them. Then they were induced or not for 5 min with 1 µM flg22 or other elicitors. Seedlings were dried, directly frozen in liquid N_2 and stored at -80 °C.

2.2.1.2. Crossing

In order to obtain double mutant plants the corresponding single mutants were crossed. One mutant was chosen as female plant. From these plants several buds were chosen and the sepals, petals and stamens were removed with forceps. Only the unfertilized carpel remained on the flower. The other mutant was taken as male plant. From this plant several mature stamens were removed with forceps and spotted onto the carpel so that pollen grains

remained on the carpel. After successful crossing, siliques were obtained that contained the F1 seeds. Successful crossing was verified by monitoring presence of the male T-DNA insertion in the F1 generation. In the F2 generation double mutants were selected by PCR-based genotyping. F3 plants were used for analysis.

2.2.1.3. Transient transformation of *N. benthamiana* by *Agrobacterium tumefaciens*

For protein expression in *N. benthamiana* transient expression via *A. tumefaciens* GV3101 was used. 5-10 ml *A. tumefaciens* cultures in LB medium supplemented with appropriate antibiotics were inoculated from LB plates and grown at 28 °C overnight. Cells were harvested the next morning by centrifugation at 4000 x *g* for 5 min. The cell pellets were resuspended in 10 mM $MgCl_2$ and washing was repeated one more time. The cultures were diluted to OD_{600}=1 with 10 mM $MgCl_2$. The strains were mixed to the same rate, also with the silencing inhibitor p19 (Voinnet et al., 2003). 150 µM acetosyringone were added (from a 150 mM stock in DMSO) and the bacteria were incubated for 2 hours at room temperature. Tobacco leaves of 3 week old plants were pricked with a cannula at the infiltration side and leaves were infiltrated with a needleless syringe. Leaves were harvested 2-3 days after infiltration and used to confirm protein expression in total protein extracts and subsequent CoIP analysis.

2.2.1.4. Seeds sterilization

Seed sterilization was performed with chlorine gas. The seeds were put into microcentrifuge tubes and the tubes were put into a glass desiccator with open lid. In the desiccator a beaker with 50 ml 12 % sodium-hypochlorite solution was placed and 2 ml 37 % HCl were added. The desiccator was closed immediately and the seeds were incubated for 4 hours to overnight. After that the tubes were placed open under the sterile bench for 30 min to allow evaporation of remaining chlorine gas.

2.2.1.5. BL and BRZ assays

Arabidopsis seeds were surface sterilized and sawn in one row on ½ MS plates with 0.8 % select agar supplement with or without 10 nM Epi-Brassinolide (BL) or 1 µM Brassinazole (BRZ). Plates were incubated for 3 days at 4°C in the dark to promote equal germination. For BL assays seedlings were grown under long day conditions horizontally for 7 days and root length was measured. For BRZ assays seedlings were grown for 7 days in the dark and

hypocotyl length was measured. Plates were photographed and root or hypocotyl length was measured using image J software.

2.2.1.6. Oxidative burst

Production of reactive oxygen species (ROS) is measured in a luminol based assay. A peroxidase catalysis the oxidation of luminol to 3-aminophthalate what is accompanied by emission of light at 428 nm. The light emission is measured in a plate reader and proportional to the released ROS. For the experiment six week old *A. thaliana* plants were used. Leaves were cut into rectangular pieces and floated in petri dishes with H_2O overnight, so that the wound response has worn off. The next day leaf pieces were put into white 96 well plates with 20 µM luminol L-012 and 2 µg/ml peroxidase. Leaf pieces were treated with 100 nM flg22 (Synthesized by Thermo Scientific) or elf18 (Synthesized by Sellekchem) and analyzed with the multiplate reader Centro LB 960 (Berthold Technologies) for 30 min. Per line 9 replicates were measured.

2.2.1.7. MAMP-induced transcriptional changes

A. thaliana seedlings were grown sterile on ½ MS plates with 0.8 % agar under long day conditions. After 5 days the seedlings were transferred into liquid ½ MS medium with 1 % sucrose and equilibrated overnight in the long day chamber. The seedlings were elicited with 100 nM flg22 or elf18 for 3 hours. Seedlings were harvested, directly frozen in liquid N_2 and stored at -80°C until RNA extraction. For one biological replicate around 20 seedlings were used.

2.2.1.8. Infections with *Alternaria brassicicola*

A. brassicicola MUCL 20297 cultivation and spore production was performed as described in (Thomma et al., 1999). For infection experiments 6 week old *A. thaliana* plants were used. A glycerol stock of *A. brassicicola* spores with of $2 * 10^7$ spores/ml was diluted with sterile water to 1×10^6 spores/ml and brought to room temperature. Two leaves per plant were inoculated with 2-4 5 µl droplets of the spore solution. Plants were randomly distributed in a tray and were kept under 100 % humidity in a short day chamber. The bonitation was done after 7, 10 and 13 days according to the following scheme: 1: no symptoms, 2: light brown spots at infection site, 3: dark brown spots at infection site, 4: spreading necrosis, 5: leaf maceration, 6:

sporulation. The disease index (DI) was calculated with the following formula: DI = Σ i * n_i. ''i'' is the symptom category, and ''n_i'' is the percentage of leaves in ''i.

For one experiment about 9 plants were used and divided into three groups for statistical analysis or the statistics were calculated over all values.

2.2.1.9. Trypan blue staining

Trypan blue staining of *A. brassicicola* inoculated leaves was performed as described in (Kemmerling et al., 2007). *A. thaliana* leaves were put into 6 well plate with 2 ml trypan blue staining solution (8 % (v/v) lactic acid, 8 % (v/v) glycerol, 8 % (v/v) Aqua-Phenol; 66 % (v/v) EtOH; 0.36 % (w/v) trypan blue) and incubated in a 100 °C water bath for 45 s - 1 min. The staining solution was then replaced with chloralhydrate solution (1 g/ml) for destaining. After 6 hours the destaining solution was replaced with fresh solution and incubated again over night. The destained leaves were placed on microscope slides with 20 % glycerol and examined under a binocular.

2.2.1.10. Hormone measurements

The analysis of SA and JA content was performed in collaboration with the ZMBP analytics department at the University of Tübingen. The measurements were performed as previously described (Lenz et al., 2011).

2.2.1.11. Bacterial growth assays

Pseudomonas syringae pv. tomato DC3000 was grown on King's B Agar plates with Rifampicin for 24 hours at 28 °C. For infection experiments overnight cultures in King's B medium with Rifampicin were inoculated and grown at 28 °C. The next morning the culture was harvested by centrifugation at 3500 rpm, 4 °C for 10 min. The pellet was carefully resuspended in 10 mM $MgCl_2$ and washing was repeated one more time. The OD_{600} was set to 0.2 (corresponds to 10^8 cfu/ml) and the bacteria were diluted to 1 * 10^4 cfu/ml for infiltration.

For infection experiments 6 week old *A. thaliana* plants were used and two leaves per plant were syringe infiltrated. Per line and time point 8 leaves were infiltrated and represent one biological replicate. Leaves were harvested after 0, 2 and 4 days and surface sterilized for 1 min in 70 % EtOH and then washed in sterile H_2O. Two leave discs were cut out with a cork borer and homogenized in 10 mM $MgCl_2$. Dilution series of this bacterial suspension were plated onto LB-plates with Rifampicin and Cycloheximide and incubated for two days at 28 °C. After two days the number of colonies was counted and the cfu/cm^2 leaf area was calculated.

2.2.2. Protein analysis

2.2.2.1. Total protein extraction from plant material

For total protein extraction from *A. thaliana* or *N. benthamiana* 100 mg leaf material were ground in liquid N_2. The ground material was mixed with 100 µl cold extraction buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.5 % Nonidet P40, proteinase inhibitor cocktail (Roche)) and incubated for 30 min on ice with occasional mixing. Afterwards the samples were centrifuged for 10 min at 14000 rpm, 4 °C and the supernatant was transferred into a fresh tube. The clear lysates were mixed with 5 x SDS loading buffer (312.5 mM Tris/HCl pH 6.8, 10 % (w/v) SDS, 25 % (v/v) β-mercaptoethanol, 50 % (v/v) glycerol, 0.05 % (w/v) bromphenol blue) and boiled for 5 min at 95 °C. Samples were directly used for immunoblot analysis or stored at -20 °C.

2.2.2.2. Protein concentration measurements

For protein concentration measurements the detergent compatible kit Biorad DC protein assay (Biorad, Hercules, USA) was used according to the manufacturer's instruction. All samples of one experiment were adjusted with extraction buffer to the sample with the lowest protein concentration.

2.2.2.3. SDS-PAGE

For SDS-PAGE analysis the Biorad Mini-PROTEAN Tetra Cell was used. 8% resolving gels consisting of 2.3 ml H_2O, 1.3 ml acrylamide-bisacrylamide mix (37.5:1), 1.3 ml 1.5 M Tris pH 8.8, 50 µl 10 % SDS, 50 µl 10 % APS and 3 µl Temed were pured between glass plates with 1 mm spacers. The surface was covered with 50 % isopropanol and the gel was let polymerize. When the gel was polymerized the isopropanol was removed and the 4 % stacking gel consisting of 1.4 ml H_2O, 0.17 ml acrylamide-bisacrylamide mix (37.5:1), 0.13 ml 1M Tris pH 6.8, 10 µl 10 % SDS, 10 µl 10 % APS and 1 µl Temed was poured on top. A comb for 10 or 15 slots was inserted and the gel was let polymerize. The gel was placed in the running tank and covered with 1 x SDS-running buffer (25 mM Tris base, 192 mM glycine, 0.1 % (w/v) SDS). The samples and 5 µl PageRuler Prestained protein ladder (Thermo Scientific) were loaded. The gels were run at 100-150 V according to the size of the proteins. The gels were removed from the glass plates, the stacking gel was discarded and the resolving gel was used for immunoblot analysis.

2.2.2.4. Immunoblot analysis

For immunoblot analysis the proteins were electroblotted from the SDS-gel on PVDF membranes (Roche) using the Biorad Tetra Blotting Module. Blotting was performed for 1 hour at 110V in 1 x transfer buffer (25 mM Tris base, 192 mM glycine). Afterwards the membranes were incubated in 5 % low fat milk powder in PBS-T (137 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4, 0.1 % Tween 20) for 1 hour at RT to block unspecific binding sites. The membranes were incubated in the primary antibody in PBS-T with 5 % milk overnight at 4 °C. The next day membranes were washed 3 times for 5 min in PBS-T and the secondary antibody was incubated in 5 % milk in PBS-T for 1 hour at RT. Afterwards the membrane was again washed 3 times for 5 min in PBS-T. The signal of the horseradish peroxidase coupled secondary antibody was detected using ECL reagent (GE Healthcare) according to the manufacturer's instructions. The signal was detected on X-ray films (CL X-posure films, Thermo Scientific).

Afterwards membranes were Coomassie stained to visualize equal protein loading. Membranes were incubated for 5 min in Coomassie staining solution (0.025 % (w/v) Coomassie Brilliant Blue R-250, 45 % (v/v) methanol, 10 % (v/v) acetic acid). Afterwards membranes were incubated in destaining solution (45 % (v/v) methanol, 10 % (v/v) acetic acid) until bands became clearly visible.

2.2.2.5. Co-immunoprecipitation experiments

For CoIP experiments 200 mg leaf material of *A. thaliana* seedlings or *N. benthamiana* were ground in liquid N_2. The powder was mixed with 200 µl extraction buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 % (v/v) Nonidet P40, proteinase inhibitor cocktail (Roche)) and incubated for 1 hour at 4°C with gentle shaking. In the same time 15 µl protein A agarose beads (Roche) or GFP-trap beads (Chromotec) were washed 3 times with the same volume of buffer (50 mM Tris pH 8.0, 150 mM NaCl). The antibody was added (e.g. 5 µl α-BAK1) to the protein A beads and the beads were incubated at 4°C with gentle shaking; The GFP-trap beads were used directly. The protein extracts were cleared by 2-3 times centrifugation for 10 min at 14000 rpm and 4 °C. 20 µl protein extract was taken as input sample, mixed with 5 x SDS- loading dye and boiled for 5 min at 95°C. The antibody beads are added to the rest of the protein extracts and the IP is incubated for 1 hour at 4 °C with gentle shaking. Afterwards the beads are washed 2 times with 50 mM Tris pH 8.0, 150 mM NaCl and one time with 50 mM Tris pH 8.0. 10 µl 5 x SDS-Loading buffer is added to the beads and they are boiled for 5 min at 95°C. Samples were directly used for immunoblot analysis or stored at -20°C.

2.2.3. DNA analysis

2.2.3.1. Transformation of *E. coli* DH5α

For transformation of *E.coli* DH5α 1 µl plasmid DNA or a cloning reaction were added to 200 µl of chemically competent cells. The cells were kept on ice for 5-30 min and then heat shocked for 90 s at 42°C in a water bath. 450 µl SOC medium was added to the cells and they were incubated for 1 hour at 37 °C with shaking. Afterwards 50 and 500 µl were plated on LB plates with appropriate antibiotics and plates were incubated over night at 37 °C. Single colonies were used for the inoculation of liquid LB cultures. From these cultures mini preps were prepared and plasmids were checked with restriction digests and glycerol stocks were prepared for long term storage.

2.2.3.2. Transformation of *Agrobacterium tumefaciens*

For transformation of *A. tumefaciens* 2 µl plasmid DNA were added to 200 µl electro-competent cells. The cells were transferred into an electroporation cuvette and electroporated at 1500 V. 300 µl LB medium were added to the cells and they were transferred back into microcentrifuge tubes. Tubes were shaken at 28 °C for 1.5 hours. The cells were plated on LB plates with appropriate antibiotics and incubated for 2 days at 28 °C.

2.2.3.3. Bacterial plasmid extraction

For bacterial plasmid extraction by alkaline lyses 5 ml overnight cultures were harvested by centrifugation for 2 min at 12000 x *g*. Cell pellets were resuspended in 100 µl lysis buffer (50 mM Tris/HCl pH 8, 50 mM EDTA pH 8, 15 % (w/v) sucrose, 10 µg/ml RNAse A). The suspension was mixed with 200 µl alkaline SDS-solution (200 mM NaOH, 1 % (w/v) SDS) and incubated for maximum 5 min until the lysate became clear. Lysis reaction was stopped by neutralisation with 150 µl potassium-acetate solution (3 M Potassium acetate, 11.5 % (v/v) acetic acid). The samples were incubated for 10 min on ice and then centrifuged for 10 min at 14000 rpm and 4°C. The supernatant was transferred to a fresh microcentrifuge tube. DNA was precipitated by adding 0.6 volumes isopropanol and centrifugation for 15 min at 14000 rpm and 4 °C. DNA pellets were washed with 500 µl 70 % EtOH and centrifuged for 5 min at 13000 rpm. Supernatants were discarded, pellets were air dried and dissolved in 50 µl 10 mM Tris/HCl pH 8. Plasmids were stored at -20 °C. Plasmid DNA obtained from alkaline lysis is applicable for restriction digests or transformation of *A. tumefaciens*. If cleaner DNA for sequencing was

needed, mini preps were performed with GeneJet plasmid mini prep kit (Thermo Scientific) according to manufacturer's instructions.

2.2.3.4. Plant genomic DNA extraction

DNA isolation for plant genotyping was performed according to (Edwards et al., 1991). A small leaf piece was homogenized in 200 µl Edwards buffer (200 mM Tris/HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, and 0.5 % (w/v) SDS). The homogenate was centrifuged at 13000 rpm for 5 min and the supernatant was transferred into a fresh microcentrifuge tube. 200 µl isopropanol were added and the DNA was precipitated for 5 min. The samples were centrifuged 10 min at 14000 rpm and 4 °C and the supernatant was discarded, The DNA pellet was washed with 70 % EtOH and centrifuged 5 min at 13000 rpm. The supernatant was discarded. The pellet was air dried and resuspended in 50 µl Tris/HCl pH 8.5. 2 µl were used for genotyping PCRs.

2.2.3.5. Polymerase Chain Reaction (PCR)

PCR reactions for plant genotyping were performed with a homemade Taq polymerase. The PCR was performed in a 20 µl mix consisting of 1 x reaction buffer (67 mM Tris, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.01 % Tween, pH 8.8), 125 µM dNTPs, 0.5 µM fwd and rev primer, 0.5 µl Taq polymerase, 2 µl DNA from Edwards protocol. The protocol was as follows: Initial denaturation for 3 min at 95 °C, 40 cycles of 30 s 95 °C denaturation, 30s at Tm -3 annealing, 1 min/kb at 72 °C elongation, 5min 72 °C final elongation.

Semiquantitative RT-PCRs were also performed with homemade Taq polymerase and the above described protocol. 1 µl cDNA were used per reaction and the cycle number was adjusted to the amplified gene.

For cloning the proofreading polymerases Pfu or Phusion (Thermo Scientific) were used according to manufacturer's instructions.

2.2.3.6. Restriction enzyme digestion of DNA

To analyze successful cloning of plasmids a restriction digest was performed using an enzyme that cuts at least one time in the insert and one time in the vector backbone. For the restriction digest about 500 ng plasmid DNA and 1 U enzyme were used as given in the manufacturer's instructions (Thermo Scientific). Fragments were analyzed by agarose gel electrophoresis.

2.2.3.7. DNA agarose gel electrophoresis

For agarose gel electrophoresis DNA samples were mixed with 5 x DNA loading buffer (10 mM Tris pH 7.5, 60 mM EDTA, 60 % (v/v) glycerol, 0.25 % bromphenol blue) and loaded on 1 % (or higher percentage if needed for short DNA fragments) agarose gels in 1 x TAE buffer (40 mM Tris, 1 mM EDTA, pH 8.5) with ethidiumbromide (0.5 µg/ml). Gels were run at 100 V in 1x TAE buffer according to the fragment size. GeneRuler 1 kb DNA ladder (Thermo Scientific) was used as standard. Bands were visualized using UV-Transilluminator (Infinity-3026 WL/26 MX, Peqlab).

2.2.3.8. Purification of DNA fragments from agarose samples

For purification of DNA from agarose gels the DNA bands were cut out and DNA was extracted using GeneJet Gel extraction kit (Thermo Scientific) according to manufacturer's instructions.

2.2.3.9. Gateway TOPO cloning

For creation of vectors the Gateway system was used (Life Technologies). Entry vectors were created with the pCR™8/GW/TOPO®TA Cloning®Kit (Life Technologies). The exact coding sequence of the gene of interest was PCR amplified with proofreading enzymes (or without codon for use with C-terminal tags). The PCR product was purified from agarose gels. An A overhang was added by incubation of 7.9 µl PCR product with 0.1 µl Taq polymerase, 1 µl 10 mM dATPs and 1 µl 10 x Taq buffer for 10 min at 72°C. 4 µl PCR fragment with A overhang were mixed with 1 µl salt solution and 1 µl TOPO vector (from the pCR™8/GW/TOPO®TA Cloning®Kit, Life Technologies) and incubated for 5 min at RT. The TOPO reaction was directly transformed into *E. coli* DH5α cells. Entry vectors were analyzed by restriction digestion and sequencing.

2.2.3.10. Gateway LR reaction

Expression vectors were created by LR reactions between Gateway entry and destination vectors using the Gateway® LR Clonase® II Enzyme mix (Life Technologies). Therefore 50-150 ng entry and destination vector were mixed in a volume of 4 µl with TE, pH 8. 1 µl LR clonase II enzyme mix were added and the reaction was incubated at RT for 1 hour to overnight. The next morning the reaction was stopped by incubation with 0.5 µl proteinase K for 10 min at 37 °C. The reaction was directly transformed into *E.coli* DH5α. Successful cloning was analyzed by restriction digestion.

2.2.3.11. DNA sequencing

For sequencing of plasmid DNA the light run service of GATC was used. Therefore 400-500 ng plasmid DNA and 25 pmol primer were mixed in a total volume of 10 µl and send for sequencing. Sequencing results were analyzed using CLC Main workbench (CLC bio).

2.2.3.12. Quantitative Real-time PCR

For qPCR experiments the iCycler with iQ5 multicolor real-time PCR detection system (Biorad) was used. For the qPCR reaction 2 µl 1:10 diluted cDNA, 0.5 µM fwd and rev primer and 10 µl Maxima SYBR Green/Fluorescein qPCR Master Mix (2X) (Thermo Scientific) were used in a 20 µl reaction. The PCR program was as follows: 95 °C for 10 min initial denaturation, 40 cycles of 95 °C for 15 s denaturation, Tm - 3 for 15 s annealing, 72 °C for 1 min/kb elongation (→ real time detection). For the melt curve samples were heated 1 min to 95 °C, 2 min cooled to Ta and then heated to 95 °C in 0.5 °C steps, 10 s per step (→ melt curve detection after each step). Relative gene expression was calculated according to the $2^{-\Delta ct}$ method (Livak and Schmittgen, 2001) to the housekeeping gene EF1α.

2.2.4. RNA analysis

2.2.4.1. RNA extraction

RNA extraction from plant material was performed with TriZOL method (Chomczynski and Sacchi, 1987). About 100 mg ground plant material was mixed with 1 ml TriZOL reagent (380 ml/l phenol, 0.8 M guanidinium thiocyanate, 0.4 M ammonium thiocyanate, 33.4 ml/l 3 M Na-acetate, pH 4.5, 5 % (v/v) glycerol) and vigorously mixed until thawn. Samples were incubated for at least 5 min at RT. 200 µl chloroform were added, samples were vigorously mixed for at least 15 s and then incubated for 3 min at RT. Samples were centrifuged at 12000 x *g* for 15 min at 4 °C. The aqueous phase was transferred to a fresh microcentrifuge tube. RNA was precipitated by adding 500 µl isopropanol and incubation for 10 min at RT. RNA was pelleted by centrifugation at 12000 x *g* for 10 min at 4 °C. Pellets were washed with 1 ml 75 % EtOH and centrifuged at 7500 x *g* for 5 min at 4 °C. Pellets were air dried and dissolved in 30 µl nuclease free H_2O. RNA was stored at -80 °C. Concentration was determined with NanoDrop 2000 Spectrophotometer (Peqlab).

2.2.4.2. DNAse treatment of RNA

For DNAse treatment 5 µg RNA in a volume of 17 µl were used. Digestion was performed with 1 U DNAse I (Thermo Scientific) and 2 µl of DNAse buffer for 30 min at 37°C. Reactions were placed on ice and stopped by adding 130 µl H_2O and 150 µl Phenol/Chloroform/Isoamylacolhol (PCI, 25:24:1). Samples were centrifuged for 5 min at 120000 rpm and RT and the aqueous phase was transferred to a fresh microcentrifuge tube. RNA was precipitated with 1/10 Vol. 3 M NaOAc pH 5.2 and 2 Vol. cold EtOH and incubation at RT for 10 min. Samples were centrifuged for 10 min at 12000 x *g* and 4°C to pellet RNA. Pellets were washed with 1 ml 75% EtOH and centrifugation for 5 min at 7500 x *g* and 4°C. Pellets were air dried and dissolved in 15 µl H_2O.

2.2.4.3. Reverse transcription

Reverse transcription (RT) of RNA into cDNA was performed with 1 µg DNAse digested RNA in a volume of 13 µl. The RNA was mixed with 1 µl 2.5 mM dNTPs and 1 µl 10 µM oligo-dT primers, incubated for 5 min at 65 °C and directly cooled on ice. To this mix 200 U RevertAid RT (Thermo Scientific) and 4 µl corresponding 5 x RT-buffer were added and reverse transcription was performed for 50 min at 42 °C and 5 min at 94 °C. cDNA was directly used in qPCR or RT-PCR experiments or stored at -20 °C.

2.2.5. Split-ubiquitin bridge assay

The split-ubiquitin bridge assay was used to determine interactions between three proteins. The assay is performed in yeast and based on the reconstitution of the two artificially cleaved halves of ubiquitin. It enables the use of full length or membrane bound proteins. One protein is fused to the N-terminal half of ubiquitin (Nub) and the second protein to the C-terminal part (Cub) with the reporter construct PLV (ProteinA-LexA-VP16). Interaction of the tow proteins leads to reconstitution of the ubiquitin upon which the PLV is cleaved off by ubiquitin-specific proteases and thus able to switch on reporter genes. The use of a repressible promotor for the Cub fusion (met25) gives more reliability by decreasing the artefacts of overexpression. Expression of an additional protein (bridge) allows detection of facilitated or enhanced interaction by this protein.

For the interaction assay the yeast THY.AP4 (*S . cerevisiae* MATa; *ade2* − , *his3* − , *leu2* − ,*trp1* − , *ura3* − ; lexA::ADE2, lexA::HIS3, lexA::lacZ) was grown in 10 ml YPD shaking over night at 30 °C. 4ml of the pre-culture were transferred into fresh 50ml YPD medium and incubated shaking

for 5 hours until an OD_{600} of 0.5 to 0.8 was reached. Cells were harvested by centrifugation for 10 min at 2000 x *g* and the supernatant was discarded. The pellets were resuspended in 20 ml sterile H_2O and centrifuged again. Cells were resuspended in 1 ml 0.1 M lithium acetate pH 7.5 (LiAc) and transferred in a 2 ml microcentrifuge tube. The tubes were centrifuged for 2 min at 1000 x *g* and the supernatant was discarded. Pellets were resuspended in 500 µl 0.1 M LiAc and incubated at RT for 30 min. Meanwhile sterile tubes with 9 µl 2 mg/ml ssDNA and 6 µl of plasmid DNA (at least 200 ng/µl, 2µl from each clone) were prepared for each transformation. A mastermix of 70 µl 50 % PEG 3350, 10 µl 1 M LiAc and 20 µl competent cells was prepared for each transformation. The mastermix was carefully mixed with the DNA and incubated for 30 min at 30 °C. After 20 min each reaction was mixed by pipetting carefully up and down. Heat shock was performed for 15 min at 43 °C. Cells were centrifuged for 5 min at 2000 x *g* and the supernatant was discarded. Pellets were washed with 100 µl sterile water and again centrifuged. Supernatants were discarded and cells were resuspended in 100 µl sterile water. Cells were plated on CSM- Leu^-, Trp^-, Ura^- plates and incubated at 30°C for 3 to 4 days.

For growth assays 5 ml CSM- Leu^-, Trp^-, Ura^- were inoculated with 5-10 colonies per transformation. Cultures were grown overnight at 30°C shaking. The OD_{600} was determined. 100 µl sample were centrifuged for 2 min at 2000 x *g,* supernatants were discarded and pellets resuspended in a volume of sterile water to get an OD_{600} of 1.0. From this cell suspension 1:10 and 1:100 dilutions were prepared in sterile water. 7 µl droplets of all dilutions were placed on CSM- Leu^-, Trp^-, Ura^- (vector selective medium) and CSM- Leu^-, Trp^-, Ura^-, Ade^-, His^- with increasing methionine concentration (interaction selective medium) plates. Plates were incubated at 30 °C until yeast growth became visible.

2.2.6. Statistical analysis

Statistical significance between two groups has been analyzed using Student's t-test. Asterisks represent significant differences (*p <0.05; **p <0.01; ***p <0.001). One-way ANOVA was performed for multiple comparisons combined with Tukey's honest significant difference (HSD) test. Different letters indicate significant differences ($p < 0.05$).

All experiments were repeated at least three times with similar results, if not indicated otherwise.

3. Results

3.1. The BAK1 interacting RLK 2 (BIR2)

The regulatory LRR-RLK BIR2 was identified in a CoIP-MS analysis for BAK1 interaction partners. It was shown by Sara Mazzotta that BAK1 and BIR2 interact constitutively and that BIR2, which itself is kinase inactive, gets phosphorylated by BAK1. In yeast-two-hybrid assays it could be shown that the interaction between BAK1 and BIR2 is dependent on BAK1 kinase activity (Mazzotta, 2012). Thierry Halter continued the characterization of BIR2 with functional analysis of *bir2* mutants. He could show that *bir2* mutants are hyperresponsive to MAMPs, show stronger cell death after infection with the necrotrophic fungus *A. brassicicola* and are more resistant to infections with the hemibiotrophic bacterium *Pseudomonas syringae pv. tomato* DC3000. In contrast to *bak1* mutants, *bir2* mutants do not show a BL phenotype. These results indicate that BIR2 differentially regulates BAK1 dependent signaling pathways (Figure 3-1). Moreover he showed first analyses addressing how BIR2 influences BAK1 complexes with ligand-binding receptors (Halter, 2014).

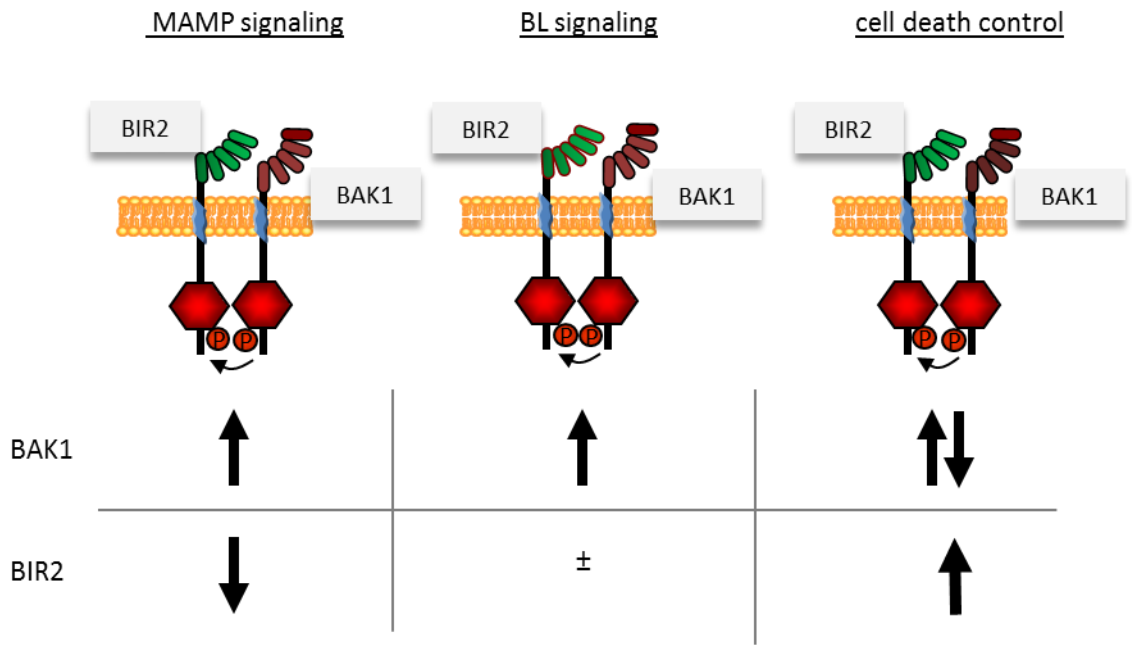

Figure 3-1: Summary of phenotypic analysis of *bir2* mutants (Halter et al., 2014)
The role of BAK1 and BIR2 proteins in MAMP signaling, BL signaling and cell death control is indicated by arrows pointing up (positive effect) or down (negative effect) or no influence (±.)

3.1.1. BIR2 overexpressors show stronger symptom development after *A. brassicicola* infection and less MAMP responses

In order to further analyze the role of BIR2 in MAMP responses and cell death regulation and to support the data obtained with knockout mutants, BIR2 overexpressing plants were used. Two independent 35S-BIR2-YFP lines were used for elf18 induced ROS burst assays. Both lines show reduced ROS production compared to Col-0 wildtype plants (Figure 3-2 A). Thus, these BIR2 overexpressing plants show the opposite phenotype to *bir2* mutants in ROS burst assays. These results confirm the negative regulatory role of BIR2 in the MAMP response pathway.

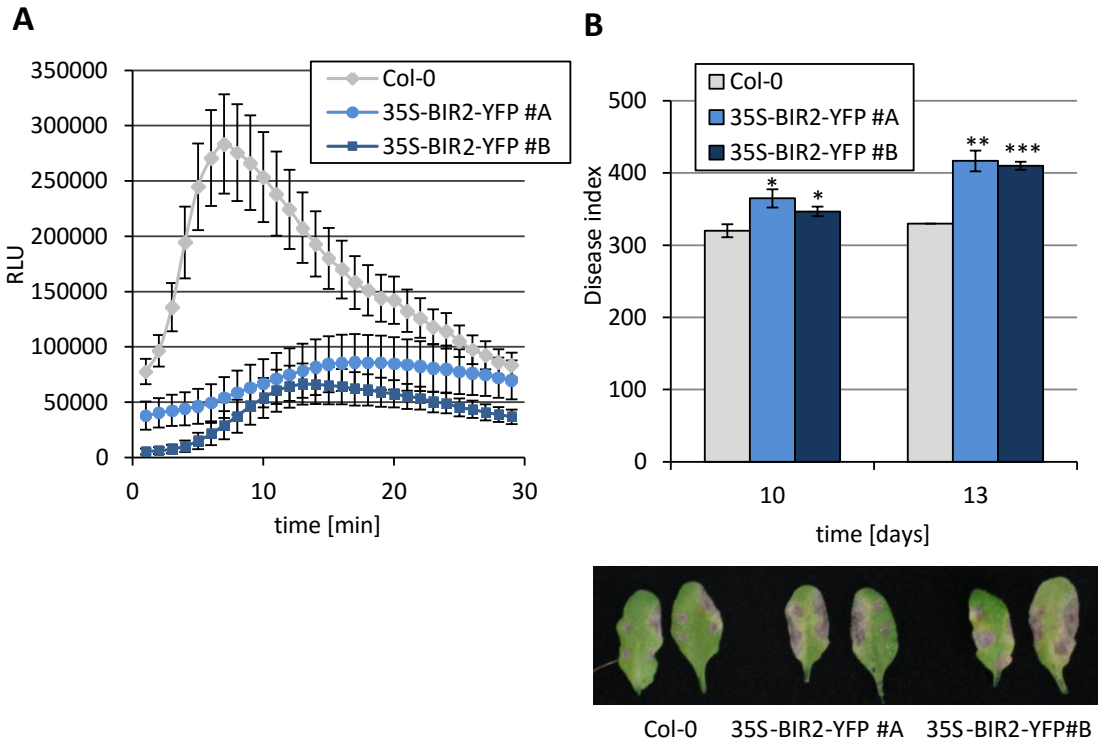

Figure 3-2: Functional analysis of 35S-BIR2-YFP plants
A: Luminol based ROS measurements over 30 min represented as relative light units (RLU) in Col-0 and two independent BIR2 overexpressor lines on leaf discs after elicitation with 100 nM elf18. Values are means ± SE (n=9).
B: Infection experiments with the necrotrophic fungal pathogen *Alternaria brassicicola* on Col-0 and two independent BIR2 overexpressor lines. Bonitation of disease symptoms was done after 10 and 13 days and the disease index was calculated. Upper chart shows the disease index as mean ± SE (n=3). Asterisks represent significant differences to Col-0 (*p<0.05; **p<0.01; ***p<0.001; Student's t-test). Lower part shows a picture of the symptom development on two representative leaves after 13 days.

35S-BIR2-YFP lines were also used for further analysis of cell death regulation and subjected to infection experiments with the necrotrophic fungus *A. brassicicola*. Surprisingly, 35-BIR2-YFP plants showed a higher disease index and stronger symptom development compared to Col-0

wildtype plants (Figure 3-2 B). This is the same phenotype as *bir2* mutant plants show. Therefore, BIR2 is a positive and negative regulator of cell death responses, likely because of the necessity of balanced protein amounts. The correct protein level of BIR2 or the correct ratio to proteins like BAK1 seems to be important for proper cell death regulation.

3.1.2. BIR2 is released from BAK1 after MAMP treatment

It was shown in phenotypic analyses that BIR2 is a negative regulator of MAMP responses. We were therefore interested how BIR2 acts mechanistically and continued with a molecular analysis of receptor complexes. It was shown that BIR2 and BAK1 interact constitutively and that BAK1 forms complexes with ligand-binding receptors such as FLS2 only after ligand-binding (Chinchilla et al., 2007; Halter et al., 2014). We hypothesized that BAK1 gets released from BIR2 after flg22 treatment to be able to interact with FLS2. To proof this hypothesis Co-IP experiments were performed, analyzing the interaction of BIR2 with BAK1 after flg22 treatment. Immunoprecipitation of BAK1 with specific antibodies in Col-0 plants leads to coimmunoprecipitation of BIR2, detected with specific BIR2 antibodies, showing the strong interaction of both proteins in wildtype Arabidopsis plants. Treatment with flg22 leads to reduced BAK1 BIR2 complex formation compared to the mock treated control showing release of BIR2 from the complex with BAK1 (Figure 3-3 A). Flg22 treatment leads to only about 30% release of BAK1 from BIR2. In parallel Thierry Halter has shown that treatment with other ligands, like BL or *At*pep1, for which BAK1 is the co-receptor, also leads to only partial release of BAK1 from BIR2. We asked whether this is an effect of accessibility or if specific ligands can address specific subpools of BAK1 that exist in preformed complexes with specific ligand-binding receptors. The latter is indeed the case. The same Co-IP experiment was performed treating plants with a cocktail of different MAMPs and BL. This treatment with a cocktail of different ligands leads to strongly increased release of BAK1 from BIR2 compared to single treatments (Figure 3-3 B). This indicates that individual BAK1 molecules can be specifically addressed by specific ligands and that BAK1 that is in complex with a ligand-binding receptor is not accessible by a different ligand.

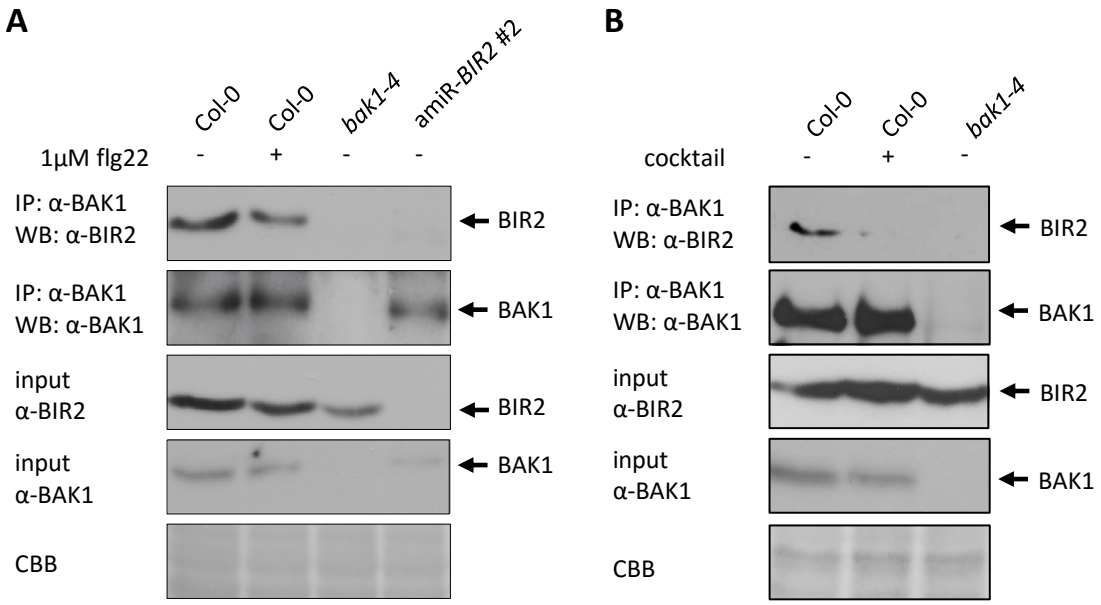

Figure 3-3: BAK1 is released from BIR2 after ligand perception
Two week old Arabidopsis seedlings of the indicated genotypes were treated with different elicitors. Immunoprecipitation (IP) was performed with specific α-BAK1 antibody. Precipitated BAK1 and coimmunoprecipitated BIR2 were detected with specific α-BAK1 and α-BIR2 antibodies respectively. Protein input is shown with Western blot (WB) analysis of protein extracts before IP and specific α-BAK1 and α-BIR2 antibodies. Coomassie brilliant blue (CBB) staining shows protein loading.
A: Seedlings were treated for 5 min with 1 µM flg22 (+) or with H_2O (-).
B: Seedlings were treated with a cocktail of 1 µM flg22, 1 µM elf18 and 1 µM *At*pep1 for 5 min and 10 nM Epi-BL for 90 min (+) or with H_2O (-).

3.1.3. BIR2 regulates BAK1 FLS2 complex formation

BIR2 interacts with BAK1 in the absence of ligands and gets released upon activation of BAK1 after ligand binding, but what is the molecular mechanism underlying the negative regulation of MAMP responses? Does BIR2 directly affect complex formation of BAK1 and PRRs? To further analyze if BIR2 is really able to regulate complex formation between BAK1 and ligand-binding receptors we analyzed BAK1 FLS2 complex formation in the presence or absence of BIR2. CoIP experiments were performed analyzing the interaction of BAK1 with FLS2. FLS2 could be coimmunoprecipitated with BAK1 only after flg22 treatment in Col-0 plants showing the ligand induced interaction of both proteins. The same experiment was performed in plants lacking BIR2 (amiR-*BIR2*) and plants overexpressing BIR2 (35S-BIR2-YFP). In amiR-*BIR2* lines more FLS2 can be coimmunoprecipitated with BAK1 after flg22 treatment as compared to wt plants while in 35S-BIR2-YFP overexpressing lines lower interaction of BAK1 with FLS2 was detected (Figure 3-4 A). This shows that BIR2 is indeed able to influence MAMP responses directly at the receptor level by negatively regulating the interaction between BAK1 and FLS2.

FLS2 protein levels seem to be enhanced in amiR-*BIR2* plants. Therefore, increased complex formation between BAK1 and FLS2 could also be caused by differential expression of *FLS2* in the BIR2 mutant lines. In order to test this possibility, FLS2 expression in amiR-*BIR2* and 35S-BIR2-YFP lines was analyzed by qRT-PCR. amiR-*BIR2* lines show indeed increased FLS2 expression levels but FLS2 expression is not altered in 35S-BIR2-YFP lines (Figure 3-4 B). This data shows that the reduced BAK1 FLS2 complex formation in 35S-BIR2 lines is indeed caused by a negative effect of BIR2 on the BAK1 FLS2 interaction and not by reduced FLS2 expression. The enhanced FLS2 expression in *bir2* mutant plants could be a secondary effect of the enhanced MAMP responses in *bir2* leading to induction of PRRs.

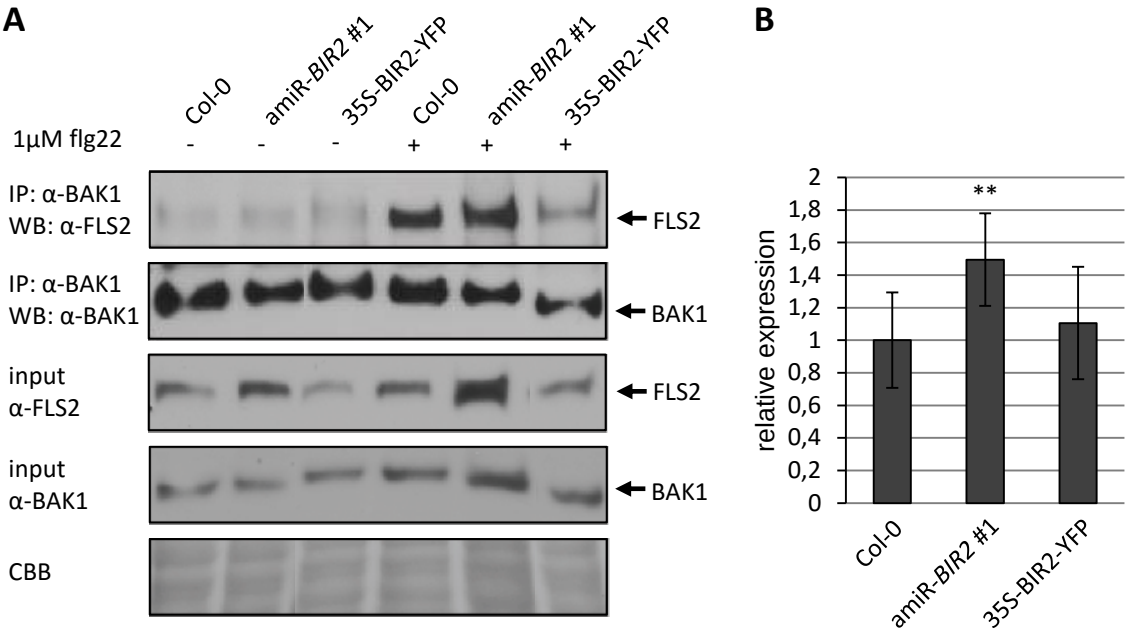

Figure 3-4: BIR2 regulates BAK1 FLS2 complex formation
A: Arabidopsis seedlings of the indicated genotype were treated for 5 min with 1 µM flg22 (+) or H_2O (-). Immunoprecipitation (IP) was performed with specific α-BAK1 antibody. Precipitated BAK1 and coimmunoprecipitated FLS2 were detected with specific α-BAK1 and α-FLS2 antibodies respectively. Protein input is shown with Western blot analysis of protein extracts before IP and specific α-BAK1 and α-FLS2 antibodies. Coomassie brilliant blue (CBB) staining shows protein loading.
B: qRT-PCR analysis of FLS2 expression in the indicated genotypes. FLS2 expression was normalized to the housekeeping gene *EF1α* and plotted relative to expression in Col-0. Results are mean ± SD (n=8). Asterisks represent significant differences according to Student's t-test (**p<0.01).

In order to further confirm the regulatory role of BIR2 on BAK1 FLS2 complexes the *bak1-5* mutant was used for Co-IP experiments. BAK1-5 has a point mutation in its kinase domain at position C408 and it was shown that the BAK1-5 protein interacts stronger with ligand-binding

receptors such as FLS2, EFR and BRI1 compared to BAK1 wildtype protein (Schwessinger et al., 2011). We therefore analyzed how the BAK1-5 mutation behaves concerning its interaction with BIR2 and performed CoIP experiments of BAK1 with BIR2 in wildtype plants compared to *bak1-5* mutants. *bak1-5* mutants still show wildtype BAK1 protein levels that can be detected by α-BAK1 antibodies. BIR2 showed strongly reduced interaction with BAK1-5 compared to the BAK1 wildtype protein (Figure 3-5). This shows that weaker interaction of BAK1 with BIR2 correlates with stronger interaction of BAK1 with FLS2. It was shown by Sara Mazzotta that kinase dead BAK1 does not interact with BIR2 in yeast-two-hybrid assays. The *bak1-5* mutant is additionally interesting because it shows reduced phosphorylation activity (Schwessinger et al., 2011) supporting a correlation of BAK1 kinase activity and interaction of BIR2 and BAK1 in vivo.

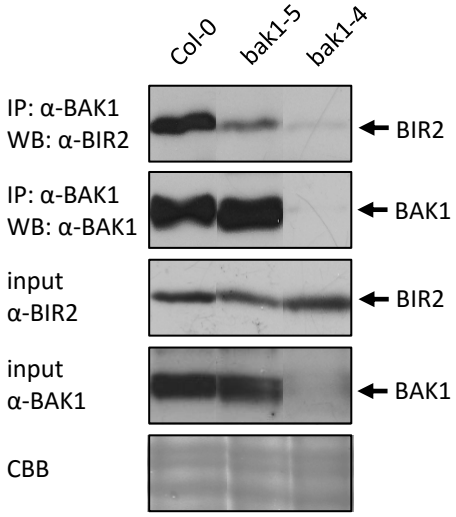

Figure 3-5: BIR2 interacts less with BAK1-5 compared to BAK1 wildtype protein
Untreated Arabidopsis seedlings of the indicated genotype were used for immunoprecipitation experiments with specific α-BAK1 antibody. Coimmunoprecipitated BIR2 was detected with specific α-BIR2 antibody. Protein input is shown with Western blot analysis of protein extracts before IP and specific α-BAK1 and α-BIR2 antibodies. Coomassie brilliant blue (CBB) staining shows protein loading.

3.1.4. BAK1 functions downstream of BIR2 in the MAMP pathway

In order to analyze the relationship of BAK1 and BIR2, double mutants of both proteins were generated. Different allele combinations of the respective single mutants were crossed and double mutants were isolated in the F2 generation by PCR based genotyping in case of *bak1-3*, *bak1-4* and *bir2-1* or Basta selection in case of amiR-*BIR2*. Homozygous *bir2-1 bak1-3* and *bak1-4* amiR-*BIR2* were used for functional analysis. In the MAMP response pathway BAK1 and BIR2 have opposite functions, thus epistasis experiments were performed to analyze which component is upstream in the signaling cascade. In ROS burst assays after flg22 treatment *bir2-1 bak1-3* mutants show the same reduced ROS production as the *bak1-3* single mutants (Figure 3-6 A). This shows that BAK1 acts downstream of BIR2 in the MAMP response pathway. The result was confirmed with *bak1-4* amiR-*BIR2* double mutants (Figure 3-6 B). Here, the ROS

production in *bak1-4* and *bak1-4* amiR-*BIR2* is even lower compared to wildtype as in *bak1-3* single and double mutants, most likely because of remaining transcript in *bak1-3 mutants*.

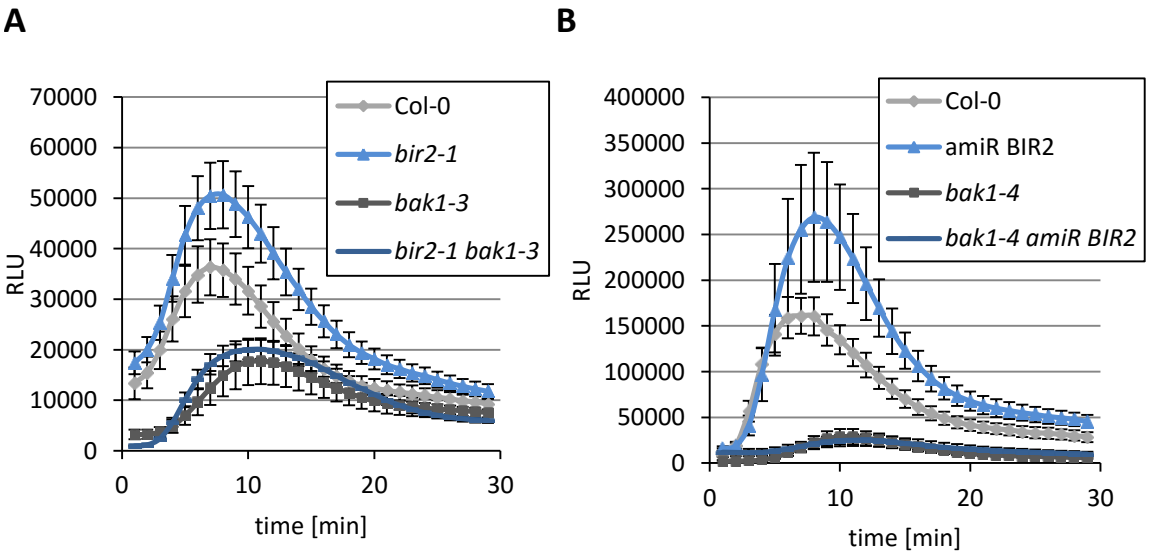

Figure 3-6: BIR2 functions upstream of BAK1 in the MAMP response pathway
A, B: ROS production was measured with a luminol based assay on leaf discs of the indicated Arabidopsis lines over a period of 30 min after elicitation with 100 nM flg22. ROS production is represented as relative light units (RLU) and results are mean ± SE (n=9).

3.1.5. Cell death in *bir2* mutants is not altered by *bak1*

bak1 and *bir2* mutants both show enhanced cell death after infection with pathogens. We therefore analyzed the cell death phenotype of the double mutant. The morphological phenotype of *bir2-1* already shows weak cell death symptoms without infection, which are decreased size and early senescence of older leaves. *bak1* mutants show shorter and wider leaves due to partial BL insensitivity. The *bir2-1 bak1-3* double mutant is even a bit smaller than *bir2-1* and also shows chlorosis in older leaves. In contrast, *bak1-4* amiR-*BIR2* mutants have an intermediate size between *bak1-4* and amiR-*BIR2* single mutants (Figure 3-7 A). In order to analyze cell death in these mutants they were inoculated with the necrotrophic fungus *A. brassicicola* and the symptom development was analyzed. *bak1* single mutants show a significant higher disease index compared to Col-0 and *bir2-1* mutants show even stronger symptom development. *bak1-3 bir2-1* single mutants show a disease index comparable to *bir2-1*, indicating that *bir2-1* already shows the highest disease index and *bak1* mutation has no additive effect (Figure 3-7 B). *bak1-4* amiR-*BIR2* double mutants show infections comparable to both single mutants (Figure 3-7 C). These results again indicate that cell death regulation depends on BIR2 protein amounts and probably balanced amounts compared to BAK1 and

thus BAK1 BIR2 complex integrity. These results also indicate that BIR2 and BAK1 are not acting up- or downstream from each other in a probable cell death regulating pathway but more likely as one complex.

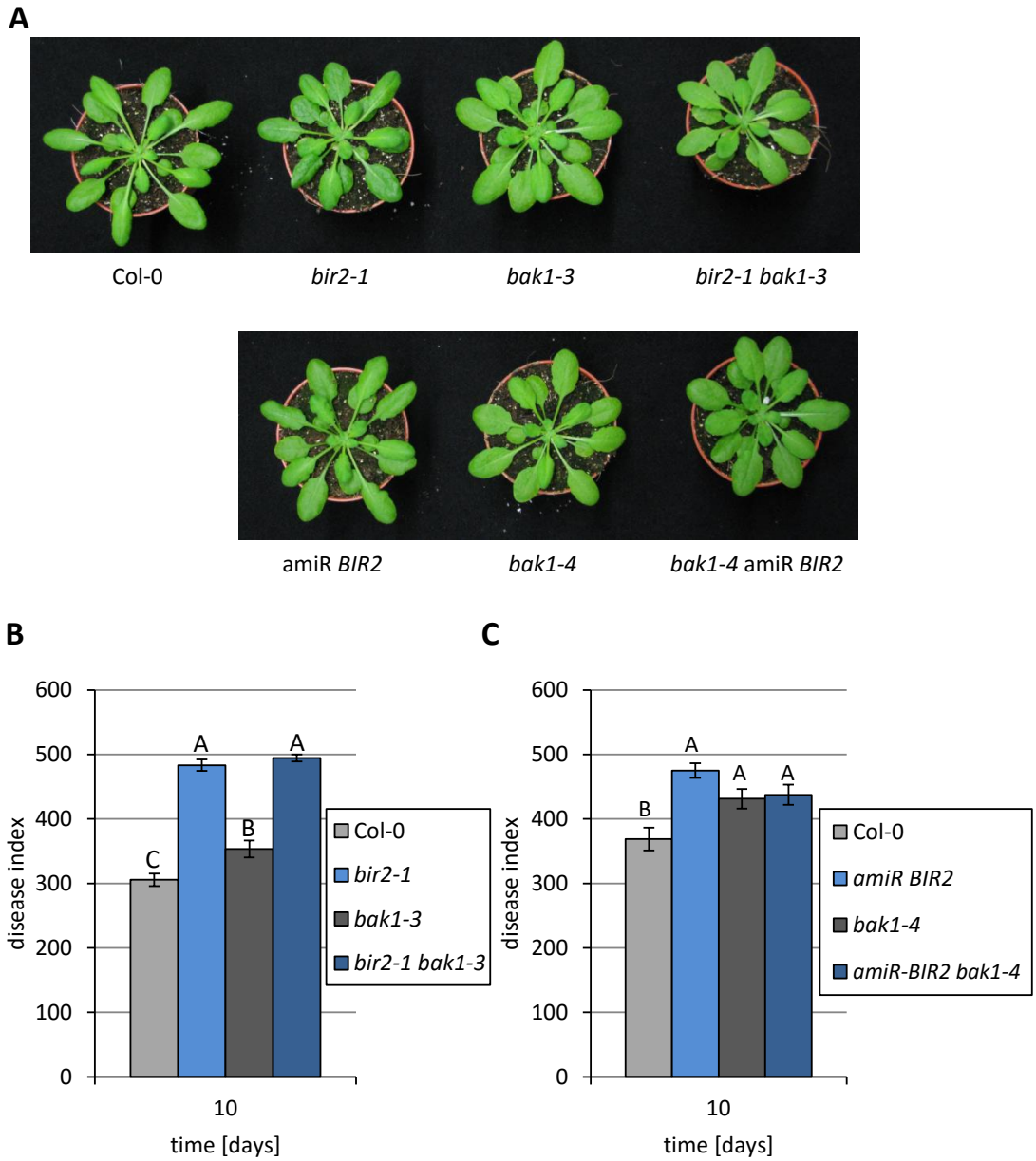

Figure 3-7: Cell death analysis with *bir2 bak1* mutants
A: Morphological phenotype of different allele combinations of *bak1 bir2* double mutants and the respective single mutants.
B, C: Infection experiments with the necrotrophic fungal pathogen *Alternaria brassicicola* on *bak1 bir2* double mutants and the respective single mutants. Upper chart shows the disease index after 7 and 10 days as mean ± SE (n=16). Different letters indicate significant differences according to one-way ANOVA.

3.1.6. Analysis of possible downstream regulators of BIR2 in the cell death control pathway

In many cell death mutants cell death is induced by activated R-proteins (Shirano et al., 2002; Zhang et al., 2003). Also for BIR1 a guarding model was proposed, because *eds1*, *pad4* and to a lower extend *ndr1* could partially rescue the *bir1* mutant phenotype (Gao et al., 2009). Additionally the mutation of one special R-protein, SNC1, can rescue the *bir1* mutant phenotype (Wang et al., 2011). Another player in cell death regulation is the phytohormone salicylic acid (SA), as SA is induced by R-gene signaling and many autoimmunity mutants show elevated SA levels (Alvarez, 2000). To analyze if cell death in *bir2* can be linked to any of these cell death signaling components different double mutants were generated. *bir2* was crossed to *nahG*, a transgene that degrades SA (Lawton et al., 1995), *pad4*, a protein that acts downstream of TIR-type NB-LRR proteins (Zhou et al., 1998; Zhang et al., 2003) and a knockout of the R-protein SNC1, *snc1-11* (Kim et al., 2010). The morphological phenotype of all double mutants resembles *bir2-1* single mutants (Figure 3-8 A). To analyze if any of these mutations can block the pathogen induced cell death in *bir2-1*, infection experiments with *A. brassicicola* were performed. *bir2-1* mutants show a much higher disease index compared to Col-0 and *nahG* single mutants show an even lower disease index. *nahG* expression in *bir2-1* mutants brings the *bir2-1* phenotype almost back to *nahG* levels showing that the cell death in *bir2-1* is mainly dependent on SA. *pad4* single mutants are also resistant to *A. brassicicola* infections but *pad4* does not alter the cell death of *bir2*-1. Therefore, *bir2-1* cell death seems not to be induced by TIR-type NB-LRR proteins. Likewise *snc1-11* does not change the cell death in *bir2-1* mutants showing that *bir2-1* cell death is independent of SNC1 (Figure 3-8 B). In summary, it could be shown that cell death in *bir2-1* is partially dependent on SA but a dependency on TIR-type NB-LRR proteins could not be shown. Further analysis with *ndr1* crosses are needed to investigate if *bir2-1* cell death is dependent on CC-type NB-LRR proteins. The fact that cell death in *bir1* can be suppressed by mutations in the R-protein signaling pathway but not the cell death in *bir2* indicates that cell death is regulated differently by different BIR proteins.

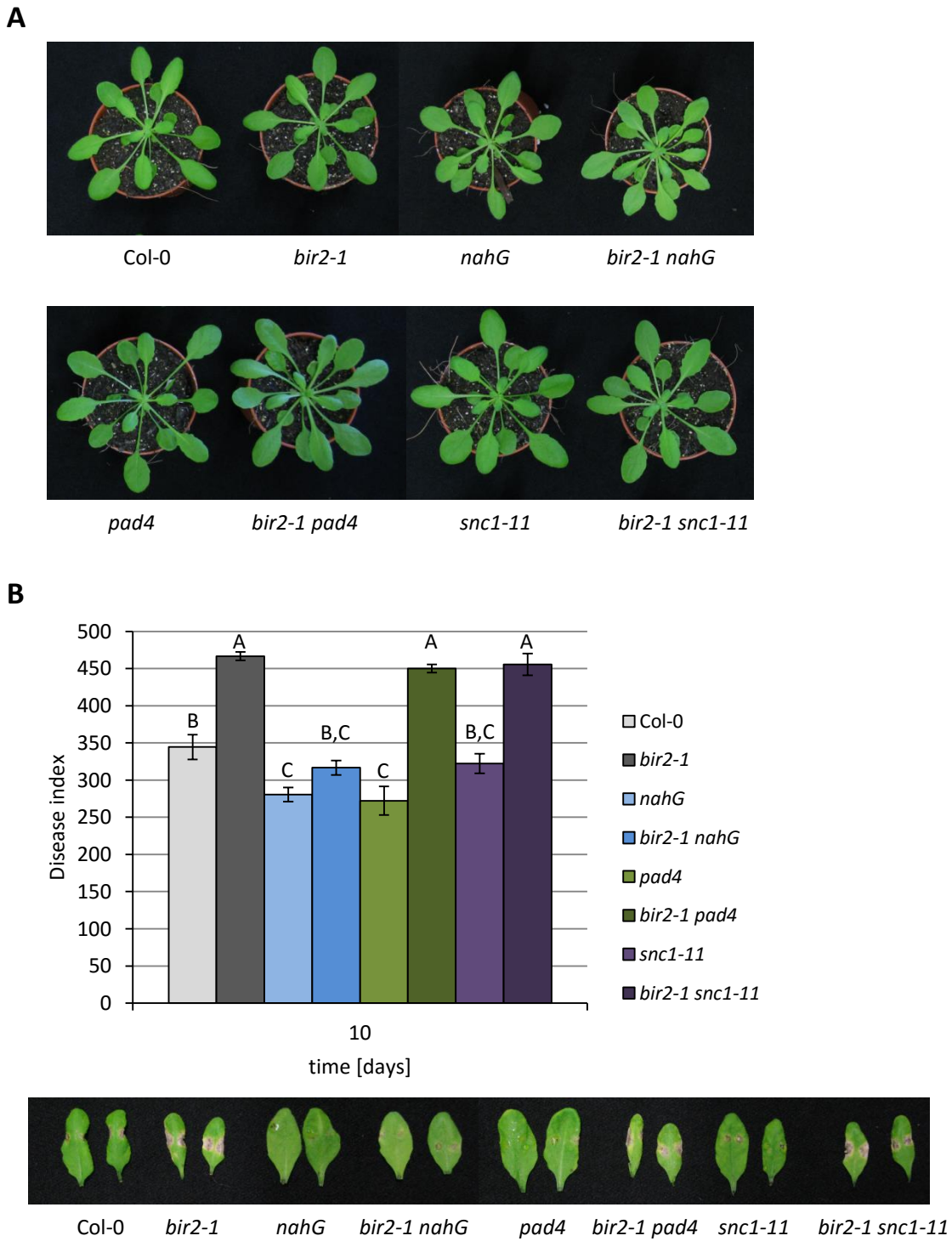

Figure 3-8: Analysis of possible downstream regulators of BIR2 in cell death induction
A: Morphological phenotype of different *bir2* double mutants and the respective single mutants.
B: Infection experiments with the necrotrophic fungal pathogen *Alternaria brassicicola* on different *bir2* double mutants and the respective single mutants. Upper chart shows the disease index after 10 days as mean ± SE (n=3) Letters indicate significant differences according to one-way ANOVA ($p<0.05$). Lower part shows a picture of the symptom development on two representative leaves after 10 days.

3.2. The BAK1-interacting RLK 3 (BIR3)

In the same BAK1 CoIP-MS analysis in which BIR2 was identified, we could also identify the closely related protein BIR3. BIR3 could also be confirmed as constitutive interactor of BAK1. BIR3 is, opposite to BIR2, transcriptionally downregulated after pathogen infection (Halter et al., 2014). BIR3 is also kinase dead and get only weakly phosphorylated by BAK1 in in vitro kinase assays (Mazzotta, 2012).

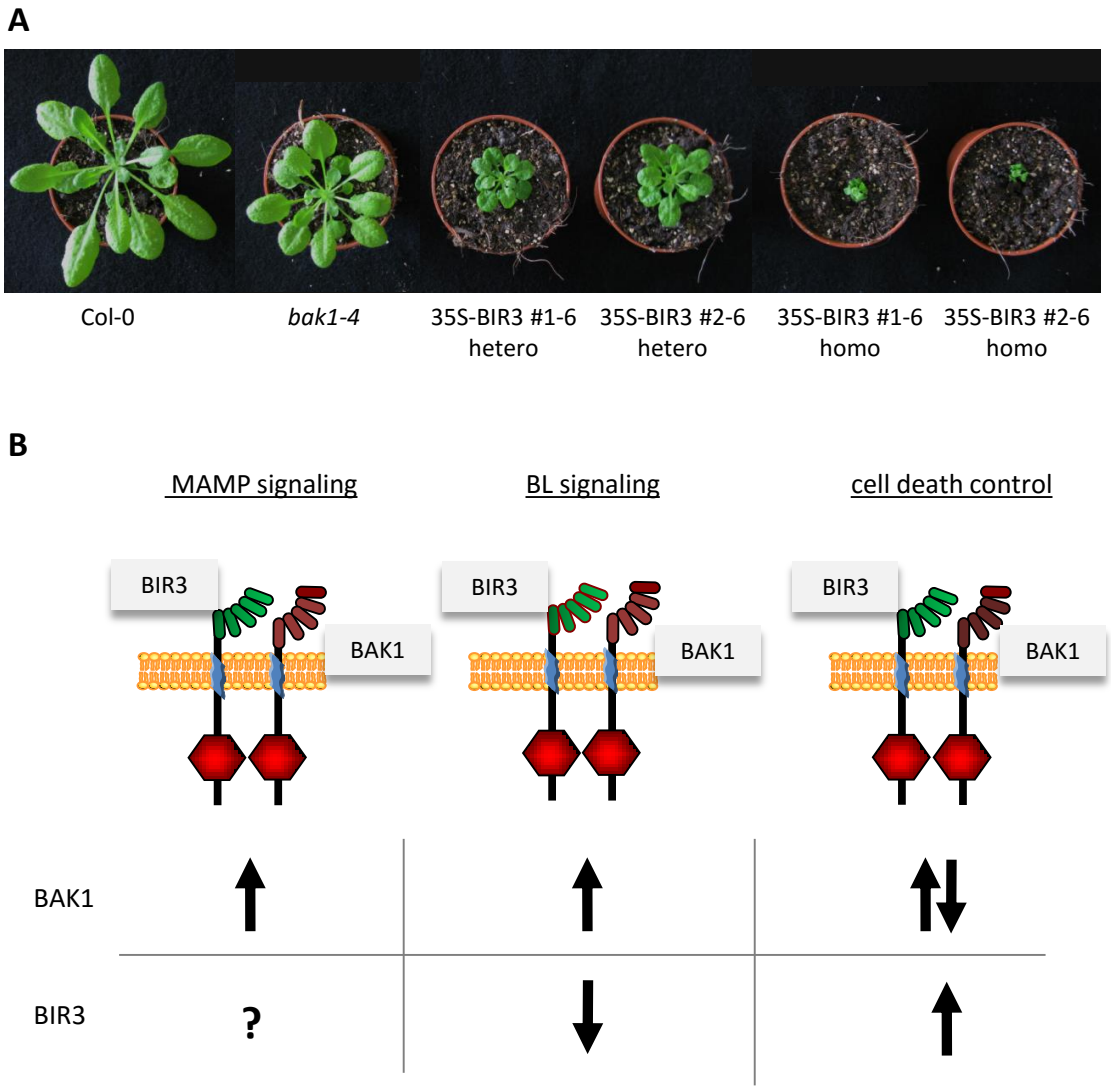

Figure 3-9: Summary of phenotypic analysis of 35S-BIR3 plants (Halter, 2014)
A: Morphological phenotype of hetero- and homozygous BIR33 overexpressing plants.
B: The role of BAK1 and BIR3 proteins in MAMP signaling, BL signaling and cell death control is indicated by arrows pointing up (positive effect) or down (negative effect).

The functional analysis of *bir3* mutants showed no impact on cell death regulation or the BL pathway. The weak hyperresponsiveness to MAMPs shown by Thierry Halter could not be shown to be significant in this work. The lack of altered phenotypes in *bir3* mutants could be due to redundancy with other BIR proteins so that BIR3 overexpressing plants were generated. These BIR3 overexpressors show, interestingly, a very strong growth phenotype resembling *bri1* mutants and it was shown that 35S-BIR3 plants are indeed BL insensitive (Figure 3-9 A). 35S-BIR3 plants show less ROS production after elf18 treatment and stronger symptom development after *A. brassicicola* infection (Figure 3-9 B). Moreover it could be shown that BIR2 and BIR3 interact with each other indicating that they could act together in regulating BAK1 dependent signaling pathways (Halter, 2014).

3.2.1. BIR3 directly interacts with BRI1

35S-BIR3 plants show a severe growth phenotype and are insensitive to BL treatment. This phenotype could be explained mechanistically by regulation of BAK1, but BIR3 could also have a direct effect on BRI1. From our cooperation partner Steve Clouse we kindly got the result that simultaneous overexpression of BRI1 in a 35S-BIR3 background rescues the 35S-BIR3 growth phenotype (Figure 3-8). This points into the direction of a direct effect of BIR3 on BRI1.

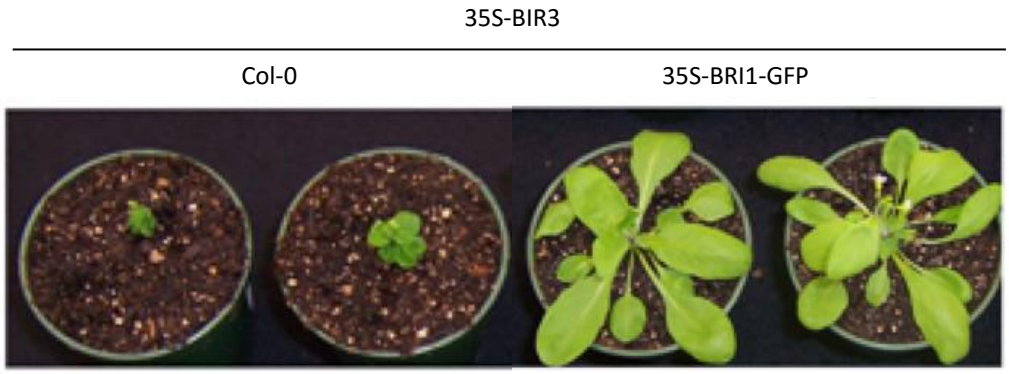

Figure 3-10: Overexpression of BRI1 rescues the 35S-BIR3 phenotype
Morphological phenotype of plants expressing 35S-BIR3 in wildtype of 35S-BRI1 background. Picture kindly provided by Steve Clouse.

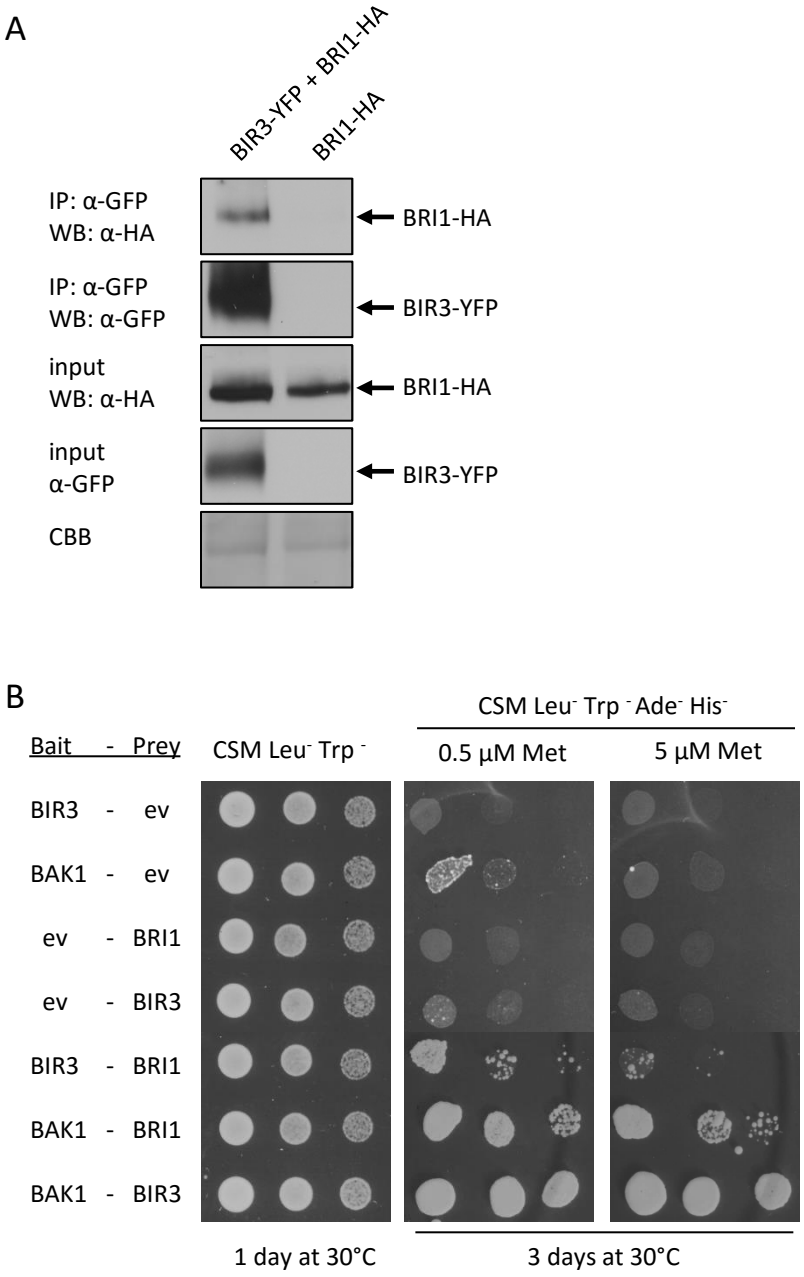

Figure 3-11: BIR3 directly interacts with BRI1

A: Indicated constructs were transiently expressed in *N. benthamiana* leaves and immunoprecipitation was performed with GFP-trap beads. Precipitated BIR3 and co-immunoprecipitated BRI1 were detected with α-GFP and α-HA antibodies respectively. Protein input is shown with Western blot analysis of protein extracts before IP and α-GFP and α-HA antibodies. Coomassie brilliant blue (CBB) staining shows protein loading.

B: Growth assay of yeast containing two plasmids ("bait" and "prey", ev = empty vector). Yeast was dropped at three different dilutions on medium selecting for vector transformation (CSM-Leu$^-$, Trp$^-$) and on medium selecting for interaction (CSM-Leu$^-$, Trp$^-$, Ade$^-$, His$^-$, with increasing methionine concentrations). Growth was monitored after 1 day for the vector-selective control plates and after 3 days for the actual interaction plates, respectively. Experiment B was repeated 2 times with identical results.

It was therefore analyzed if BIR3 is able to directly interact with BRI1. 35S-BIR3-YFP and 35S-BRI1-HA constructs were transiently expressed in *Nicotiana benthamiana* and immunoprecipitation was performed using GFP-trap beads. After immunoprecipitation of BIR3-GFP coimmunoprecipitated BRI1-HA was detected showing the interaction of both proteins (Figure 3-11 A). This indicates that BIR3 might have not only an effect on BAK1 but also directly on BRI1. However, in this transient expression system it cannot be excluded that other proteins like the *N. benthamiana* homologue of BAK1 influences the complex formation. Thus the interaction of BIR3 with BRI1 could also be indirect via BAK1. To confirm that the interaction between BIR3 and BRI1 is direct, a yeast split ubiquitin assay was used. In this assay full length constructs of membrane proteins are expressed in yeast and interaction of the proteins leads to reporter gene activation that allows growth on selective medium. Negative controls of co-expression of one protein and an empty vector does not lead to significant yeast growth on medium that selects for interaction. As positive control BAK1 and BIR3 were used that show strong interaction and thus functionality of the constructs. Co-expression of BAK1 and BRI1 also leads to weak yeast growth showing the interaction of both proteins in this system already without BL treatment. Co-expression of BIR3 with BRI1 also leads to yeast growth confirming the direct interaction of both proteins (Figure 3-11 B).

As BIR3 is able to interact with both BAK1 and BRI1 it should be tested if these proteins build a tripartite complex. A split-ubiquitin bridge assay was used that allows testing the interaction of three proteins. Transformation of yeast with BAK1 and BRI1 constructs allows growth on medium that selects for interaction, showing again that BAK1 and BRI1 interact in this yeast system already without BL treatment. Additional expression of BIR3 as bridge between the two proteins does not enhance the growth of yeast cells and thus the interaction of BAK1 and BRI1 (Figure 3-12). Therefore, existence of a tripartite complex of BAK1-BIR3-BRI1 could not be shown. Further analyses are needed to elucidate the composition of receptor complexes containing BRI1, BAK1 and BIR3.

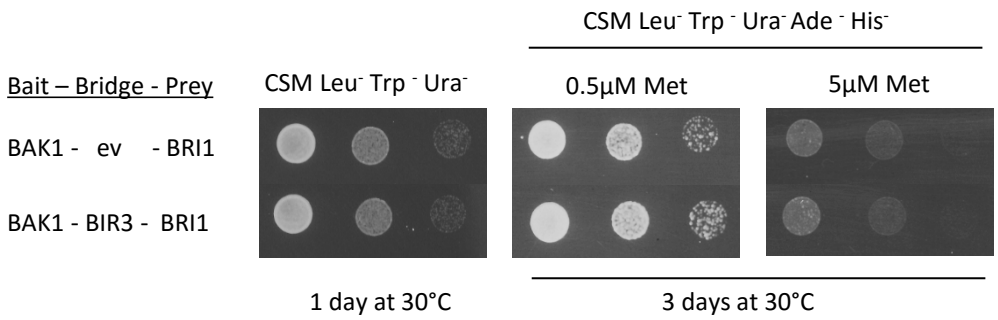

Figure 3-12: Analyses of a possible tripartite complex of BRI1, BIR3 and BAK1
Growth assay of yeast containing three plasmids ("bait,""bridge," and "prey", ev = empty vector). Yeast was dropped at three different dilutions on medium selecting for vector transformation (CSM-Leu⁻, Trp⁻, Ura⁻) and on medium selecting for interaction (CSM-Leu⁻, Trp⁻, Ura⁻, Ade⁻, His⁻, with increasing methionine concentrations). Growth was monitored after 1 day for the vector-selective control plates and after 3 days for the actual interaction plates, respectively. The experiment was repeated two times with identical results.

3.2.2. BIR3 shows interaction with other ligand-binding receptors such as FLS2

As it was shown that BIR3 is able to directly interact with BRI1 it was tested if BIR3 also interacts with other ligand-binding receptors from other pathways like the MAMP receptor FLS2. 35S-BIR3-YFP and 35S-FLS2-Myc constructs were transiently expressed in *N. benthamiana* and BIR3-YFP was immunoprecipitated with GFP-trap beads. Coimmunoprecipitated FLS2-myc could be detected using α-myc antibodies showing the interaction of BIR3 with FLS2 (Figure 3-13). These findings indicate that BIR3 is not only able to directly interact with BRI1 but also interacts with another ligand-binding receptor involved in a different pathway.

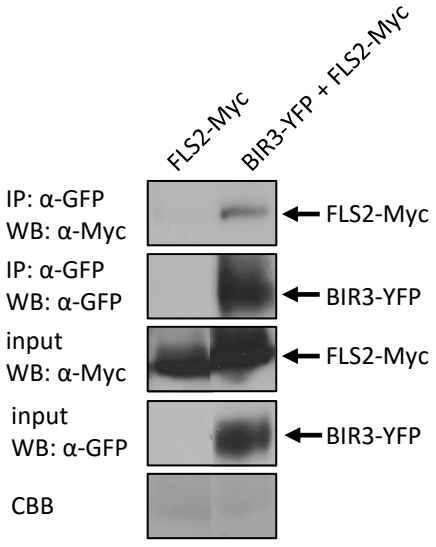

Figure 3-13: BIR3 interacts with FLS2
Indicated constructs were transiently expressed in *N. benthamiana* leaves and immunoprecipitation was performed with GFP-trap beads. Precipitated BIR3-YFP and co-immunoprecipitated FLS2-myc were detected with α-GFP and α-Myc antibodies respectively. Protein input is shown with Western blot analysis of protein extracts before IP and α-GFP and α-Myc antibodies. Coomassie brilliant blue (CBB) staining shows protein loading. Blots in one row show cutouts from the same Western blot film.

3.2.3. BIR3 overexpressors show less MAMP responses but are not affected in susceptibility to *Pto* DC3000

The results, that BIR3 interacts with FLS2 indicates that BIR3 not only regulates BL responses but is also involved in regulation of MAMP responses. Moreover, Thierry Halter has already shown that 35S-BIR3 plants show reduced ROS production after treatment with the MAMP elf18. To support this finding we performed ROS burst assays with the MAMP flg22 and tested also a second readout - *FRK1* marker gene expression. For ROS burst assays hetero- and homozygous plants of two independent 35S-BIR3 lines were used and elicited with the MAMP flg22. Heterozygous 35S-BIR3 plants already show reduced MAMP responses compared to Col-0 wildtype. In homozygous plants the ROS production is even lower but the plants are not as insensitive as the *bak1-4* control (Figure 3-14 A). This shows a quantitative negative effect of BIR3 on flg22 signaling. As an independent readout expression of the marker gene *FRK1* was analyzed in homozygous 35S-BIR3 plants. flg22 treatment leads to induction of *FRK1* in Col-0 wildtype plants. In 35S-BIR3 plants the induction is significantly lower, however, again not as low as in *bak1-4* control plants (Figure 3-14 B). With two independent methods we have shown that BIR3 is indeed a negative regulator of flg22 responses. It was further analyzed how the reduced MAMP responses influence the resistance against infection with bacterial pathogens. Heterozygous 35S-BIR3 plants were inoculated with the virulent hemibiotrophic pathogen *Pseudomonas syringae pv. tomato* DC3000 and bacterial growth was monitored 2 and 4 days after inoculation. At both time points 35S-BIR3 plants do not show significant differences compared to growth in wildtype (Figure 3-14 C). This could be explained by the fact that BIR3 overexpressors show reduced MAMP responses favoring growth of biotrophic pathogens but stronger cell death that antagonizes growth of these pathogens. As a result the growth of *Pto* DC3000 bacteria would not be altered in 35S-BIR3 plants, as it was described for *bak1* mutants (Kemmerling et al., 2007).

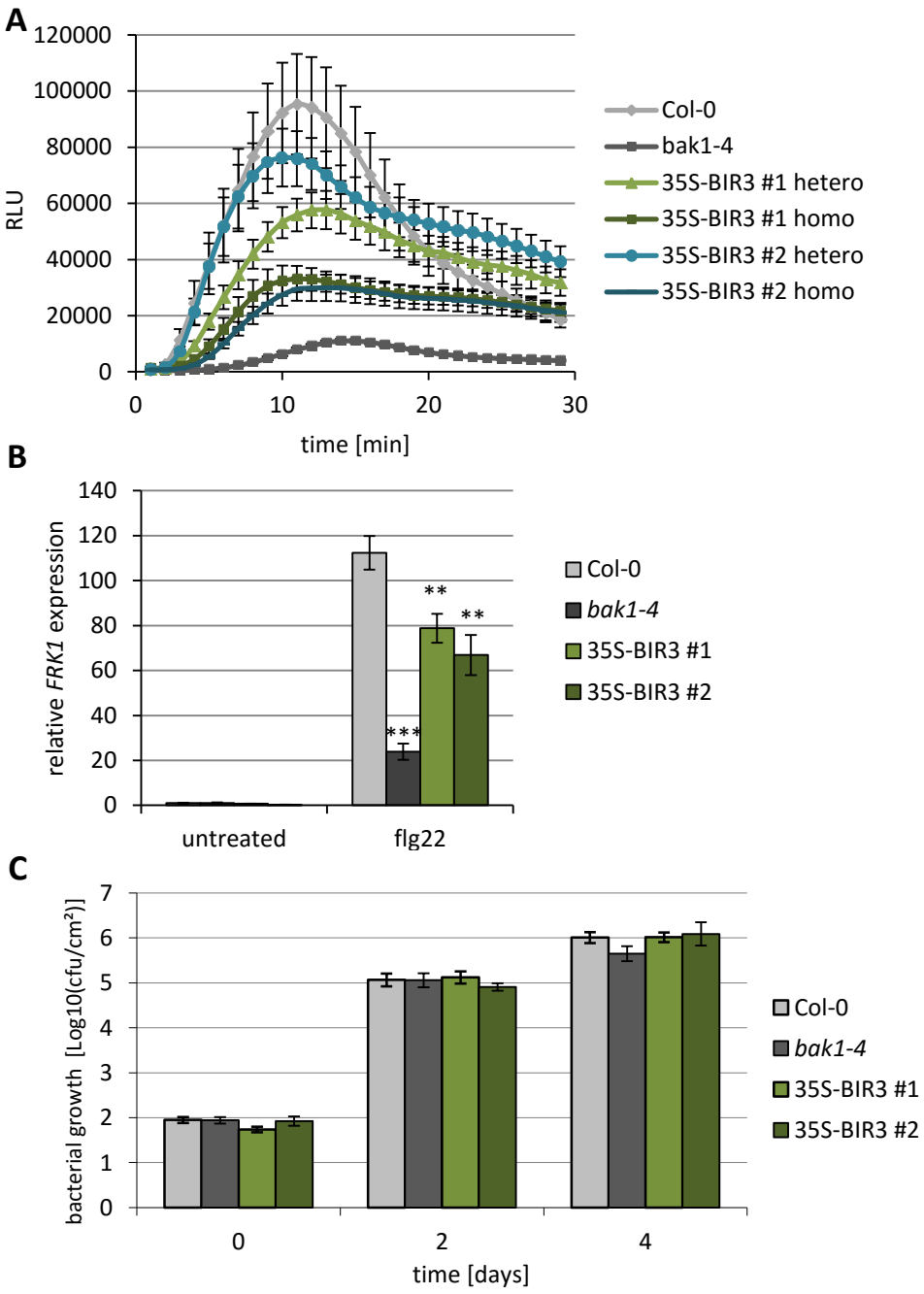

Figure 3-14: BIR3 is a negative regulator of MAMP responses

A: ROS production measured over a period of 30min with Col-0, *bak1-4* and two hetero- and homozygous 35S-BIR3 lines, represented as relative light units (RLU) after elicitation with 100 nM flg22. Results are means ± SE (n=9).

B: *FRK1* marker gene expression in Col-0, *bak1-4* and homozygous 35S-BIR3 plants was measured by qRT-PCR analysis 3 hours after flg22 treatment. *FRK1* expression was normalized to *EF1α* and plotted relative to Col-0 untreated. Results are mean ± SE (n=8). Asterisks represent significant differences to wildtype (**p<0.01; ***p<0.001; Student's t-test).

C: Col-0, *bak1-4* and two heterozygous 35S-BIR3 lines were infiltrated with 10^4 cfu/ml of the virulent bacterial pathogen *Pto* DC3000. Growth of bacteria was monitored at the indicated time points. Results are mean ± SE (n=8). No significant differences according to Student's t-test.

3.2.4. BIR3 influences BAK1-FLS2 complex formation

BIR3 negatively regulates MAMP responses but the molecular mechanism underlying this phenomenon was unclear. BIR3 negatively regulates MAMP responses after flg22 treatment and interacts with BAK1, thus it could influence complex formation between FLS2 and BAK1. To test this hypothesis BAK1 was immunoprecipitated form Col-0 wildtype plants. Coimmunoprecipitated FLS2 could only be detected after flg22 treatment, showing ligand induced complex formation. In the absence of BIR3 (*bir3-2* mutants) flg22 treatment leads to stronger complex formation while with higher BIR3 levels (35S-BIR3) BAK1 FLS2 interaction is almost abolished. This shows that BIR3 is indeed able to negatively regulate BAK1 FLS2 complex formation (Figure 3-15). These data indicate that BIR3 regulates MAMP responses by preventing BAK1 FLS2 complex formation in the absence of the ligand.

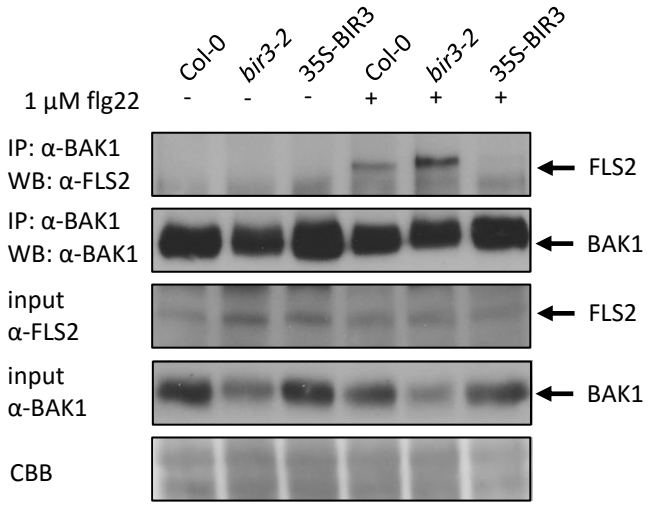

Figure 3-15: BIR3 regulates BAK1 FLS2 complex formation
Arabidopsis seedlings of the indicated genotypes were treated for 5 min with 1 µM flg22 (+) or H2O (-). Immunoprecipitation (IP) was performed with specific α-BAK1 antibody. Precipitated BAK1 and coimmunoprecipitated FLS2 were detected with specific α-BAK1 and α-FLS2 antibodies respectively. Protein input is shown with Western blot analysis of protein extracts before IP and specific α-BAK1 and α-FLS2 antibodies. Coomassie brilliant blue (CBB) staining shows protein loading.

BAK1 and BIR3 interact constitutively and flg22 treatment leads to a ligand induced complex of BAK1 with FLS2. In order to analyze if ligand-binding indeed releases BAK1 from BIR3, BAK1 BIR3 interaction was analyzed after flg22 treatment. So far no specific BIR3 antibody could be generated so that plants expressing BIR3-GFP under its native promotor in the *bir3-2* mutant background were used. Immunoprecipitation was performed with GFP-trap beads against the BIR3-GFP protein and coimmunoprecipitated BAK1 was detected with specific BAK1 antibody. In the untreated state strong interaction of BAK1 with BIR3 was observed, flg22 treatment leads to the release of BIR3 and less complex formation with BAK1 (Figure 3-16). This suggests that BAK1 is indeed released from BIR3 after ligand-binding.

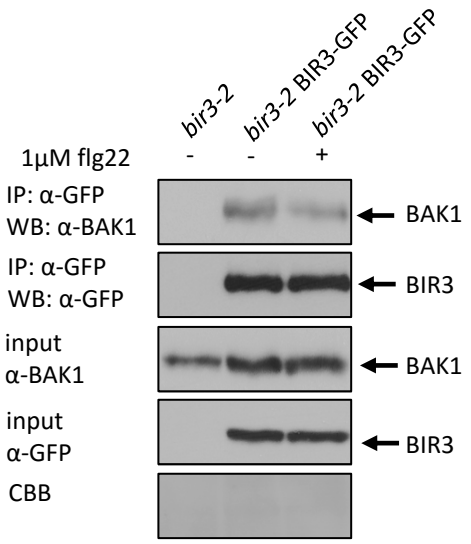

Figure 3-16: BAK1 is released from BIR3 after flg22 treatment
Arabidopsis seedlings of the indicated genotypes were treated for 5 min with 1 µM flg22 (+) or H_2O (-). Immunoprecipitation (IP) was performed with GFP-trap beads. Precipitated BIR3-GFP and coimmunoprecipitated BAK1 were detected with specific α-GFP and α-BAK1 antibodies respectively. Protein input is shown with Western blot analysis of protein extracts before IP and α-BAK1 and α-GFP antibodies. Coomassie brilliant blue (CBB) staining shows protein loading.

3.2.5. BIR3 is upstream of BAK1 in the MAMP response pathway

bak1 bir3 double mutants were used to analyze epistasis in the MAMP response pathway. Double mutants of *bir3-2* with *bak1-4* or *bak1-5*, an allele which is only affected in the MAMP response pathway, were used for flg22 induced ROS burst assays.

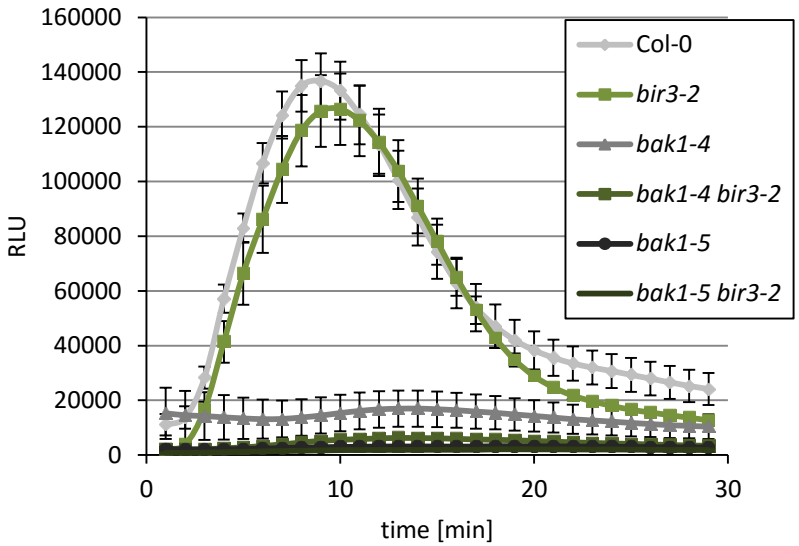

Figure 3-17: BAK1 is downstream of BIR3 in the MAMP response pathway
ROS production measured over 30 min represented as relative light units (RLU) in indicated *bir3* double mutant lines on leaf discs after elicitation with 100nM elf18.

Col-0 shows production of ROS peaking at 10 min after elicitation, *bir3-2* mutants behave like wildtype and the *bak1-4* and *bak1-5* single mutants show very little ROS production and are thus almost insensitive to flg22 treatment. Both double mutant combinations *bak1-4 bir3-2* and *bak1-5 bir3-2* behave like *bak1* mutants. This indicates that BIR3 is upstream of BAK1 in the MAMP response pathway and acts at a very early time point in the MAMP signaling pathway, directly at the receptor level (Figure 3-17).

3.2.6. *bir2 bir3* double mutants resemble *bir2* single mutants

BIR2 and BIR3 are closely related proteins, both interact with BAK1 and regulate different BAK1 dependent processes. Therefore, the question arises if they have redundant functions. In order to analyze the relationship of BIR2 and BIR3, double mutants were generated by crossing of the corresponding single mutants. Homozygous *bir2-1 bir3-2* mutants were used for different functional analyses. The *bir2-1 bir3-2* mutant does not show any altered morphological phenotype compared to wildtype plants (Figure 3-18 A). In order to analyze MAMP responses the double mutants and corresponding single mutants were subjected to elf18 induced ROS burst assays. As described before, *bir2-1* mutants show higher ROS production as wildtype while *bir3* single mutants are not significantly different to wildtype. Thierry Halter has detected enhanced ROS levels in *bir3* mutants. In my experiments *bir3* mutants sometimes also showed slightly altered MAMP responses but in most of the cases they were not significantly different from wildtype indicating that the MAMP pathway is only weakly affected in *bir3* mutants. The *bir2-1 bir3-2* double mutant shows higher ROS production, comparable to *bir2-1* levels (Figure 3-18 B). In order to analyze resistance against biotrophic pathogens the mutants were inoculated with *Pto* DC3000 bacteria and bacterial growth was monitored two days after infection. *bir2-1* mutants show less bacterial growth, *bir3-2* mutants behave as wildtype and *bir2-1 bir3-2* double mutants show less bacterial growth, comparable to *bir2-1* single mutants (Figure 3-18 C). This shows that *bir2-1 bir3-2* double mutants are more resistant to infections with *Pto* DC3000 bacteria resembling the behavior of *bir2-1* single mutants and showing that *bir3* mutation is not blocking the *bir2* immunity phenotype. Cell death analyses were performed using infection experiments with the necrotrophic fungus *A. brassicicola*. *bir3-2* single mutants behave like wildtype, *bir2-1* single mutants show higher disease index and *bir2-1 bir3-2* double mutants behave like *bir2-1* single mutants (Figure 3-18 D). In summary the *bir2-1 bir3-2* double mutant behaves in all pathogen and MAMP assays as the *bir2-1* single mutant. From this data it seems that BIR2 can already maximally block the responses in these pathways or if they act in one pathway BIR2

would be downstream. However, as *bir3* single mutants do not show altered phenotypes the conclusion from these double mutant analyses is that lack of *bir3* does not enhance (or rescue) the *bir2* phenotypes.

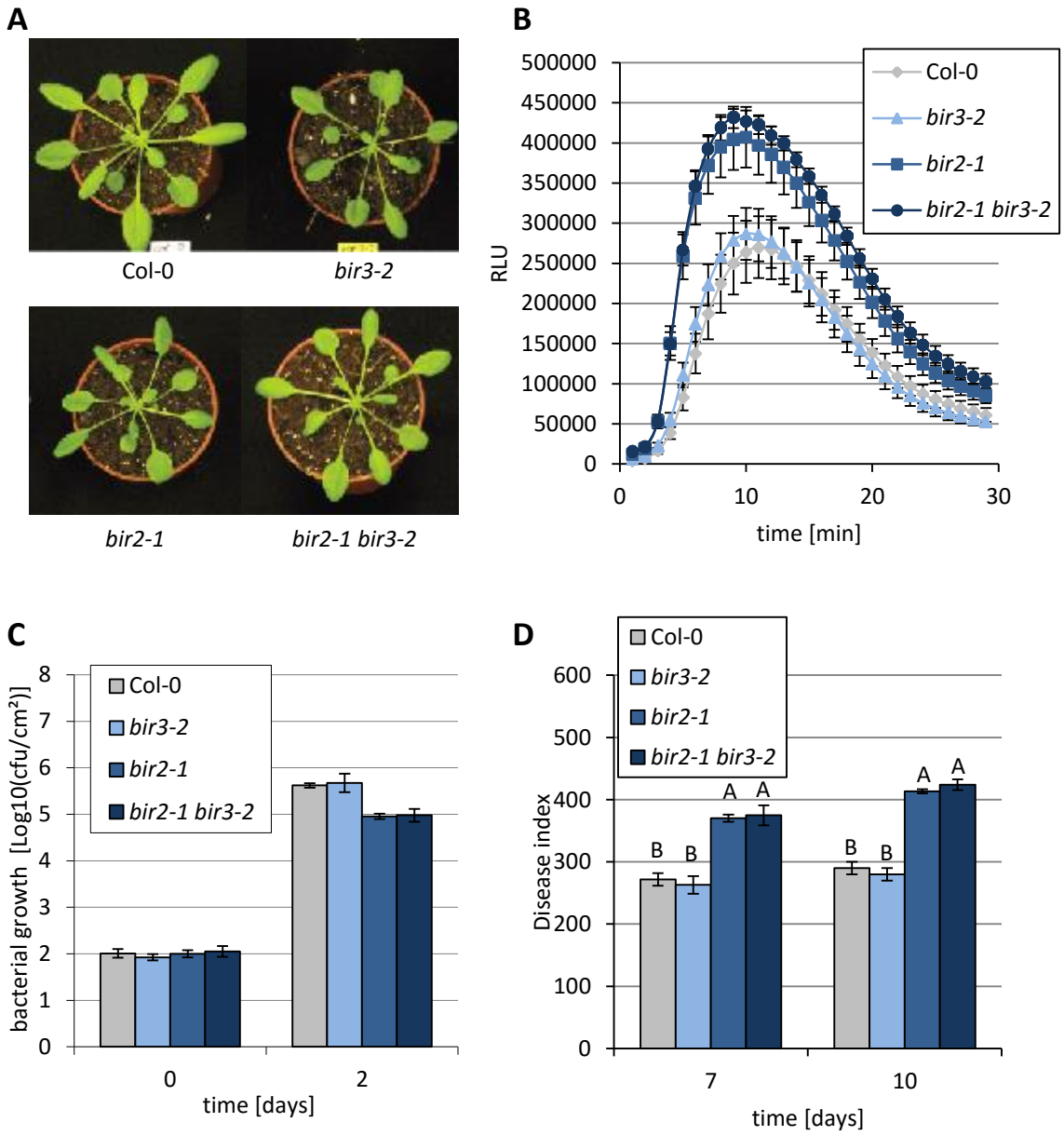

Figure 3-18: Characterization of *bir2 bir3* double mutants
A: Morphological phenotype of Col-0, *bir3-2*, *bir2-1* and *bir2-1 bir3-2* mutant plants.
B: ROS production measured with a luminol based assay over a period of 30min with indicated lines after elicitation with 100 nM elf18. Results are represented as relative light units (RLU) and are means ± SE (n=9).
C: Indicated Arabidopsis lines were infiltrated with 10^4 cfu/ml of the virulent bacterial pathogen *Pto* DC3000. Growth of bacteria was monitored at the indicated time points. Results are mean ± SE (n=8).
D: Infection experiments with the necrotrophic fungal pathogen *Alternaria brassicicola* on indicated Arabidopsis lines. The disease index after 7 and 10 days is shown as mean ± SE (n=3). Different letters indicate significant differences according to one-way ANOVA ($p<0.05$).

3.2.7. Analysis of *bir2-1* 35S-BIR3 mutant plants

In the previous chapter *bir2 bir3* double mutants were used to analyze the relationship between the two proteins. As *bir3* single mutants are not showing a strong phenotype and probably therefore the double mutant behaves in all assays like *bir2-1* it was difficult to draw conclusions. Because more drastical phenotypes were observed with BIR3 overexpressors, *bir2-1* mutants were crossed with these BIR3 overexpressors. In the following analysis *bir2-1* mutants with heterozygous 35S-BIR3 expressing plants were used because the very small homozygous 35S-BIR3 lines are not accessible to infection assays. Figure 3-19 A shows the typical morphology of *bir2-1* and 35S-BIR3 plants. *bir2-1* mutants are a little bit smaller than wt plants with slim leaves, 35S-BIR3 plants show a more stunted phenotype with wide leaves. The double mutant interestingly has a comparable diameter to the small 35S-BIR3 plants but slimmer leaves. This could mean that mutation of *BIR2* partially rescues the BL insensitivity of 35S-BIR3 plants. On the other hand one could also conclude that overexpression of BIR3 does not rescue the morphological phenotype of *bir2-1* mutants, which is most likely caused by cell death, because the double mutant does not have wildtype like morphology. These results show that lack of BIR2 cannot simply be replaced by overexpression of BIR3 and both proteins have differential functions. The double mutants were additionally used to analyze the role of both proteins in the MAMP response pathway. In flg22 induced ROS burst assays *bir2-1* mutants show higher ROS production and 35-BIR3 plants lower ROS production compared to wildtype. *bir2-1* 35S-BIR3 plants show also higher ROS production as wildtype but not exactly as high as *bir2-1* (Figure 3-19 B). This could mean that BIR3 overexpression slightly reduces the MAMP hyperresponsiveness of *bir2-1*. But the ROS production in the double mutant is still much higher than in wildtype what could mean that BIR2 is the major MAMP response regulator and BIR3 only plays a minor role in the pathway. Taking into account that BIR2 and BIR3 can interact with each other it could mean that both proteins act in one pathway and BIR2 is downstream. In order to investigate the relationship of both proteins in cell death regulation the double mutants were used for infection experiments with *A. brassicicola*. *bir2-1* mutants show a much higher disease index with leaf maceration already after 10 days and 35S-BIR3 plants also show stronger symptom development, but not as severe as in *bir2-1* mutants. The double mutant shows slightly weaker disease symptoms than *bir2-1*, there is no additive effect in the *bir2-1* 35S-BIR3 plants (Figure 3-19 C).

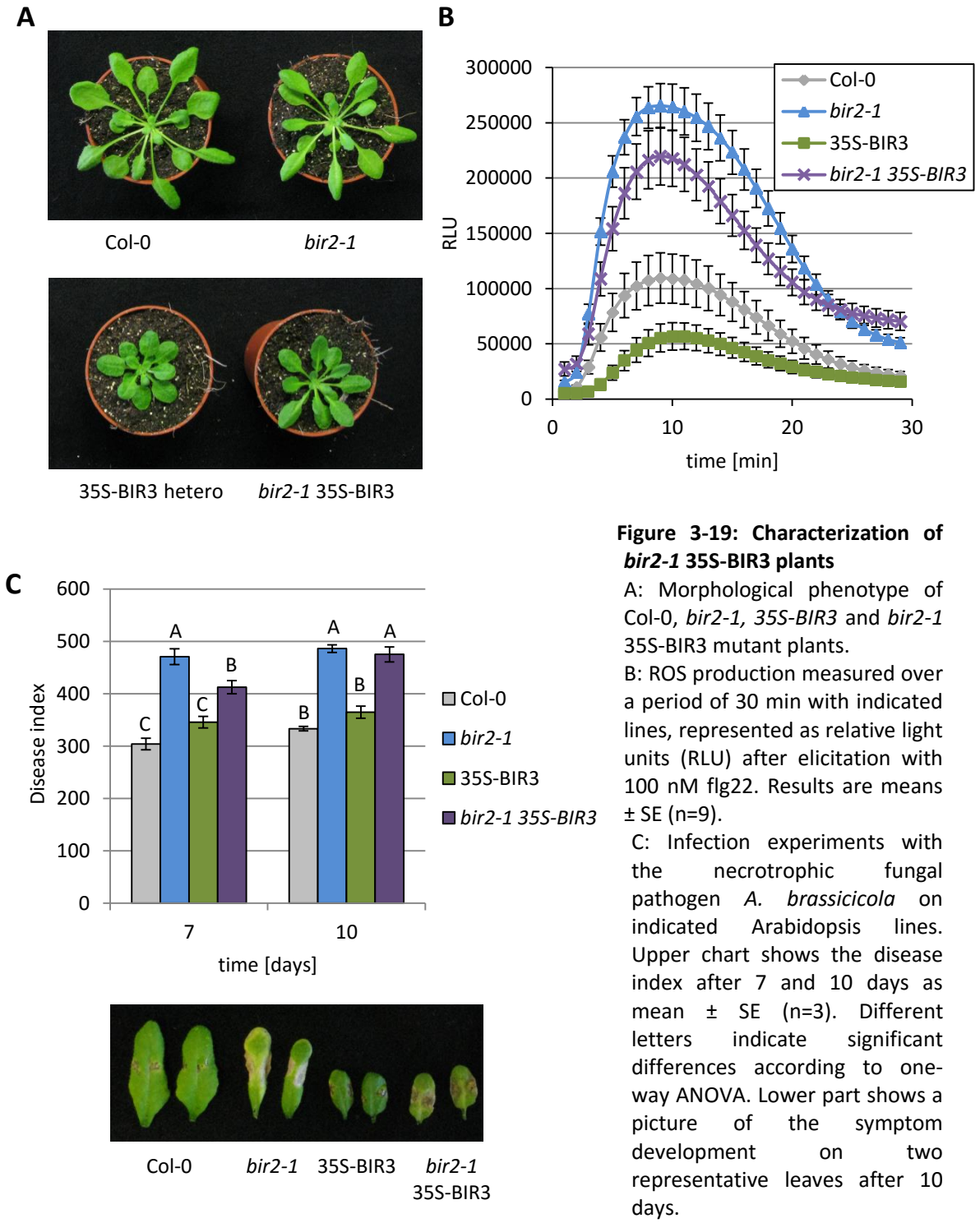

Figure 3-19: Characterization of *bir2-1* 35S-BIR3 plants

A: Morphological phenotype of Col-0, *bir2-1, 35S-BIR3* and *bir2-1* 35S-BIR3 mutant plants.

B: ROS production measured over a period of 30 min with indicated lines, represented as relative light units (RLU) after elicitation with 100 nM flg22. Results are means ± SE (n=9).

C: Infection experiments with the necrotrophic fungal pathogen *A. brassicicola* on indicated Arabidopsis lines. Upper chart shows the disease index after 7 and 10 days as mean ± SE (n=3). Different letters indicate significant differences according to one-way ANOVA. Lower part shows a picture of the symptom development on two representative leaves after 10 days.

The experiments were repeated twice with similar results.

Taken together it was shown that BIR2 and BIR3 are partially redundant. *bir2-1* mutant plants have more severe MAMP and cell death phenotypes compared to *bir3* mutants and also BIR3 overexpressors. Overexpression of BIR3 partially complements the ROS burst of *bir2-1* mutants and weakly also the cell death phenotype, supporting the model that both BIR2 and BIR3 negatively regulate MAMP responses and that cell death is triggered by an imbalance between

BAK1 and BIR protein levels and complex formation. Therefore, BIR2 likely complements the function of BIR3 in a wildtype situation. Because of the partially redundant function of BIR2 and BIR3, the function of BIR3 becomes only visible in the BIR3 overexpressing plants.

3.2.8. Relationship of BIR family proteins

All so far described BIR proteins, BIR1, BIR2 and BIR3 are involved in cell death regulation but each mutant shows a different cell death phenotype. *bir1* mutants show strong spontaneous cell death and are seedling lethal when grown at 22°C. *bir2* mutants only show a marginal cell death phenotype without infection but infection with necrotrophic pathogens leads to severe disease symptoms with spreading necrosis throughout the whole leaf. *bir3* single mutants do not show a cell death phenotype, but BIR3 overexpressors show spreading cell death after infection. We therefore addressed the relationship of different BIR proteins in cell death control. *bir1* shows the strongest cell death phenotype of all *bir* mutants. It should be analyzed if cell death in *bir1* could be altered by mutations in BAK1 or other BIR proteins. All shown double mutant combinations were grown at 28°C long day conditions so that *bir1* single mutants survive and the morphological phenotypes could be compared. Crossing with *bak1-4,* surprisingly, leads to rescue of cell death in *bir1-1,* the plants grow bigger and show less leaf necrosis (Figure 3-20 A). Thus mutation of BAK1, which normally induces cell death, leads in this double mutant combination with *bir1* to a rescue of the cell death phenotype. Crossing of *bir1-1* with *bir3* or *bir2* knockouts does not alter the *bir1* mutant phenotype, there is no rescue and there are no additive effects (Figure 3-20 B, C). Interestingly, overexpression of BIR3 in *bir1-1* leads to a partial rescue of the *bir1* mutant phenotype (Figure 3-20 D). This points into the direction that BIR3 can partially take over the role of BIR1 in cell death regulation and that these proteins have partially redundant functions.

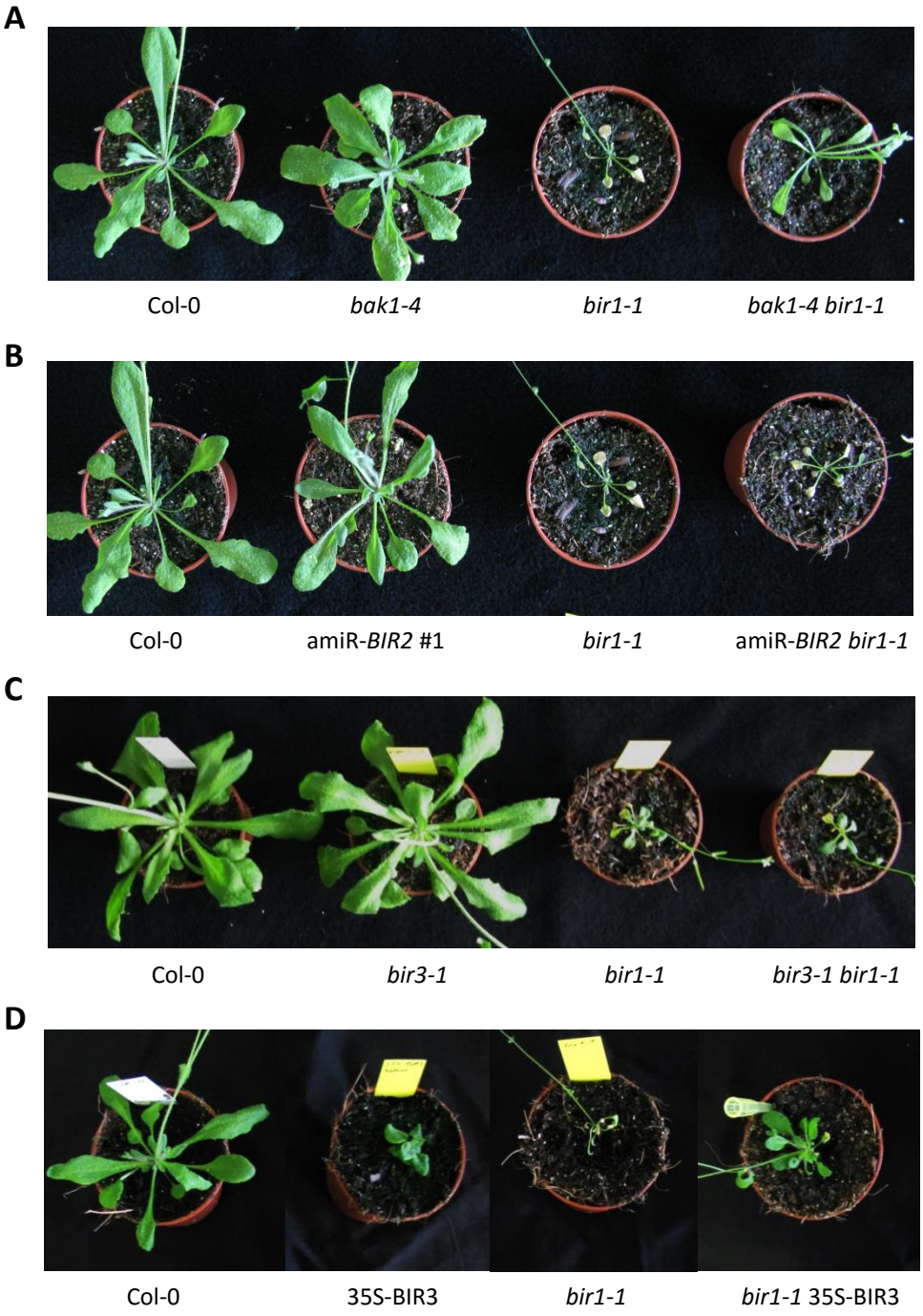

Figure 3-20: *bir1* double mutants
A to D: Morphological phenotype of different *bir1* mutant combinations and the respective single mutants grown at 28°C long day.

3.2.9. *bak1 bir3* double mutants show an enhanced growth phenotype

To further analyze the function of BIR3 and the relationship between BIR3 and BAK1 double mutants were generated. Different allele combinations were crossed and three different homozygous double mutants were obtained, *bak1-3 bir3-1*, *bak1-3 bir3-2* and *bak1-4 bir3-2*. Interestingly, these double mutants already show a severe growth phenotype. *bir3* single mutants have wildtype like morphology, *bak1* single mutants have a weak BL insensitive phenotype but the double mutants *bak1-3 bir3-1* and *bak1-4 bir3-2* are much smaller, have slim and curly leaves and show spontaneous necrotic lesions at later developmental stages (Figure 3-21). Thus, a combination of two single mutants with no or marginal growth phenotypes leads to a double mutant with a severe growth phenotype. This is in contrast to the *bak1 bir1* double mutant, where *bak1* can partially rescue the *bir1* mutant phenotype. The double mutant combination of *bir3-2* with the *bak1-4* null allele has the most severe phenotype and the weaker *bak1-3 bir3-2* mutant combination does not show this phenotype but sometimes even grows a bit bigger than wildtype. *bak1-4* is a null mutant, but *bak1-3* is a T-DNA line with an intron insertion, so that alternative splicing sometimes leads to residual full length transcript. This could explain why the *bak1-3* mutant combination shows a less severe phenotype. In case of the *bir3* knockout lines, both lines show residual transcript depending on the position of the primers used for the RT-PCR analysis, but *bir3-2* shows no full length transcripts (Halter, 2014).

Figure 3-21: *bak1 bir3* double mutants show an enhanced growth phenotype compared to the single mutants
A: Morphological phenotype of different allele combinations of *bak1 bir3* double mutants and the respective single mutants.

3.2.10. *bak1 bir3* double mutants can be complemented by BAK1 or BIR3

As *bir3* single mutants were not significantly affected in MAMP or pathogen responses it was not possible to use these mutants for complementation assays. Therefore, the *bak1 bir3* double mutant was used and transformed with either BAK1-GFP or BIR3-GFP under their

endogenous promotor. Expression of both constructs leads to complementation of the growth phenotype of *bak1-4 bir3-2*. Transformation with BAK1-GFP leads to plants with relatively slim leaves, transformation with BIR3-GFP results in a typical *bak1-4* mutant phenotype (Figure 3-22 A). Expression of the constructs was confirmed in immunoblot analysis with α-GFP antibody (Figure 3-22 B). The fact that both constructs alone can complement the *bak1-4 bir3-2* mutant phenotype shows that only this mutant combination causes the severe phenotype. Moreover, it confirms that mutation of BIR3 has an impact on cell death control that remains hidden in the single mutant but becomes obvious in the double mutant combination with *bak1*.

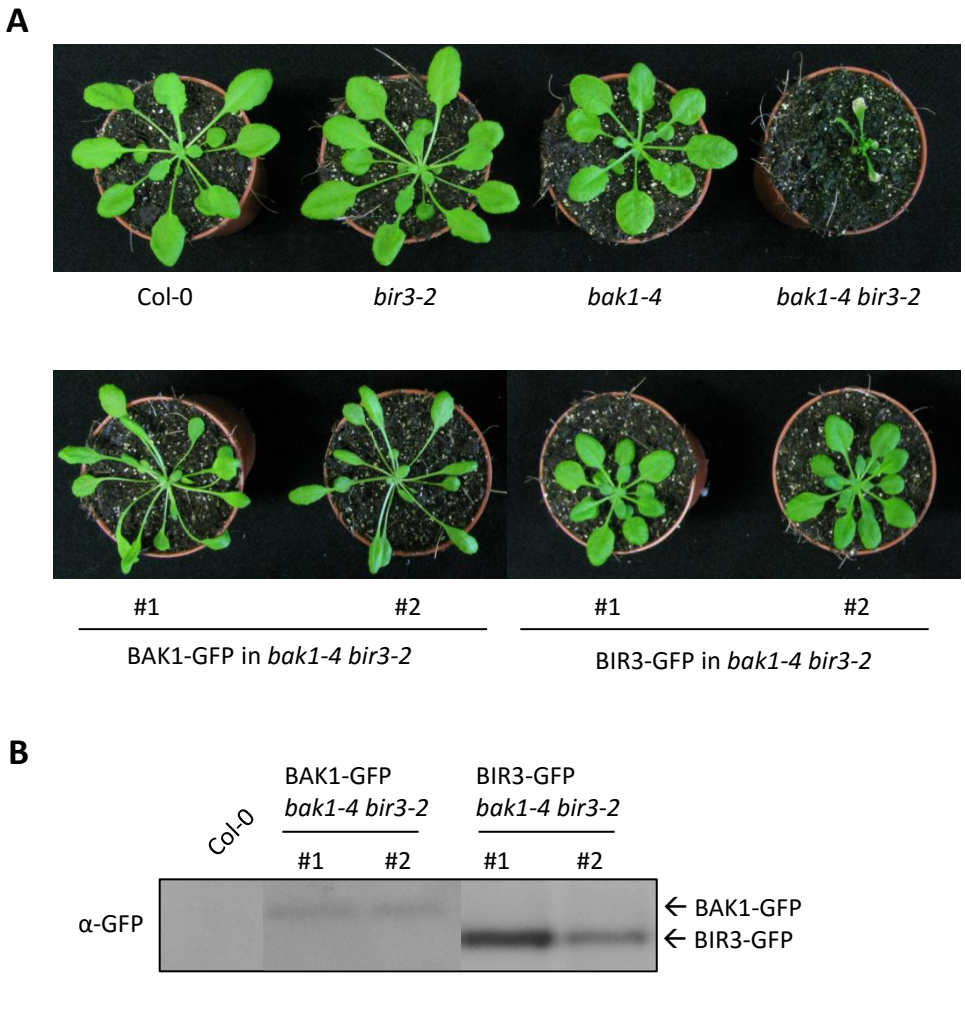

Figure 3-22: *bak1 bir3* double mutants can be complemented by expression of BAK1 or BIR3
A: Morphological phenotype of Col-0, *bak1-4* and *bir3-2* single mutants, *bak1-4 bir3-2* double mutant and 2 lines of *bak1-4 bir3-2* mutant complemented with either BAK1-GFP or BIR3-GFP.
B: Western blot analysis of expression levels of BAK1-GFP or BIR3-GFP in *bak1-4 bir3-2* in two lines. Expression is detected with α-GFP antibody. The blot shows cutouts from different Western blots, thus protein amounts cannot be compared.

3.2.11. *bak1 bir3* double mutants show an enhanced cell death phenotype

In order to analyze cell death regulation in *bak1 bir3* mutants the double mutants and corresponding single mutants were inoculated with *A. brassicicola*. 4 days post inoculation infected and uninfected leaves were subjected to trypan blue staining to visualize dead cells. In case of *bak1-3 bir3-1* and *bak1-4 bir3-2* already uninfected leaves show cell death patches and after infection the cell death spreads through the whole leave (Figure 3-23). This shows that the double mutants have an enhanced cell death phenotype and show in contrast to *bak1* single mutants spontaneous cell death without infection.

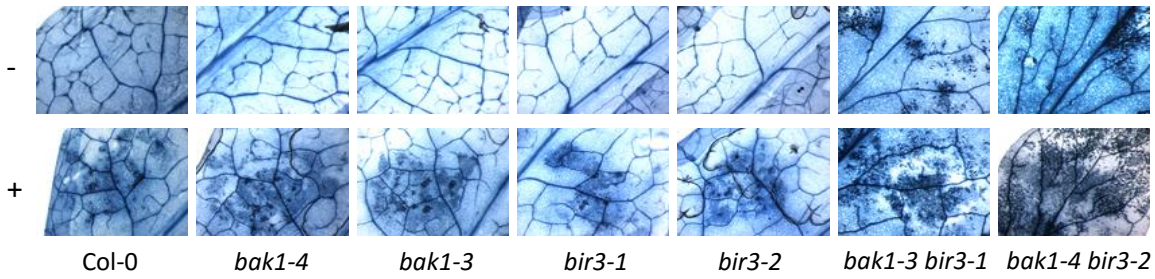

Figure 3-23: *bak1 bir3* double mutants show enhanced cell death
Trypan blue staining to stain dead cells of leaves of the indicated genotypes 4 days after inoculation with *Alternaria brasscicola* (+) or untreated (-).

3.2.12. *bak1 bir3* double mutants show higher SA and JA levels

Cell death mutants typically show enhanced levels of the phytohormones salicylic acid and jasmonic acid at the same time (Mur et al., 2006). In order to confirm the enhanced cell death phenotype in *bak1 bir3* mutants SA and JA hormone levels and gene expression of the respective marker genes *PR1* and *PDF1.2* were analyzed in untreated plants. *bak1-4* single mutants already show moderately higher SA levels and the SA content in *bak1-4 bir3-2* double mutants is even higher (Figure 3-24 A). This could be confirmed by enhanced *PR1* marker gene expression in *bak1-4 bir3-2* double mutants compared to *bak1-4*. Here the effect is even more pronounced compared to the total SA amounts (Figure 3-24 B). *bak1* single mutants do not show enhanced JA levels but the *bak1-3 bir3-1* and *bak1-4 bir3-2* double mutants show enhanced JA levels (Figure 3-24 C). This phenotype correlates with enhanced *PDF1.2* marker gene expression in *bak1-4 bir3-2* (Figure 3-24 D). In summary it was shown that the severe morphological phenotype of *bak1 bir3* double mutants is linked to spontaneous cell death with typically elevated SA and JA levels.

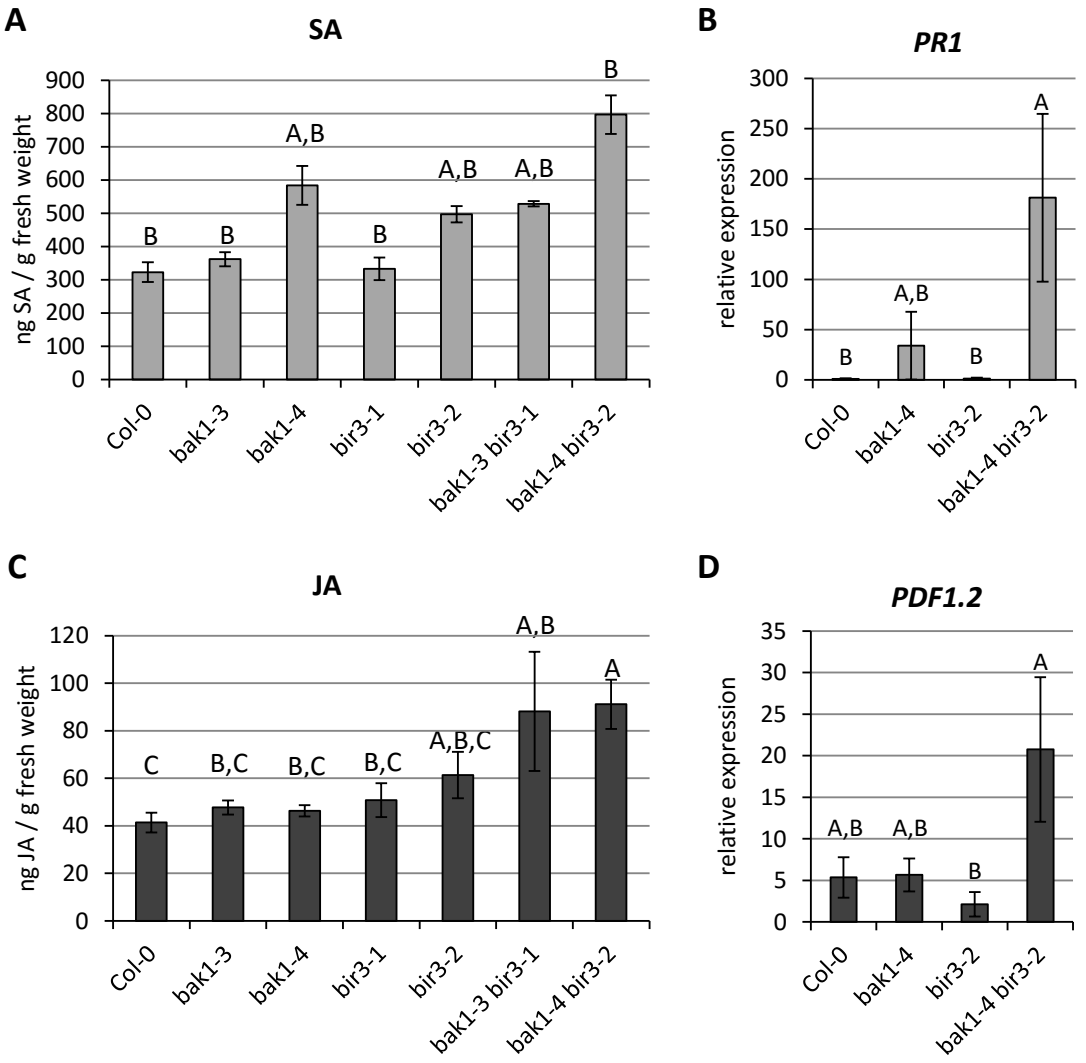

Figure 3-24: Hormone levels and marker gene expression in *bak1 bir3* mutants
A: and C: Gas-chromatography-MS quantification of SA and JA content in 5 week old leaves of untreated Arabidopsis plants of the indicated genotypes. Results are presented as mean ± SE (n=6). Letters indicate significant differences according to one-way ANOVA.
B and D: qRT-PCRs to analyze *PR1* and *PDF1.2* marker gene expression in leaves of untreated Arabidopsis plants of the indicated genotypes. Gene expression was normalized to the housekeeping gene *EF1α* and is plotted relative to Col-0. Results are mean ± SE (n=6).
Hormone levels (A and C) were measured once.

3.2.13. *bak1 bir3* mutants do not show enhanced BL insensitivity

bak1 bir3 mutants have a severe dwarfism phenotype that correlates with spontaneous cell death but the severe growth phenotype could additionally be caused by defects in BL signaling. Therefore the mutants were subjected to different BL assays. On the one hand seedlings were treated with the BL biosynthesis inhibitor brassinazole (BRZ), grown in the dark and the hypocotyl length was measured.

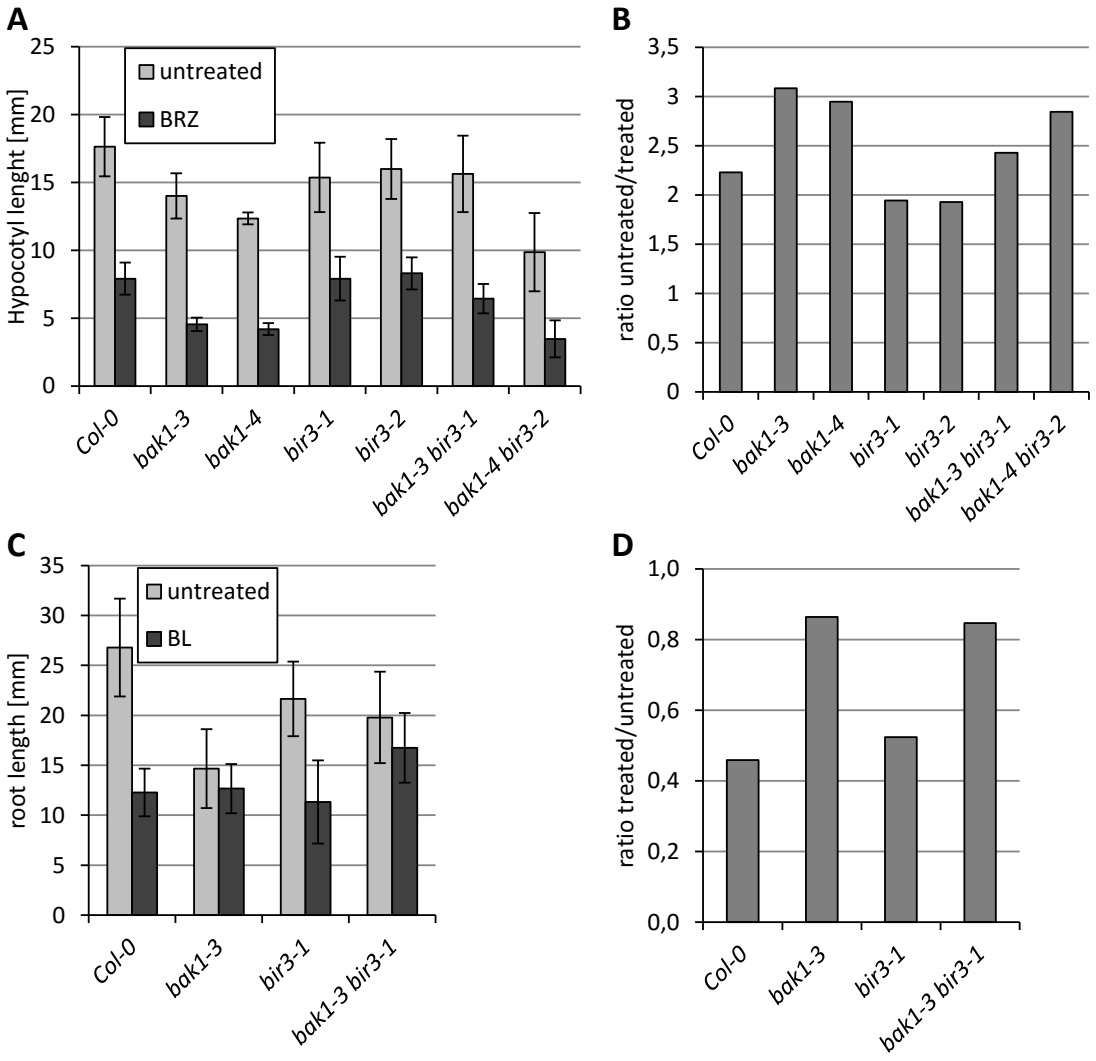

Figure 3-25: BL and BRZ assays with *bak1 bir3* mutants
A: Indicated mutant lines were grown for 7 days in the dark vertically on ½ MS plates supplemented or not with 1 µM BRZ. Hypocotyl length was measured on pictures using Image J software and is represented as mean ± SD.
B: Results from A are represented as ratio untreated/treated.
C: Indicated mutant lines were grown for 8 days under long day conditions vertically on ½ MS plates supplemented or not with 10 nM Epi-BL. Pictures were taken and root length was measured using Image J software and is represented as mean ± SD.
D: Results from C are represented as ratio treated/untreated.

BRZ treatment leads to shorter hypocotyl in wildtype plants, *bak1* mutants are less sensitive to the treatment and the hypocotyl remains relatively longer. The double mutant plants also show BRZ insensitivity, comparable to *bak1* single mutants or a bit less (Figure 3-25 A, B). In the second experiment seedlings were treated with 10 nM epi-BL, grown in the light and the root length was measured. Epi-BL treatment at concentrations of 10 nM leads to reduced root growth in wildtype plants. *bak1-3* mutants are insensitive to BL treatment. Roots are already

shorter in untreated state and there is no reduction in root length. *bak1 bir3* double mutants have a root length between Col-0 and *bak1-3* in the untreated state but show almost no reduction in root length after BL treatment (Figure 3-25 C, D). The experiments show that *bak1 bir3* double mutants are less sensitive to BL, comparable to *bak1* single mutants indicating that the BL insensitivity in the double mutants is caused by *bak1* mutation only. It is unlikely that the enhanced morphological phenotype is caused by defects in BL sensitivity.

3.2.14. *bir3 bkk1* double mutants are not sufficient to show enhanced cell death

The here described *bak1 bir3* double mutants resemble *bak1-3 bkk1-1* double mutants with the stunted morphology and spontaneous cell death (Albrecht et al., 2008). In general BAK1 and BKK1 are the two SERK family members which are involved in cell death control (He et al., 2007). Therefore it should be analyzed how BKK1 influences cell death control by BIR3. Double mutants of *bkk1* and *bir3* were generated by crossing the respective single mutants. These double mutants were used to analyze if *bkk1* mutation also leads to an enhanced cell death phenotype in double mutant combinations with *bir3. bkk1-1 bir3-2* mutants do not show an obvious morphological phenotype (Figure 3-26 A). *bak1-4 bir3-2* and *bkk1 bir3-2* were inoculated with the necrotrophic pathogen *A. brassicicola* and the disease symptoms were monitored. *bak1-4* mutants show a higher disease index compared to Col-0, and the *bak1-4 bir3-2* double mutant shows even stronger symptoms with complete leaf maceration after 13 days. The *bkk1-1* single mutant shows in this experiment also a slightly increased disease index after 13 days but the *bkk1-1 bir3-2* double mutant does not show stronger symptoms compared to *bkk1-1* (Figure 3-26 B). In contrast to *bak1* mutants, *bkk1* mutants show no cell death in single mutants. These results indicate that lack of BKK1 is not sufficient to show deregulated cell death in combination with *bir3* mutants.

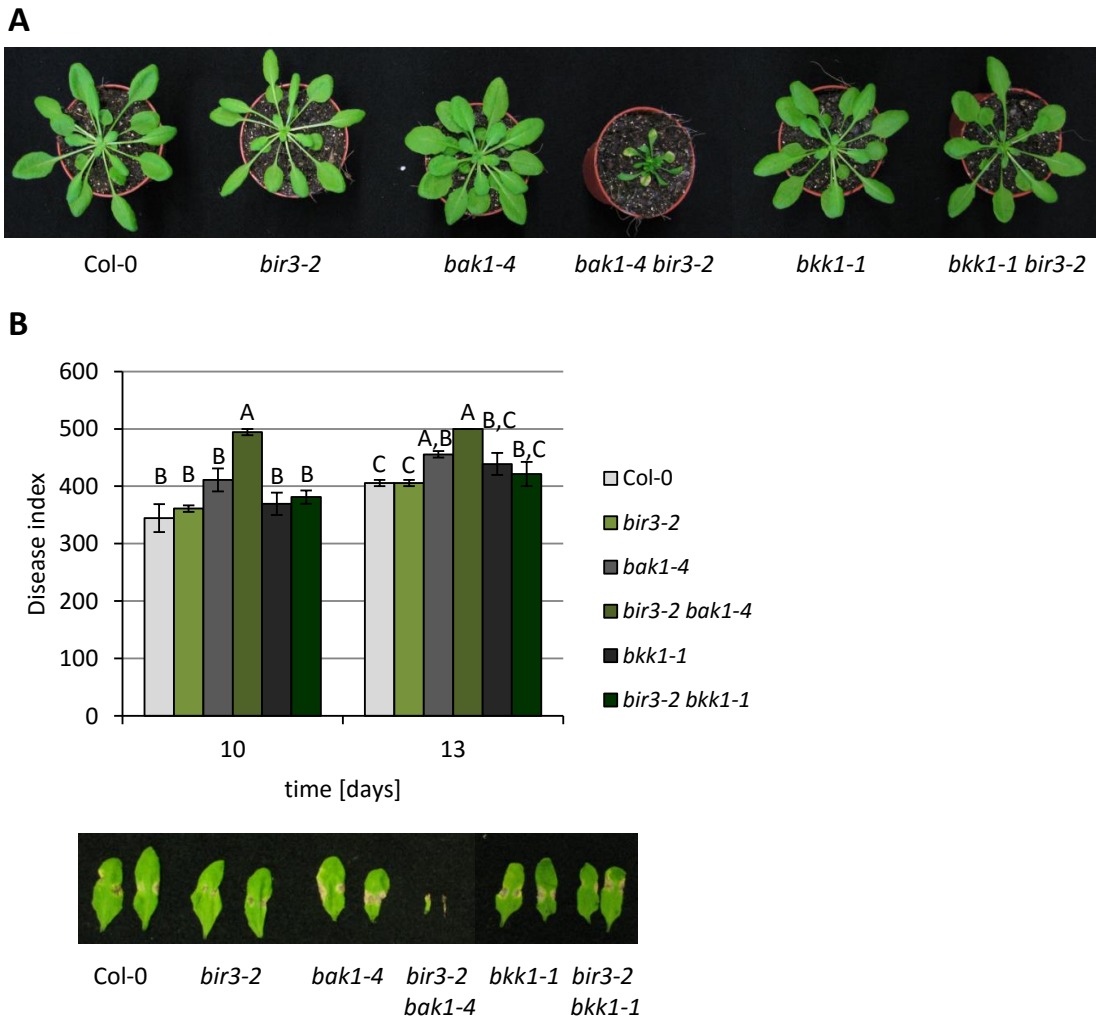

Figure 3-26: Influence of BKK1 on cell death regulation by BIR3
A: Morphological phenotype of *bir3-2 bak1-4* and *bir3-2 bkk1-1* double mutants and the respective single mutants.
B: Infection experiments with the necrotrophic fungal pathogen *Alternaria brassicicola* on different *bir3* double mutants and the respective single mutants. Upper chart shows the disease index after 10 and 13 days as mean ± SE (n=3). Lower part shows a picture of the symptom development on two representative leaves after 13 days.

3.2.15. *bir3* mutants show reduced BAK1 protein levels

In previous experiments like the CoIP in Figure 3-15 it was observed that *bir3* mutants show lower BAK1 protein levels. To confirm this observation immunoblot analysis with protein extracts from untreated Col-0, *bir3-1* and *bir3-2* plants were performed. BAK1 protein levels were detected using specific α-BAK1 antibody and both *bir3* mutant alleles indeed showed lower BAK1 protein amounts (Figure 3-27 A). In order to analyze if these reduced protein amounts are caused by reduced *BAK1* transcription semiquantitative RT-PCRs were performed. The RT-PCR analysis did not show altered BAK1 transcript amounts in *bir3* mutants compared

to Col-0 (Figure 3-27 B). This indicates that altered BAK1 protein amounts are not caused by differential expression. In transient expression experiments in *N. benthamiana* it was observed that expression of BAK1 alone leads to protein degradation visible as several bands on Western blots. Co-expression of BIR proteins led to stabilization of BAK1, the truncated versions were no longer visible.

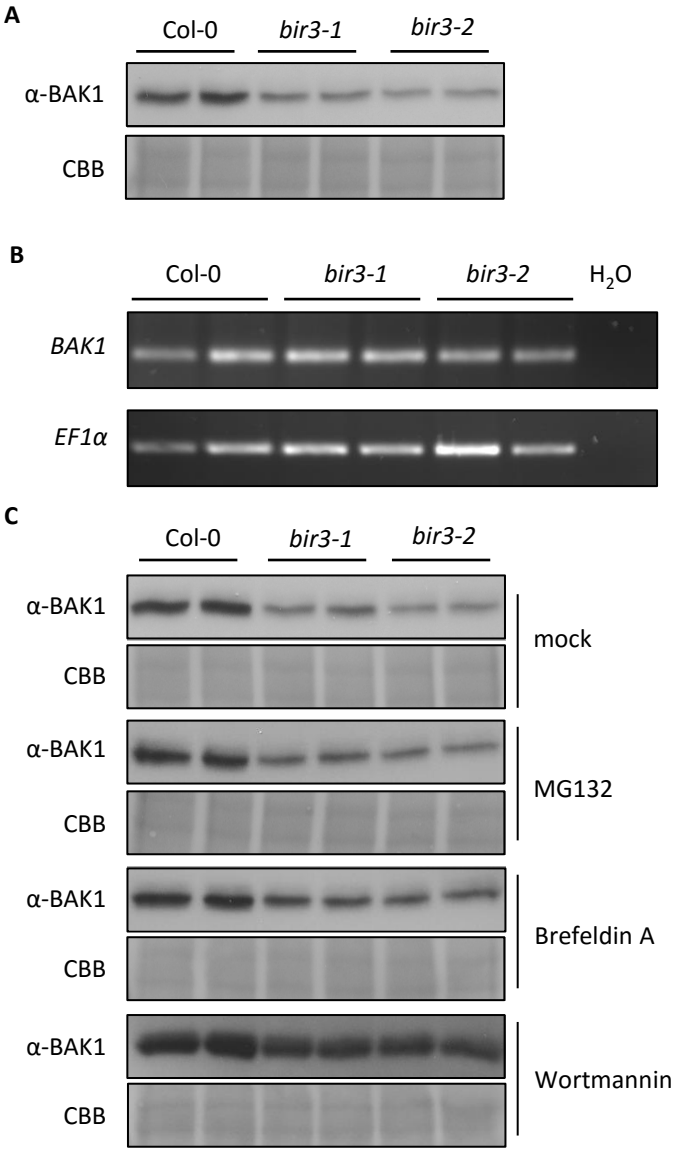

Figure 3-27: *bir3* mutants show reduced BAK1 protein levels
A: Immunoblot analysis of BAK1 protein amounts in untreated Col-0, *bir3-1* and *bir3-2* plants.
B: Semiquantitative RT-PCR analysis of *BAK1* expression in Col-0 and *bir3* mutants. Expression of the housekeeping gene *EF1α* is shown as loading control.
C: Immunoblot analysis of total protein extracts from Col-0 and *bir3* mutant plants after treatment with different chemicals. Seedlings were vacuum infiltrated with 50 µM MG 132 (from 50 mM stock in DMSO), 30 µM Brefeldin A (from 10 mM stock in DMSO), 30 µM Wortmannin (from 10 mM stock in DMSO) or mock and incubated for 4 hours. CBB staining shows equal loading. The experiment in C was done once.

Therefore, it was hypothesized that BAK1 protein amounts in *bir3* may be reduced due to lack of stabilization and degradation. To test this hypothesis, plants were treated with the proteasome inhibitor MG132 to suppress proteasome mediated protein degradation. However, the immunoblot analysis after MG132 treatment still shows reduced BAK1 protein amounts in *bir3*. This shows that reduced BAK1 levels in *bir3* mutants are not caused by enhanced degradation by the 26S-proteasome. As receptors undergo endocytosis after activation and also constant cycling between the plasma membrane and early endosomes it was tested if BAK1 gets endocytosed in *bir3* mutants. The endocytosis inhibitor Brefeldin A inhibits constitutive cycling of FLS2 between plasma membrane and early endosomes and Wortmannin was described to prevent endocytosis of FLS2 after activation (Beck et al., 2012). Both treatments did not lead to rescued BAK1 protein amounts in *bir3* mutants (Figure 3-27 C). Thus it remains unclear what causes the reduced BAK1 protein amounts in *bir3* mutants and further analysis are needed to address this question.

3.2.16. BIR3 interacts with and stabilizes BKK1

In order to further investigate the role of BKK1 in BIR3-mediated cell death it was analyzed if BIR3 is able to interact with BKK1. 35S-BIR3-YFP and 35S-BKK1-HA constructs were transiently expressed in *N. benthamiana* and CoIP experiments were performed. Immunoprecipitation of BIR3 leads to clear coimmunoprecipitation and thus interaction with BKK1 (Figure 3-28). This experiment shows that BIR3 cannot only interact with BAK1 but also with the close homologue BKK1.

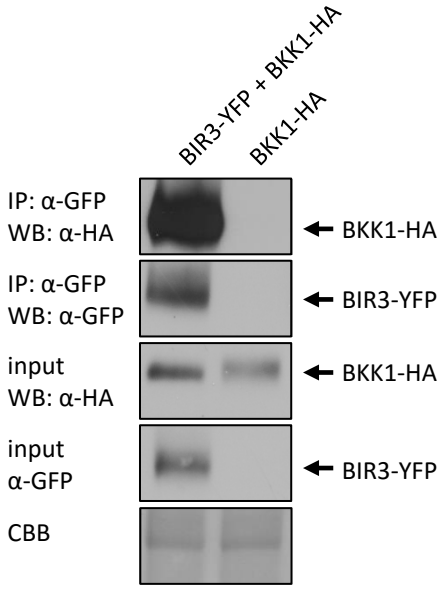

Figure 3-28: BIR3 interacts with BKK1
Indicated constructs were transiently expressed in *N. benthamiana* leaves and immunoprecipitation (IP) was performed with GFP-trap beads. Precipitated BIR3-YFP and co-immunoprecipitated BKK1-HA were detected with α-GFP and α-HA antibodies respectively. Protein input is shown with Western blot (WB) analysis of protein extracts before IP and α-GFP and α-HA antibodies. Coomassie brilliant blue (CBB) staining shows protein loading.

As BKK1 interacts with BIR3 it was analyzed if BKK1 gets also stabilized by BIR3. As no specific BKK1 antibody was available, tagged versions of both proteins were transiently expressed in *N. benthamiana*. The Western blot analysis shows that co-expression of BIR3 with BKK1 leads to higher BKK1 proteins amounts compared to BKK1 expression alone (figure 3-29). This experiment indicates that BIR3 not only stabilizes BAK1 but also BKK1.

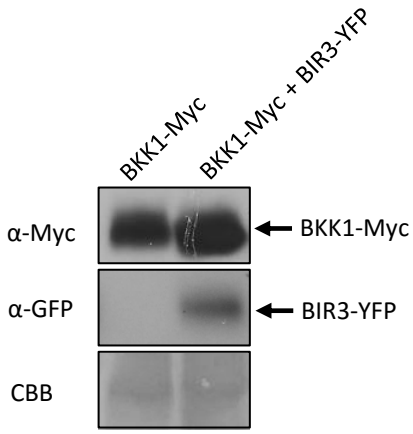

Figure 3-29: BIR3 stabilizes BKK1
BKK1-Myc and BIR3-YFP constructs were transiently expressed in *N. benthamiana*. Western-blot analysis on total protein extracts with α-GFP and α-Myc antibodies shows BKK1-Myc and BIR3-YFP protein amounts. Coomassie brilliant blue (CBB) staining shows protein loading.

4. Discussion

BAK1 is a multifunctional co-receptor that regulates BR, MAMP and DAMP signaling by interaction with the respective ligand-binding receptors BRI1, EFR or FLS2 and PEPR1 and PEPR2. Moreover, BAK1 is involved in regulation of cell death containment. However, it is poorly understood how BAK1 itself is regulated. Therefore in vivo BAK1 complexes were purified and two new interactors, BIR2 and BIR3, were identified (Halter et al., 2014). Functional analysis showed that BIR2 and BIR3 differentially regulate BAK1 dependent processes (Halter, 2014). This work focuses on the regulation of receptor complex formation by BIR2 and BIR3, analysis of the relationship of different BIR proteins and sheds further light on cell death regulation by BIR proteins.

4.1. Receptor complexes in MAMP and BL signaling

4.1.1. BIR2 influences MAMP responses by regulating BAK1-PRR complex formation

BIR2 is a negative regulator of MAMP responses, as BIR2 mutants are hyperresponsive to MAMP treatment (Halter, 2014). In this work the negative regulatory role of BIR2 in the MAMP response pathway was confirmed using BIR2 overexpressing plants that showed reduced MAMP responses. We were interested how BIR2 negatively regulates MAMP responses on a molecular level. BIR2 constitutively interacts with BAK1 in an untreated state and treatment with individual ligands, for which BAK1 is the co-receptor, leads to a fast release of BAK1 from BIR2 (Halter, 2014). This indicates that BIR2 regulates MAMP responses by preventing interaction of BAK1 with ligand-binding receptors in the untreated state. But the question remains how this release is achieved. On the one hand it could be shown that BAK1 interacts with FLS2 only after ligand-binding (Chinchilla et al., 2007). Binding of flg22 to FLS2 creates an additional interaction interface for BAK1 and flg22 acts as “molecular glue” between BAK1 and FLS2 extracellular domains. There are no conformational changes in the FLS2 structure (Sun et al., 2013). This could mean that flg22 binding creates an additional binding site and increases the affinity of FLS2 to BAK1 above the affinity between BIR2 and BAK1 and this affinity shift leads to a preferential binding of BAK1 to FLS2. Another question is, if protein interactions can be seen as static as it was done here so far. BAK1 constitutively interacts with BIR2. However, does that mean that one specific BIR2 and BAK1 molecule are glued together? The situation could also be more dynamic and proteins could be under constant movement in the lipid

bilayer. In this model, BAK1 in the untreated state would bind preferentially or more often and longer to BIR2. After ligand-binding the equilibrium is shifted into the direction of FLS2.

It could also be that BIR2 is actively released from BAK1 by e.g. phosphorylation of specific residues that would affect complex formation with BAK1. A phosphorylation is imaginable by BAK1 and/or FLS2 or even by BIK1 (Lu et al., 2010). This active release model would need an additional direct interaction between FLS2/BIK1 and BIR2. In our first experiments a direct interaction between BIR2 and FLS2 could not be shown (Halter et al., 2014). However, this negative experimental result does not exclude an interaction between FLS2 and BIR2. It is probably a transient and very short interaction which is experimentally difficult to detect. Ligand-binding of flg22 to FLS2 could first recruit the BAK1 BIR2 complex and then lead to an immediate release of BIR2. The release could probably be initiated by phosphorylation. It should therefore be tested if FLS2 is able to phosphorylate BIR2 and if phosphorylation at specific residues hinders interaction of BIR2 with BAK1. This work is in progress by another PhD student in the group. BAK1 constitutively phosphorylates BIR2 in vitro and the BAK1 kinase activity seems to be necessary for the interaction (Halter et al., 2014). However, it could also be possible that phosphorylation of specific residues at BIR2 by BAK1 leads to their release. Binding of flg22 to FLS2 leads to activation of FLS2 and phosphorylation of BAK1. This phosphorylation of BAK1 could also be a signal for BIR2 release.

It could be shown that BAK1 gets released from BIR2 after flg22 treatment, but only about 30% of the BAK1 pool was released. The plants were treated with a saturating concentration of flg22 (1µM), thus, this does not seem a result of partial flg22 pathway activation. It indeed makes sense that treatment with only flg22 does not recruit the whole BAK1 pool to FLS2, because BAK1 acts as co-receptor for different ligand-binding receptors. We therefore treated the plants with a cocktail of different ligands, for which BAK1 is the co-receptor and this led to a drastic increase in complex release compared to the treatment with individual ligands. This result supports the hypothesis of preformed receptor complexes. This means that BAK1, individual ligand-binding receptors and probably other components already exist in so called nanoclusters, compartments that contain receptor complexes in close vicinity before ligand-binding (Bücherl et al., 2013). BAK1 is quickly released from BIR2 in a ligand dependent manner. Binding of flg22 to FLS2 would recruit only BAK1 within this nanocluster, it would be unable to recruit a BAK1 protein that is e.g. preassembled with BRI1. The interaction of BAK1 with FLS2 occurs within seconds (Schulze et al., 2010). This indicates that both proteins are already in close vicinity in the untreated state. For BAK1 and BRI1 it could be shown with microscopic analysis that both proteins co-localize (Bücherl et al., 2013). BAK1 seems not to be a limiting factor between BR and flg22 signaling, as treatment with BRs or

overexpression of BRI1 does not influence the interaction of BAK1 with FLS2 after flg22 treatment (Albrecht et al., 2012; Lozano-Duran et al., 2013). All these observations together support the theory of preformed receptor complexes.

In the same study where BAK1-BRI1 colocalization was shown, it was also shown that about 7% of the BRI1 pool interacts constitutively with BAK1. Other studies also report that they have seen weak interaction of BAK1 with ligand-binding receptors in the untreated state (Schulze et al., 2010; Roux et al., 2011; Schwessinger et al., 2011). The BAK1 FLS2 co-crystal has also shown that the FLS2 and BAK1 extracellular domains have an interaction surface independent of flg22 and therefore might directly interact weakly with each other in the absence of the ligand. Flg22 binding simply creates an additional interaction surface and thus strengthens the interaction leading to activation and downstream signaling.

bir2 mutants are hyperresponsive to MAMPs and BIR2 overexpressors show weaker MAMP responses showing that BIR2 has a negative regulatory role in MTI. To analyze the underlying molecular mechanism it was tested if BIR2 has a direct regulatory effect on the complex formation between BAK1 and FLS2. In CoIP experiments BIR2 overexpressing plants showed weaker complex formation between BAK1 and FLS2 and in the opposite experiment amiR-*BIR2* lines showed stronger complex formation. This explains the results of the functional analysis, and shows that BIR2 has indeed an immediate and direct role on the receptor complex formation. Epistasis analyses have shown that BIR2 is upstream of BAK1 supporting this direct mechanism at this very early point in the signaling cascade.

To support these results the *bak1-5* mutant was used. *bak1-5* is a point mutation in the BAK1 kinase domain that leads to stronger interaction of BAK1 with FLS2 and other ligand-binding receptors and interaction even in the absence of the ligands. This suggests that the affinity of BAK1-5 to ligand-binding receptors is higher or a negative regulation is less active (Schwessinger et al., 2011). BAK1-5 protein showed little interaction with BIR2 compared to wildtype BAK1, indicating that the affinity is altered in the opposite way as compared to all ligand-binding receptors tested. This correlation nicely supports that BIR2 is the negative regulator of BAK1 that keeps BAK1 in check in the resting state, however, when the affinity to BIR2 is altered (in BAK1-5 and after flg22 treatment) and BAK1 binds less to BIR2 it is able to interact stronger with FLS2. The *bak1-5* mutation is additionally interesting because it was shown that BAK1-5 protein is less phosphorylation active (Schwessinger et al., 2011). We could show that BAK1 unidirectionally phosphorylates BIR2 and that BAK1 phosphorylation activity is necessary for interaction in yeast-two-hybrid assays (Halter et al., 2014). The fact that BAK1-5 which is less phosphorylation active interacts less with BIR2 compared to BAK1 wildtype

protein gives an in vivo hint that BAK1 kinase activity might be necessary for interaction with BIR2.

During these interaction experiments it was observed that *bir2* mutants show higher FLS2 protein levels and qPCR analysis confirmed higher expression of FLS2 in amiR-*BIR2*. As several RLKs are induced by MAMPs, this enhanced FLS2 expression could be a secondary effect of the enhanced MAMP responses in *bir2* leading to induction of PRRs (Zipfel et al., 2006). Thus, in case of *bir2* mutants the detected higher BAK1-FLS2 complexes could also be caused by higher FLS2 expression. However, two other independent experiments indicate that BIR2 regulates MAMP responses at the level of receptor complexes. First, reduced interactions of FLS2 and BAK1 were observed in 35S-BIR2-YFP plants that are not altered in FLS2 expression. Second, in *bak1-5* interaction of BAK1 with BIR2 is strongly reduced mimicking the effect of BAK1 release from BIR2 by flg22 treatment and leading to enhanced interaction with ligand-binding receptors, confirming that alterations in BIR2/BAK1 interaction directly affect complex formation of BAK1 with FLS2. In *bir2* mutants the complex formation between BAK1 and FLS2 after ligand-binding is lower compared to wildtype, however, there is no detectable interaction without the ligand. This could on the one hand be explained by redundancies with other BIR proteins. In the absence of BIR2, BAK1 is still kept in check by other BIR proteins, which were shown to be able to interact with BAK1. On the other hand it seems to be not enough to have free BAK1 to induce MAMP responses, only flg22 binding leads to a signaling active complex. Negative regulation by BIR proteins in the absence of the ligand could be an additional backup to prevent possible low amounts of signaling from MAMP receptors without ligand-binding.

BAK1 not only interacts with the different ligand-binding receptors FLS2, EFR, BRI1, PEPR1 and PEPR2 and the small regulatory RLKs BIR1 – BIR4 but with several other proteins. These are: The BRI1 homologues BRL1 and BRL3 (Fabregas et al., 2013), BIK1 and the homologues PBL1 and PBL2 (Zhang et al., 2010; Lin et al., 2013), BSK1 (Shi et al., 2013), IOS1 (Chen et al., 2014), KAPP (Gomez-Gomez et al., 2001), PUB12 and PUB13 (Lu et al., 2011), PP2A (Segonzac et al., 2014), 14-3-3 proteins (Chang et al., 2009), the glutaredoxin *At*GRXC2 (Bender et al., 2015). It is hard to imagine how all these proteins interact with BAK1 at the same time, first of all because of space reasons. Further studies are needed to analyze the time and spatial resolution of receptor complexes in different situations.

4.1.2 BIR3 interacts with BAK1 and shows additional direct interaction with PRRs and BRI1

After publishing the BIR2 manuscript (Halter et al., 2014) we concentrated on the characterization of BIR3. BIR3 is, as BIR2, a negative regulator of BAK1 in the MAMP response pathway (Halter, 2014). To further support these findings different MAMP assays were performed with BIR3 overexpressors in this work. For ROS burst assays, a fast MAMP response already peaking after 10 min, hetero- and homozygous BIR3 overexpressing plants were used. The heterozygous plants already showed reduced MAMP responses compared to wildtype and the response in the homozygous plants was even lower. These results show the quantitative effect of BIR3 on BAK1-regulated MAMP responses. The results were confirmed by *FRK1* marker gene expression, an independent readout for MAMP responses.

BIR3 plays additionally a role in BL responses as BIR3 overexpressors are dwarf and BL insensitive (Halter, 2014). The fact that the BIR3 overexpression can be rescued by BRI1 overexpression let to the hypothesis that BIR3 might directly regulate BRI1. It was analyzed on the molecular level how BIR3 regulates BL responses. We have shown in CoIP experiments after transient expression in *N. benthamiana* that BIR3 is able to interact with BRI1. With this transient expression system it cannot be differentiated if the interaction is direct or via other proteins such as the *N. benthamiana* homologue of BAK1. Therefore, a yeast system was used, the split ubiquitin system, that allows the use of full length membrane proteins, to analyze if the interaction is direct. In this assay it could indeed be shown that BIR3 directly interacts with BRI1.

Because BIR3 is able to interact with BRI1 it was tested if BIR3 is also able to interact with other ligand-binding receptors in other pathways and it could be shown that BIR3 can also interact with FLS2. BIR2 regulates receptor complexes by interaction with BAK1 (Halter et al., 2014). The here described direct interaction of BIR3 with ligand-binding receptors adds an additional new regulatory mechanism by a direct molecular influence on ligand-binding receptors. A similar behavior was described for BIK1 that interacts with BAK1 in the MAMP pathway but directly interacts with BRI1 in the BL pathway and exert different functions by this different mechanism (Lin et al., 2013). The complementation of the BIR3 overexpression phenotype by overexpression of BRI1 supports the idea that BIR3 has a direct effect on BRI1 receptor complexes and points to a competitive inhibition of BRI1 by BIR3. BIR3 has evolved an additional mechanism to regulate BAK1-mediated processes by directly affecting not only BAK1 but also the ligand-binding receptors. *bak1 bir3* double mutants were used for epistasis experiments in the MAMP response pathway. It could be shown that BIR3 acts upstream of

BAK1, confirming the role of BIR3 at the very early time point in MAMP signaling, directly at the receptor level.

Besides direct interaction with FLS2, BIR3 additionally regulates complex formation between BAK1 and FLS2. In BIR3 overexpressing plants complex formation of BAK1 and FLS2 is almost abolished, while in *bir3* mutants the interaction is enhanced. The stronger effect of BIR3 overexpression compared to BIR2 overexpression can be explained by the additional direct effect of BIR3 on the ligand-binding receptors themselves or by the stronger interaction of BIR3 with BAK1. BAK1 is released from BIR3 after flg22 treatment indicating that BIR3 is not necessary for downstream signaling but that BAK1 is freed for stable binding to ligand-binding receptors. As BIR3 is not necessary for downstream signaling it is likely that it gets also released from FLS2, but this has to be proven experimentally.

As BIR3 is able to interact with both BAK1 and BRI1 in the untreated state, it was tested with a split ubiquitin bridge assay if the proteins build a tripartite complex. In the yeast system BAK1 and BRI1 already showed weak interaction and additional expression of BIR3 as a bridge did not enhance the complex formation. This experiment indicates that BIR3 is not the "molecular glue" between BAK1 and BRI1 but that BIR3 interacts individually with both proteins. In the direct split-ubiquitin assay BRI1 and BIR3 showed weaker interaction compared to the other tested interactions, BRI1 - BAK1 and BAK1 - BIR3. Because of the weak BIR3 BRI1 interaction it is probably technically not possible to show enhancement of BAK1 BRI1 interaction by BIR3 in the bridge assay and a tripartite complex cannot be excluded. BIR3 might act as a scaffold protein stabilizing preformed receptor complexes between BAK1 and PRRs. Ligand-binding releases BIR3 and allows direct interaction between BAK1 and the PRR. The actual interaction interface of BIR3 with other RLKs is so far not known.

Taken together, it was shown that the newly identified LRR-RLK BIR3 is a constitutive interactor of BAK1. Functional analyses with BIR3 overexpressor lines have shown that BIR3 is a negative regulator of the MAMP and BL response pathways. On the molecular level BIR3 constitutively interacts with the co-receptor BAK1 but also directly with the ligand-binding receptors BRI1 and FLS2. It is not yet clear if these three components build a tripartite complex or if different BIR3 proteins interact with either BAK1 or ligand-binding receptors (Figure 4-1, left part). If it is a tripartite complex, BIR3 could act as scaffold that keeps BAK1 and the ligand-binding receptor in a preformed complex but prevents direct interaction. BIR3 has a negative regulatory role on MAMP and BL signaling by preventing interaction of BAK1 with the ligand-binding receptor in the uninduced state. Ligand-binding (Figure 4-1, right part) leads to release of BAK1 from BIR3 and thus enables BAK1 to interact with the ligand-binding receptor and to induce downstream signaling.

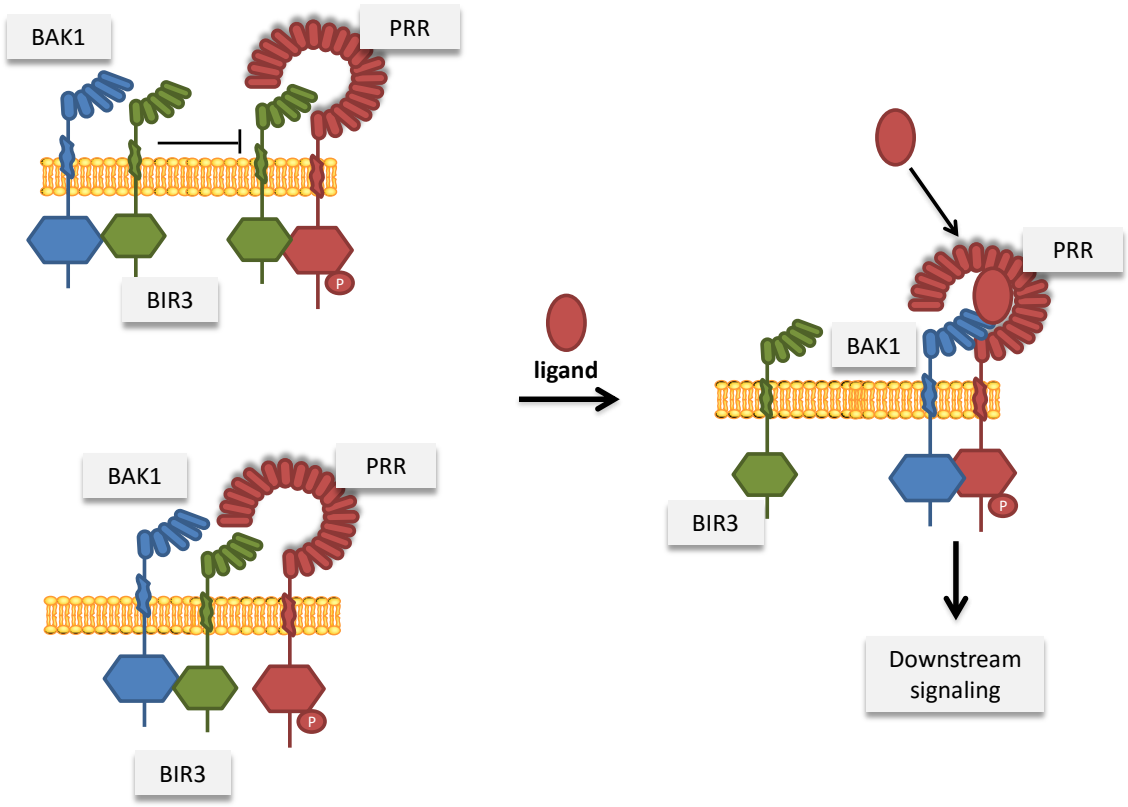

Figure 4-1: Model of receptor complexes in MAMP and BL signaling

4.2. Cell death regulation by BAK1 and BIR proteins

4.2.1. Cell death regulation by BIR2

BAK1 was described as a cell death regulator, because *bak1* mutants show spreading cell death after infection with microbial pathogens (Kemmerling et al., 2007). Moreover, double mutants of *bak1* and the closest homologue *bkk1* show spontaneous cell death and are seedling lethal (He et al., 2007). In later studies it was described that not only BAK1 mutants but also BAK1 overexpressing plants are defective in cell death regulation (Belkhadir et al., 2012; Dominguez-Ferreras et al., 2015). BAK1 overexpression leads to plants similar to lesion mimic mutants with spontaneous cell death. The cell death phenotype could be antagonized by simultaneous overexpression of BRI1. This indicates that the amount of BAK1 to its interaction partners needs to be proportional (Belkhadir et al., 2012). Another manuscript focused on BAK1 overexpressing plants and showed that inducible expression of BAK1 leads to activation of

defense responses and resistance against *Pto* DC3000 (Dominguez-Ferreras et al., 2015). These results indicate that cell death regulation by BAK1 is dosage dependent.

Similar phenotypes were described for BIR2. *bir2* knockouts show a weak autoimmunity phenotype with slightly smaller morphology and early senescence. They show spreading cell death after infection with necrotrophic fungi, even stronger as compared to *bak1* (Halter et al., 2014). Here, BIR2 overexpressing plants were analyzed and it could be shown that overexpression of BIR2 leads to loss of cell death containment, resulting in higher disease symptoms after *Alternaria brassicicola* infections. Therefore, BIR2 can also not be classified as a positive or negative regulator of cell death, but the relative amount of BIR2 protein is crucial for cell death containment after infections. In order to investigate the relationship between BIR2 and BAK1 in cell death regulation, double mutants were subjected to infection experiments with *A. brassicicola*. Double mutants showed disease symptoms comparable to both single mutants, no additive effect was observed. This indicates that both proteins cooperate on regulating cell death. The fact that knockout or overexpression of one complex component leads to cell death, suggests that the proper ratio of both proteins and complex integrity is needed. These results would fit to a guarding model, where an R-protein would sense the integrity of BAK1 BIR2 complexes and triggers cell death when disturbance of the guardee is detected. An example for such a mechanism is the R-protein RPS2 which guards RIN4. The *P. syringae* effector AvrRpt2 is a cysteine protease that cleaves RIN4 and RIN4 disappearance leads to activation of RPS2 and induction of defense responses (Axtell and Staskawicz, 2003; Mackey et al., 2003). Another example is the small LRR-RLK SRF3 that is structurally similar to BIR proteins and has been shown to be involved in autoimmunity cell death induced by the resistance protein RPP1. SRF3 exists in different variants in different Arabidopsis ecotypes. Some of these mutant versions are sensed by RPP1 from Ler, RPP1 gets activated and cell death occurs if the corresponding ecotypes are crossed. These crossings lead to autoimmunity cell death and hybrid incompatibility between these Arabidopsis ecotypes, phenotypes that are very similar to the observed *bir* mutant phenotypes (Alcazar et al., 2010).

In order to analyze if BIR2 is an object of guarding and to identify possible downstream components in cell death regulation by BIR2, double mutants with known cell death pathway components were generated. Expression of *nahG*, a salicylate hydroxylase, led to partial rescue of the *bir2-1* cell death phenotype. This indicates that cell death in *bir2-1* depends on SA. Mutations in *bir1* can also be partially rescued by knockout of the SA biosynthesis enzymes SID2 or EDS5, showing that cell death in both mutants relies on SA (Gao et al., 2009).

Cell death in *bir1* could also be partially rescued by *eds1*, *pad4* or *ndr1* (Gao et al., 2009). These proteins act downstream of R-proteins in ETI, so that a guarding model for BIR1

was proposed. However, mutation of *PAD4* does not rescue the *bir2* mutant phenotype, showing that BIR2 might not be guarded by a TIR-type NB-LRR protein. As complementation of *bir1* by the ETI mutants is only partial, the weaker cell-death phenotype in *bir2* mutants might not allow detection of quantitative effects of these mutants on *bir2*-mediated cell death responses. Time limits prevented the analysis of the role of BIR2 in other known R-protein pathways. Further analysis if *bir2-1* can be rescued by *ndr1-1* or other known cell death pathways should be performed in the future to analyze if BIR2 is involved in other autoimmune pathways.

bir1-1 and *bon1-1*, knockouts of BAK1 interactors that lead to cell death, can both be specifically rescued by the TIR-type NB-LRR protein SNC1 (Wang et al., 2011). BIR2 cell death is not dependent on SNC1, as crosses with the SNC1 knockout allele *snc1-11* showed. This was already assumed before, because BIR2 and also BAK1 knockouts in Ws-0 background, an ecotype that does not contain SNC1, also show a cell death phenotype (Halter et al., 2014).

Cell death induction by BAK1 or BIR proteins does not necessarily need guarding. BAK1 could also directly activate components of a cell death pathway by phosphorylation. BAK1 is a constitutively active kinase that could phosphorylate a downstream component of the cell death pathway and (in)activate it. If the phosphorylation activity is altered, e.g. by mutation or overexpression of BAK1 or by mutation of the BAK1 target BIR2, this could activate this cell death executioner. In plants different cell death pathways that control development and immune responses exist. It was recently suggested that HR-like cell death is induced by two different pathways, autophagy and the metacaspases *At*MC1 (Coll et al., 2014) which might be further candidates to explain BIR2 mediated cell death control.

4.2.2. Cell death regulation by BIR3

Functional analysis of BIR3 first revealed that BIR3 does not play a role in cell death regulation, as *bir3* mutants do not show spontaneous cell death or defects in cell death containment after pathogen infection. However, BIR3 overexpressors show stronger symptom development and cell death after *A. brassicicola* infection, indicating that BIR3 has a role in cell death regulation (Halter, 2014). One problem of analyzing cell death with BIR3 overexpressors could be the dwarf phenotype of these plants. The small leaves could be more susceptible to infections because the spore droplet for inoculation is already quite huge compared to the whole leave size. However, *bir2-1* 35S-BIR3 plants show an even higher disease index as 35S-BIR3 although the leaves are a bit bigger. This result shows that the altered genotype and not the leaf size causes cell death. Moreover, the dwarf 35S-BIR3 phenotype is caused by BL insensitivity and it

could be shown that BL insensitivity and the resulting morphological alterations have no impact on cell death after *A. brassicicola* infections (Kemmerling et al., 2007).

The result that BIR3 is involved in cell death becomes more obvious in double mutants with *bak1*. Both single mutants, *bak1* and *bir3*, do not show a severe morphological phenotype. Yet the double mutant shows a surprisingly severe growth phenotype with spontaneous lesion formation. Cell death spreading after *A. brassicicola* infections is strongly enhanced as chlorosis is spreading quickly throughout the whole leaves. Trypan blue staining of uninfected leaves and leaves at early time points after *A. brassicicola* infections, show the spontaneous and spreading cell death in these mutants. The cell death is accompanied by simultaneously elevated SA and JA levels in untreated plants. These double mutants show that BIR3 is indeed involved in cell death regulation, an effect that is hidden in the single mutants. This is similar to *bkk1* mutants that do not show cell death alterations as single mutants but strongly enhance *bak1* mediated cell death in double mutants (He et al., 2007). The strong phenotype of the *bak1 bir3* double mutant is due to the combination of *bak1* and *bir3* knockouts, as expression of either of the proteins can complement the mutant phenotype. These results indicate that both proteins cooperate on regulating cell death. It was tested if the strong growth phenotype of the double mutants is additionally caused by defects in BL sensitivity but this is not the case. Double mutants phenocopy *bak1-4* single mutants showing no additional effect on the BL pathway by the additional *bir3* mutation.

We observed that *bir3* mutants show reduced BAK1 protein levels. It is not yet clear what causes these reduced BAK1 levels, as no transcriptional repression is observed and the reduced protein amounts could not be rescued by inhibiting the 26S-proteasom with the inhibitor MG 132. Also endocytosis inhibitors such as Brefeldin A or Wortmannin, that would prevent faster internalization of BAK1 in the absence of BIR3, do not increase the BAK1 levels in *bir3* mutants more than in wt plants. The lower BAK1 amounts can still be caused by other endocytosis routes or protein degradation pathways. One phenomenon that could explain the reduced BAK1 levels in *bir3* is substrate stabilization. It was observed for other enzymes that they are stabilized by their substrate or that they are more stable in their active conformation (Villaume et al., 1990). As BIR proteins are a substrate for BAK1 kinase activity, binding of BIRs might bring BAK1 kinase domain into its active conformation and thus stabilize the protein. Absence of the substrate BIR3 in *bir3* knockouts could lead to destabilization of BAK1.

This protein stabilization model could help to explain the strong *bak1 bir3* mutant phenotype. Phenotypically *bak1 bir3* mutants resemble *bak1-3 bkk1* mutants (Albrecht et al., 2012). It was therefore hypothesized that BKK1 might be stabilized by BIR3 and destabilization of BKK1 would result in a mimic of a weak *bak1 bkk1* mutant. It could be shown that BKK1 can

indeed interact with BIR3 and that co-expression of both proteins in N. benthamiana leads to BKK1 stabilization. Unfortunately no BKK1 antibody exists to test the destabilization of BKK1 in *bir3* mutants. Moreover, we tested if *bir3-2 bkk1* mutants show the same phenotype but they do not show enhanced cell death compared to wildtype. However, *bkk1* has a weaker cell death phenotype than *bak1*, itself it has no effect in cell death and therefore reducing BKK1 might not be enough to resemble the *bak1 bir3* phenotype. Involvement of BKK1 in BIR3 dependent cell death should be tested by analyzing if BKK1 ectopic overexpression can rescue the *bak1 bir3* double mutant phenotype.

4.2.3. Cell death regulation by BIR1

BIR1 seems to be the strongest cell death regulator within the BIR family, as *bir1* mutants are very dwarf, show spontaneous cell death and are seedling lethal. Mutation of BIR2 or BIR3 do not enhance the cell death in *bir1*, thus no synergies in the lines can be detected. However, *bir1* mutants could already show the maximum amount of cell death, so that additional knockout of a partially redundant protein would not enhance it. Overexpression of BIR3 partially rescues the *bir1* mutant phenotype indicating that BIR3 can take over at least some functions of BIR1. The fact that *bir2* shows no suppression by SNC1 or other TIR-type NB-LRR proteins points to different cell death pathways induced by *bir1* and *bir2* . A different molecular function of *bir2* and 3 compared to *bir1* is supported by the fact that BIR2 is kinase inactive but BIR1 is an active kinase and kinase activity is partially needed for cell death regulation (Gao et al., 2009).

Double mutants of *bir1* and *bak1* surprisingly led to a partial rescue of the *bir1* mutant phenotype. If BAK1 BIR1 complexes would be guarded and cell death would be activated by sensing imbalances of the protein complex, it would make sense that the double mutant, where the complex is completely missing shows less cell death compared to *bir1* single mutants. The behavior of the *bir1 bak1* single mutant is interestingly opposite to that of *bak1 bir3* double mutants. In case of *bir1*, knockout of *bak1* rescues the phenotype, in case of *bir3* it enhances the phenotype suggesting two different underlying mechanisms. In *bir3* mutants destabilization of SERKs might mimicking *serk* double mutants in addition to a potential guarding of the BAK1/BIR3 complex.

4.2.4. Model of BIR proteins in cell death control

In the cell death pathway different BIR proteins and BAK1 cooperate to regulate cell death containment (Figure 4-2). Different overexpressing lines, single and double mutants of BAK1 and BIR proteins have shown that the correct amount and relative abundance of these proteins is important for cell death control. This leads to a model that complex integrity between different BIR proteins, BAK1 and probably also BKK1 is sensed by the plant. Alterations in the receptor complexes could activate a guard protein that in turn activates via so far unknown downstream components a cell death reaction.

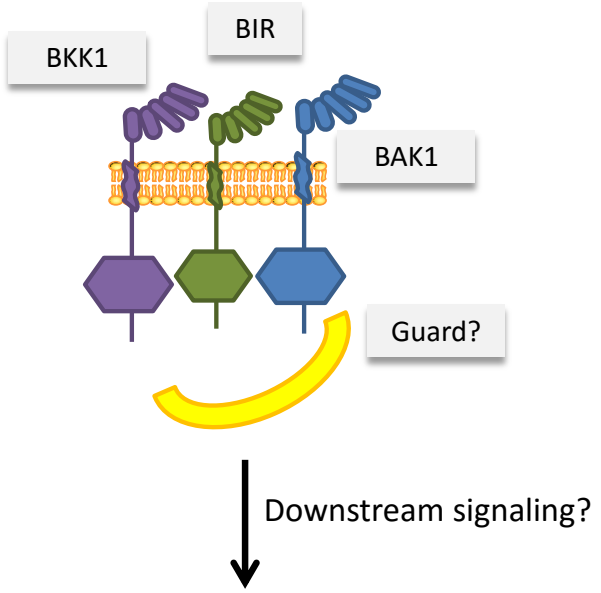

Figure 4-2: Model of receptor complexes in cell death control

4.3. Relationship of different BIR proteins

BIR2 and BIR3 are two closely related RLKs and both constitutively interact with BAK1. However, analysis of both proteins soon showed that they are not completely redundant. Microarray analysis showed that BIR1 and BIR2 are upregulated after treatment with avirulent or non-pathogenic *Pseudomonas syringae* bacteria. In contrast, BIR3 is downregulated at late time points after *Pseudomonas* infection (Halter et al., 2014). This data gave a first hint that there might be differences between BIR2 and BIR3. BIR1 is an active kinase and its kinase activity is at least partially necessary for its functions (Gao et al., 2009). In contrast to that, BIR2 and BIR3 are both kinase inactive. BIR2 is constitutively phosphorylated by BAK1 but BIR3 shows only very weak phosphorylation by BAK1 (Mazzotta, 2012). Among these three BIR

proteins, BIR1 showed the weakest interaction, BIR2 interacted stronger, and BIR3 showed the strongest interaction with BAK1 in FRET-FLIM assays (Halter et al., 2014). BIR1 is the evolutionary oldest BIR protein; duplication events evolved BIR2 and then BIR3 and BIR4. The examples of interaction strength and kinase activity indicate a gradual change in the function of BIR proteins during evolution. The fact that BIR1 has no MAMP or BL phenotype indicates that the other BIR proteins evolved completely new functions.

The BIR family analysis in this work focused on the relationship between BIR2 and BIR3. First *bir2 bir3* double mutants were used for the functional analysis. However, these double mutants behaved in all assays performed as *bir2* single mutants and it could only be concluded that mutation of *bir3* does not enhance or rescue the *bir2* mutant phenotype.

Moreover, *bir2* mutants were crossed with BIR3 overexpressors. Overexpression of BIR3 could not rescue the *bir2* mutant phenotype back to wildtype levels in morphology, MAMP responses or cell death phenotype. This indicating that lack of BIR2 cannot be replaced by overexpression of BIR3 and that both proteins are not completely redundant. Nevertheless a more detailed view is necessary to understand the functions of both proteins. In case of the morphology, the slight *bir2* phenotype is most likely caused by cell death as it can be complemented by *nahG* expression and thus the phenotype could be caused by enhanced SA dependent defense responses. The dwarf 35S-BIR3 phenotype is caused by BL insensitivity and is slightly reduced in the bir2-1 35S-BIR3 plants. Thus BIR2 probably also has a role in BL signaling, which is masked in the single mutant. This fits to the observation that BAK1 is released from BIR2 after BL treatment (Halter et al., 2014). In the performed ROS assays, the overexpression of BIR3 in the *bir2* mutant background showed ROS production a little bit lower than *bir2-1*. This indicates that BIR3 overexpression can partially complement the *bir2* hypersensitive phenotype. This can be caused by the fact that BIR2 and BIR3 are not fully redundant and that the proteins cannot fully replaced by each other, but also by expression levels or functionality of the transgene. It seems that both proteins are able to execute the same function in regulating MAMP responses and use the same molecular mechanisms. BIR2 and BIR3 are partially redundant with BIR2 complementing the BIR3 function in the wildtype or *bir3* mutant situation, the BIR3 function becomes only visible in the overexpression situation. In case of cell death control, both single mutants, *bir2-1* and 35S-BIR3, show enhanced cell death compared to Col-0. Again, the cell death phenotype of *bir2-1* is stronger compared to 35S-BIR3. The double mutant shows cell death comparable to *bir2-1*. Therefore both proteins could act in the same cell death pathway or there are no additive effects because *bir2-1* already shows the maximum amount of cell death. Another layer of complexity was introduced when it was shown that BIR2 and BIR3 can interact with each other (Halter, 2014). This data

indicates that both proteins really act together in one pathway. As the double knockout resembles *bir2-1*, BIR2 would be downstream and regulated by BIR3. The interaction could so far only be shown in overexpression systems in *N. benthamiana*. However, both proteins are expressed in all plant tissues what makes an interaction also possible in Arabidopsis. Further studies are needed to understand how BIR proteins cooperate on regulating different plant signaling pathways.

SERK proteins are also a family of structurally redundant proteins that act in different pathways. SERK proteins are only partially redundant, their functions overlap but each SERK protein performs a specific subset of signaling roles (Aan den Toorn et al., 2015). A similar model could be true for BIR proteins. BIR proteins have distinct and also overlapping function, e.g. BIR1 is the strongest cell death regulator and BIR3 overexpressors show a significant BL phenotype. Within the BIR family cell death regulatory activity decreases while BAK1 interaction and effects on BAK1 complexes increase from BIR1 to BIR3. This suggests that new functions have been developed during evolution within the BIR family and ancient and new function create partially redundant and specific function of the individual BIR proteins.

SERKs are ancient genes and it was suggested that they have been recruited as co-receptors to newly evolved signaling pathways. BIR1 homologues even reach back to *Physcomitrella patens*. Further analysis is needed to show what the ancient function of BIR proteins was and how they evolved together with SERKs.

BIR proteins are signaling components shared between different signaling pathways. Therefore, the question arises how signaling specificity is achieved. For BAK1, a model of preformed complexes was proposed (Bücherl et al., 2013) . BIR proteins fit into this model, because complexes of BAK1 with BRI1 could preferentially contain BIR3 and complexes with FLS2 or EFR could preferentially contain BIR2. Another question is, if BIR proteins are a point of crosstalk between different signaling pathways. However, some studies analyzing the role of BAK1 in crosstalk between BL and MAMP responses, suggest that the crosstalk takes place downstream of the receptor level (Lozano-Duran and Zipfel, 2015).

BIR proteins were identified as BAK1 interactors and we so far focused on the regulation of BAK1 by BIR proteins. However, BIR proteins could have additional BAK1 independent roles. BIR3 interacts with different ligand-binding receptors and could interact with receptors that do not use BAK1 as a co-receptor. Candidates would be CERK1 or LYK5, because chitin signaling is BAK1 independent.

Taken together, it was shown that BIR proteins are a family of closely related LRR-RLKs that differentially regulate BAK1 dependent processes. The BIR proteins are partially redundant and fulfill partially overlapping functions. However, there are gradual differences in

the importance of BIR proteins in the different pathways and each individual BIR proteins has evolved a specific function (Figure 4-3).

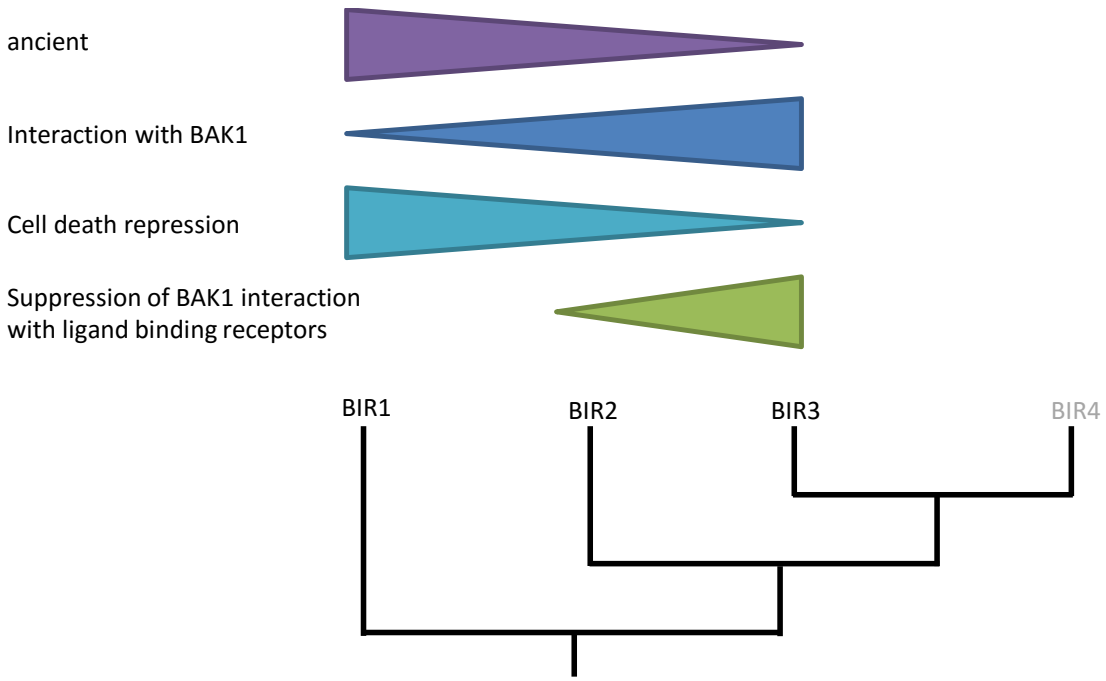

Figure 4-3: Gradual differences in the function of BIR family proteins in different pathways

5. Summary

BAK1 is a multifunctional co-receptor that positively regulates multiple plant signaling pathways by interacting with the corresponding ligand-binding receptors. It plays a role in BL signaling by interacting with BRI1, in MAMP signaling by interacting with FLS2 or EFR and in DAMP signaling by interacting with PEPR1 and PEPR2. Moreover, BAK1 plays a role in cell death control. In order to investigate how BAK1 is regulated we purified in vivo BAK1 complexes and identified two new BAK1 interactors, the BAK1 interacting RLKs 2 and 3 (BIR2 and BIR3). Functional analysis of *bir2* mutants showed that they are hyperresponsive to MAMP treatment, show cell death spreading after pathogen infection but are not impaired in BL signaling.

In this study the molecular mechanism how BIR2 regulates MAMP responses was further investigated. With Co-IP analysis in Arabidopsis wildtype plants it was shown that treatment with flg22 leads to a partial release of BAK1 from BIR2. Treatment with a cocktail of flg22, elf18, BL and *At*pep1 leads to increased release of BAK1 from BIR2 compared to single treatments. This indicates that BAK1 exists in preformed complexes with different ligand-binding receptors and BIR2 prevents interaction of BAK1 with ligand-binding receptors in the absence of the ligand.

Functional analyses of 35S-BIR3 plants have shown that BIR3 is a negative regulator of BL responses, MAMP responses and cell death. In this study the role of BIR3 in BL responses was further investigated and it could be shown that BIR3 not only interacts with BAK1 but also shows direct interaction with the ligand-binding receptor BRI1. The involvement of BIR3 in MAMP responses was confirmed with different functional assays. On the molecular level it was shown that BIR3 interacts with BAK1 and FLS2 and regulates BAK1-FLS2 complex formation. *bak1 bir3* double mutants show a strong growth phenotype with spontaneous lesion formation and strong cell death spreading after *A. brassicicola* infections. Analysis of different *bak1 bir* double mutants and overexpressors has shown that the right amount of BAK1 and BIR proteins or their relative abundance and thus complex integrity seems to be important for cell death control.

To investigate the relationship of the closely related proteins BIR2 and BIR3, *bir2-1* 35S-BIR3 plants were used. It was shown that BIR2 and BIR3 are partially redundant but BIR2 masks the BIR3 function in the wildtype situation and thus seems to have a stronger influence on the MAPMP and cell death pathway.

6. Zusammenfassung

BAK1 ist ein multifunktionaler Korezeptor der mehrere pflanzliche Signalwege positiv reguliert indem er mit den jeweiligen Liganden-bindenden Rezeptoren interagiert. BAK1 spielt eine Rolle im BL Signalweg indem es mit BRI1 interagiert, im MAMP Signalweg durch Interaktion mit FLS2 und EFR und im DAMP Signalweg durch Interaktion mit PEPR1 und PEPR2. Außerdem spielt BAK1 eine Rolle in der Zelltodkontrolle. Um zu untersuchen wie BAK1 reguliert wird haben wir in vivo BAK1 Komplexe aufgereinigt und konnten zwei neue BAK1 Interaktoren identifizieren, die BAK1 interagierenden RLKs 2 und 3 (BIR2 und BIR3). Funktionelle Analysen mit *bir2* Mutanten haben gezeigt, dass sie hyperresponsiv gegenüber MAMPs sind, Zelltodausbreitung nach Pathogeninfektion zeigen aber nicht in im BL Signalweg beeinträchtigt sind.

In dieser Arbeit wurde der molekulare Mechanismus wie BIR2 MAMP Antworten reguliert weiter untersucht. Mithilfe von Co-IP Analysen in Arabidopsis wildtyp Pflanzen konnte gezeigt werden, dass Behandlung mit flg22 zu einer partiellen Freisetzung von BAK1 von BIR2 führt. Behandlung mit einer Mischung aus flg22, elf18, BL und *At*pep1 führt zu einer verstärkten Freisetzung von BAK1 von BIR2 gegenüber der Einzelbehandlung. Dies weist darauf hin, dass BAK1 in vorgeformten Komplexen mit verschiedenen Liganden-bindenden Rezeptoren vorliegt. BIR2 verhindert die Interaktion von BAK1 mit Liganden-bindenden Rezeptoren in Abwesenheit des Liganden.

Funktionelle Analyse von 35S-BIR3 Pflanzen hat gezeigt, dass BIR3 ein negativer Regulator von BL Antworten, MAMP Antworten und Zelltod ist. In dieser Arbeit wurde die Rolle von BIR3 in BL Antworten weiter untersucht. Es konnte gezeigt werden, dass BIR3 nicht nur mit BAK1 interagiert sondern auch direkt mit dem Rezeptor BRI1. Die Beteiligung von BIR3 in MAMP Antworten konnte mit verschiedenen funktionellen Analysen bestätigt werden. Auf molekularer Ebene konnte gezeigt werden, dass BIR3 mit BAK1 und FLS2 interagiert und die Interaktion von BAK1 mit FLS2 reguliert. *bak1 bir3* Doppelmutanten zeigen einen starken Wachstumsphänotypen mit spontanen Zelltod Läsionen und starker Zelltod Ausbreitung nach *A. brassicicola* Infektionen. Untersuchung verschiedener *bak1 bir* Dopplemutanten und Überexpriemierer hat gezeigt, dass die richtige Menge von BAK1 und BIR Proteinen oder ihre relative Häufigkeit und somit Komplexintegrität wichtig für die Zelltodkontrolle ist.

Um das Verhältnis der eng verwandten Proteine BIR2 und BIR3 zu untersuchen, wurden *bir2-1* 35S-BIR3 Pflanzen genutzt. Es wurde gezeigt, dass BIR2 und BIR3 partiell redundant sind aber BIR2 in der wildtyp Situation die Funktion von BIR3 überdeckt und somit einen stärkeren Einfluss auf den MAMP und Zelltod Signalweg zu haben scheint.

7. References

Aan den Toorn, M., Albrecht, C., and de Vries, S. (2015). On the Origin of SERKs: Bioinformatics analysis of the Somatic Embryogenesis Receptor Kinases. Molecular plant **8,** 762-782.

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J., and Parker, J.E. (1998). Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. **95,** 10306-10311.

Adrain, C., and Martin, S.J. (2001). The mitochondrial apoptosome: a killer unleashed by the cytochrome seas. Trends Biochem. Sci. **26,** 390-397.

Albrecht, C., Russinova, E., Kemmerling, B., Kwaaitaal, M., and de Vries, S.C. (2008). Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE proteins serve brassinosteroid-dependent and -independent signaling pathways. Plant Physiol. **148,** 611-619.

Albrecht, C., Boutrot, F., Segonzac, C., Schwessinger, B., Gimenez-Ibanez, S., Chinchilla, D., Rathjen, J.P., de Vries, S.C., and Zipfel, C. (2012). Brassinosteroids inhibit pathogen-associated molecular pattern-triggered immune signaling independent of the receptor kinase BAK1. Proc. Natl. Acad. Sci. U. S. A. **109,** 303-308.

Alcazar, R., Garcia, A.V., Kronholm, I., de Meaux, J., Koornneef, M., Parker, J.E., and Reymond, M. (2010). Natural variation at Strubbelig Receptor Kinase 3 drives immune-triggered incompatibilities between Arabidopsis thaliana accessions. Nat. Genet. **42,** 1135-1139.

Alvarez, M.E. (2000). Salicylic acid in the machinery of hypersensitive cell death and disease resistance. Plant Mol. Biol. **44,** 429-442.

Andreasson, E., Jenkins, T., Brodersen, P., Thorgrimsen, S., Petersen, N.H., Zhu, S., Qiu, J.L., Micheelsen, P., Rocher, A., Petersen, M., Newman, M.A., Bjorn Nielsen, H., Hirt, H., Somssich, I., Mattsson, O., and Mundy, J. (2005). The MAP kinase substrate MKS1 is a regulator of plant defense responses. EMBO J. **24,** 2579-2589.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., and Sheen, J. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. Nature **415,** 977-983.

Axtell, M.J., and Staskawicz, B.J. (2003). Initiation of RPS2-specified disease resistance in Arabidopsis is coupled to the AvrRpt2-directed elimination of RIN4. Cell **112,** 369-377.

Bai, M.Y., Shang, J.X., Oh, E., Fan, M., Bai, Y., Zentella, R., Sun, T.P., and Wang, Z.Y. (2012). Brassinosteroid, gibberellin and phytochrome impinge on a common transcription module in Arabidopsis. Nat. Cell Biol. **14,** 810-817.

Balk, J., Leaver, C.J., and McCabe, P.F. (1999). Translocation of cytochrome c from the mitochondria to the cytosol occurs during heat-induced programmed cell death in cucumber plants. FEBS Lett. **463,** 151-154.

Baltrus, D.A., Nishimura, M.T., Romanchuk, A., Chang, J.H., Mukhtar, M.S., Cherkis, K., Roach, J., Grant, S.R., Jones, C.D., and Dangl, J.L. (2011). Dynamic evolution of pathogenicity revealed by sequencing and comparative genomics of 19 Pseudomonas syringae isolates. PLoS Pathog. **7,** e1002132.

Beck, M., Zhou, J., Faulkner, C., MacLean, D., and Robatzek, S. (2012). Spatio-temporal cellular dynamics of the Arabidopsis flagellin receptor reveal activation status-dependent endosomal sorting. Plant Cell **24,** 4205-4219.

Belkhadir, Y., Jaillais, Y., Epple, P., Balsemao-Pires, E., Dangl, J.L., and Chory, J. (2012). Brassinosteroids modulate the efficiency of plant immune responses to microbe-associated molecular patterns. Proc. Natl. Acad. Sci. U. S. A. **109,** 297-302.

Bender, K.W., Wang, X., Cheng, G.B., Kim, H.S., Zielinski, R.E., and Huber, S.C. (2015). Glutaredoxin AtGRXC2 catalyses inhibitory glutathionylation of Arabidopsis BRI1-associated receptor-like kinase 1 (BAK1) in vitro. Biochem. J. **467,** 399-413.

Blaum, B.S., Mazzotta, S., Noldeke, E.R., Halter, T., Madlung, J., Kemmerling, B., and Stehle, T. (2014). Structure of the pseudokinase domain of BIR2, a regulator of BAK1-mediated immune signaling in Arabidopsis. J. Struct. Biol. **186,** 112-121.

Böhm, H., Albert, I., Fan, L., Reinhard, A., and Nürnberger, T. (2014a). Immune receptor complexes at the plant cell surface. Curr. Opin. Plant Biol. **20,** 47-54.

Böhm, H., Albert, I., Oome, S., Raaymakers, T.M., Van den Ackerveken, G., and Nürnberger, T. (2014b). A conserved Peptide pattern from a widespread microbial virulence factor triggers pattern-induced immunity in Arabidopsis. PLoS Pathog. **10,** e1004491.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu. Rev. Plant Biol. **60,** 379-406.

Bomblies, K., and Weigel, D. (2007). Hybrid necrosis: autoimmunity as a potential gene-flow barrier in plant species. Nat Rev Genet **8,** 382-393.

Bomblies, K., Lempe, J., Epple, P., Warthmann, N., Lanz, C., Dangl, J.L., and Weigel, D. (2007). Autoimmune response as a mechanism for a Dobzhansky-Muller-type incompatibility syndrome in plants. PLoS Biol. **5,** e236.

Boudsocq, M., and Sheen, J. (2013). CDPKs in immune and stress signaling. Trends in plant science **18,** 30-40.

Bruggeman, Q., Raynaud, C., Benhamed, M., and Delarue, M. (2015). To die or not to die? Lessons from lesion mimic mutants. Front Plant Sci **6,** 24.

Bücherl, C.A., van Esse, G.W., Kruis, A., Luchtenberg, J., Westphal, A.H., Aker, J., van Hoek, A., Albrecht, C., Borst, J.W., and de Vries, S.C. (2013). Visualization of BRI1 and BAK1(SERK3) membrane receptor heterooligomers during brassinosteroid signaling. Plant Physiol. **162,** 1911-1925.

Cao, Y., Liang, Y., Tanaka, K., Nguyen, C.T., Jedrzejczak, R.P., Joachimiak, A., and Stacey, G. (2014). The kinase LYK5 is a major chitin receptor in Arabidopsis and forms a chitin-induced complex with related kinase CERK1. Elife **3**.

Century, K.S., Shapiro, A.D., Repetti, P.P., Dahlbeck, D., Holub, E., and Staskawicz, B.J. (1997). NDR1, a pathogen-induced component required for Arabidopsis disease resistance. Science **278,** 1963-1965.

Chang, I.F., Curran, A., Woolsey, R., Quilici, D., Cushman, J.C., Mittler, R., Harmon, A., and Harper, J.F. (2009). Proteomic profiling of tandem affinity purified 14-3-3 protein complexes in Arabidopsis thaliana. Proteomics **9,** 2967-2985.

Chen, C.W., Panzeri, D., Yeh, Y.H., Kadota, Y., Huang, P.Y., Tao, C.N., Roux, M., Chien, S.C., Chin, T.C., Chu, P.W., Zipfel, C., and Zimmerli, L. (2014). The Arabidopsis malectin-like leucine-rich repeat receptor-like kinase IOS1 associates with the pattern recognition receptors FLS2 and EFR and is critical for priming of pattern-triggered immunity. Plant Cell **26,** 3201-3219.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J.D., Felix, G., and Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature **448,** 497-500.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical biochemistry **162,** 156-159.

Choquer, M., Fournier, E., Kunz, C., Levis, C., Pradier, J.M., Simon, A., and Viaud, M. (2007). Botrytis cinerea virulence factors: new insights into a necrotrophic and polyphageous pathogen. FEMS Microbiol. Lett. **277,** 1-10.

Clouse, S.D., Langford, M., and McMorris, T.C. (1996). A brassinosteroid-insensitive mutant in Arabidopsis thaliana exhibits multiple defects in growth and development. Plant Physiol. **111,** 671-678.

Coll, N.S., Epple, P., and Dangl, J.L. (2011). Programmed cell death in the plant immune system. Cell Death Differ. **18,** 1247-1256.

Coll, N.S., Smidler, A., Puigvert, M., Popa, C., Valls, M., and Dangl, J.L. (2014). The plant metacaspase AtMC1 in pathogen-triggered programmed cell death and aging: functional linkage with autophagy. Cell Death Differ. **21,** 1399-1408.

Coll, N.S., Vercammen, D., Smidler, A., Clover, C., Van Breusegem, F., Dangl, J.L., and Epple, P. (2010). Arabidopsis type I metacaspases control cell death. Science **330,** 1393-1397.

Dangl, J.L., Horvath, D.M., and Staskawicz, B.J. (2013). Pivoting the plant immune system from dissection to deployment. Science **341,** 746-751.

Danon, A., Rotari, V.I., Gordon, A., Mailhac, N., and Gallois, P. (2004). Ultraviolet-C overexposure induces programmed cell death in Arabidopsis, which is mediated by caspase-like activities and which can be suppressed by caspase inhibitors, p35 and Defender against Apoptotic Death. J. Biol. Chem. **279,** 779-787.

Davidsson, P.R., Kariola, T., Niemi, O., and Palva, E.T. (2013). Pathogenicity of and plant immunity to soft rot pectobacteria. Front Plant Sci **4,** 191.

Dominguez-Ferreras, A., Kiss-Papp, M., Jehle, A.K., Felix, G., and Chinchilla, D. (2015). An Overdose of the Arabidopsis Coreceptor BRASSINOSTEROID INSENSITIVE1-ASSOCIATED RECEPTOR KINASE1 or Its Ectodomain Causes Autoimmunity in a SUPPRESSOR OF BIR1-1-Dependent Manner. Plant Physiol. **168,** 1106-1121.

Droillard, M.J., Boudsocq, M., Barbier-Brygoo, H., and Lauriere, C. (2004). Involvement of MPK4 in osmotic stress response pathways in cell suspensions and plantlets of Arabidopsis thaliana: activation by hypoosmolarity and negative role in hyperosmolarity tolerance. FEBS Lett. **574,** 42-48.

Durrant, W.E., and Dong, X. (2004). Systemic acquired resistance. Annu Rev Phytopathol **42,** 185-209.

Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. **19,** 1349.

Eigenbrode, S.D., Stoner, K.A., Shelton, A.M., and Kain, W.C. (1991). Characteristics of Glossy Leaf Waxes Associated with Resistance to Diamondback Moth (Lepidoptera, Plutellidae) in Brassica-Oleracea. J. Econ. Entomol. **84,** 1609-1618.

Ellinger, D., and Voigt, C.A. (2014). Callose biosynthesis in Arabidopsis with a focus on pathogen response: what we have learned within the last decade. Annals of botany **114,** 1349-1358.

Fabregas, N., Li, N., Boeren, S., Nash, T.E., Goshe, M.B., Clouse, S.D., de Vries, S., and Cano-Delgado, A.I. (2013). The brassinosteroid insensitive1-like3 signalosome complex regulates Arabidopsis root development. Plant Cell **25,** 3377-3388.

Fan, M., Bai, M.Y., Kim, J.G., Wang, T., Oh, E., Chen, L., Park, C.H., Son, S.H., Kim, S.K., Mudgett, M.B., and Wang, Z.Y. (2014). The bHLH transcription factor HBI1 mediates the trade-off between growth and pathogen-associated molecular pattern-triggered immunity in Arabidopsis. Plant Cell **26,** 828-841.

Felix, G., Regenass, M., and Boller, T. (1993). Specific Perception of Subnanomolar Concentrations of Chitin Fragments by Tomato Cells - Induction of Extracellular Alkalinization, Changes in Protein-Phosphorylation, and Establishment of a Refractory State. Plant J. **4,** 307-316.

Felix, G., Duran, J.D., Volko, S., and Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J. **18,** 265-276.

Flor, H.H. (1971). Current Status of Gene-for-Gene Concept. Annu Rev Phytopathol **9,** 275-+.

Fridman, Y., and Savaldi-Goldstein, S. (2013). Brassinosteroids in growth control: how, when and where. Plant Sci. **209,** 24-31.

Gampala, S.S., Kim, T.W., He, J.X., Tang, W., Deng, Z., Bai, M.Y., Guan, S., Lalonde, S., Sun, Y., Gendron, J.M., Chen, H., Shibagaki, N., Ferl, R.J., Ehrhardt, D., Chong, K., Burlingame, A.L., and Wang, Z.Y. (2007). An essential role for 14-3-3 proteins in brassinosteroid signal transduction in Arabidopsis. Dev. Cell **13,** 177-189.

Gao, M., Wang, X., Wang, D., Xu, F., Ding, X., Zhang, Z., Bi, D., Cheng, Y.T., Chen, S., Li, X., and Zhang, Y. (2009). Regulation of cell death and innate immunity by two receptor-like kinases in Arabidopsis. Cell host & microbe **6,** 34-44.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol **43,** 205-227.

Glazebrook, J., Rogers, E.E., and Ausubel, F.M. (1996). Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. Genetics **143,** 973-982.

Gomez-Gomez, L., and Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. Mol. Cell **5,** 1003-1011.

Gomez-Gomez, L., Bauer, Z., and Boller, T. (2001). Both the extracellular leucine-rich repeat domain and the kinase activity of FSL2 are required for flagellin binding and signaling in Arabidopsis. Plant Cell **13,** 1155-1163.

Gou, X., Yin, H., He, K., Du, J., Yi, J., Xu, S., Lin, H., Clouse, S.D., and Li, J. (2012). Genetic evidence for an indispensable role of somatic embryogenesis receptor kinases in brassinosteroid signaling. PLoS Genet **8,** e1002452.

Govrin, E.M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen Botrytis cinerea. Curr. Biol. **10,** 751-757.

Gupta, R., and Chakrabarty, S.K. (2013). Gibberellic acid in plant: still a mystery unresolved. Plant signaling & behavior **8**.

Gust, A.A., and Felix, G. (2014). Receptor like proteins associate with SOBIR1-type of adaptors to form bimolecular receptor kinases. Curr. Opin. Plant Biol. **21,** 104-111.

Halter, T. (2014). Modulation of multiple BAK1- dependent signaling pathways by two atypical receptor-like kinases Dissertation.

Halter, T., Imkampe, J., Mazzotta, S., Wierzba, M., Postel, S., Bücherl, C., Kiefer, C., Stahl, M., Chinchilla, D., Wang, X., Nürnberger, T., Zipfel, C., Clouse, S., Borst, J.W., Boeren, S., de Vries, S.C., Tax, F., and Kemmerling, B. (2014). The leucine-rich repeat receptor kinase BIR2 is a negative regulator of BAK1 in plant immunity. Curr. Biol. **24,** 134-143.

Hatsugai, N., Kuroyanagi, M., Yamada, K., Meshi, T., Tsuda, S., Kondo, M., Nishimura, M., and Hara-Nishimura, I. (2004). A plant vacuolar protease, VPE, mediates virus-induced hypersensitive cell death. Science **305,** 855-858.

He, J.X., Gendron, J.M., Sun, Y., Gampala, S.S., Gendron, N., Sun, C.Q., and Wang, Z.Y. (2005). BZR1 is a transcriptional repressor with dual roles in brassinosteroid homeostasis and growth responses. Science **307,** 1634-1638.

He, K., Gou, X., Yuan, T., Lin, H., Asami, T., Yoshida, S., Russell, S.D., and Li, J. (2007). BAK1 and BKK1 regulate brassinosteroid-dependent growth and brassinosteroid-independent cell-death pathways. Curr. Biol. **17,** 1109-1115.

Hecht, V., Vielle-Calzada, J.P., Hartog, M.V., Schmidt, E.D., Boutilier, K., Grossniklaus, U., and de Vries, S.C. (2001). The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol. **127,** 803-816.

Heese, A., Hann, D.R., Gimenez-Ibanez, S., Jones, A.M., He, K., Li, J., Schroeder, J.I., Peck, S.C., and Rathjen, J.P. (2007). The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. Proc. Natl. Acad. Sci. U. S. A. **104,** 12217-12222.

Hofius, D., Munch, D., Bressendorff, S., Mundy, J., and Petersen, M. (2011). Role of autophagy in disease resistance and hypersensitive response-associated cell death. Cell Death Differ. **18,** 1257-1262.

Hofius, D., Schultz-Larsen, T., Joensen, J., Tsitsigiannis, D.I., Petersen, N.H., Mattsson, O., Jorgensen, L.B., Jones, J.D., Mundy, J., and Petersen, M. (2009). Autophagic components contribute to hypersensitive cell death in Arabidopsis. Cell **137,** 773-783.

Hothorn, M., Belkhadir, Y., Dreux, M., Dabi, T., Noel, J.P., Wilson, I.A., and Chory, J. (2011). Structural basis of steroid hormone perception by the receptor kinase BRI1. Nature **474,** 467-471.

Hua, J., Grisafi, P., Cheng, S.H., and Fink, G.R. (2001). Plant growth homeostasis is controlled by the Arabidopsis BON1 and BAP1 genes. Genes Dev. **15,** 2263-2272.

Huffaker, A., Pearce, G., and Ryan, C.A. (2006). An endogenous peptide signal in Arabidopsis activates components of the innate immune response. Proc. Natl. Acad. Sci. U. S. A. **103,** 10098-10103.

Huot, B., Yao, J., Montgomery, B.L., and He, S.Y. (2014). Growth-defense tradeoffs in plants: a balancing act to optimize fitness. Molecular plant **7,** 1267-1287.

Igarashi, D., Tsuda, K., and Katagiri, F. (2012). The peptide growth factor, phytosulfokine, attenuates pattern-triggered immunity. Plant J. **71,** 194-204.

Jehle, A.K., Lipschis, M., Albert, M., Fallahzadeh-Mamaghani, V., Fürst, U., Mueller, K., and Felix, G. (2013). The receptor-like protein ReMAX of Arabidopsis detects the microbe-associated molecular pattern eMax from Xanthomonas. Plant Cell **25,** 2330-2340.

Jelenska, J., Yao, N., Vinatzer, B.A., Wright, C.M., Brodsky, J.L., and Greenberg, J.T. (2007). A J domain virulence effector of Pseudomonas syringae remodels host chloroplasts and suppresses defenses. Curr. Biol. **17,** 499-508.

Jiang, J., Zhang, C., and Wang, X. (2013). Ligand perception, activation, and early signaling of plant steroid receptor brassinosteroid insensitive 1. J. Integr. Plant Biol. **55,** 1198-1211.

Jiang, J., Zhang, C., and Wang, X. (2015). A recently evolved isoform of the transcription factor BES1 promotes brassinosteroid signaling and development in Arabidopsis thaliana. Plant Cell **27,** 361-374.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. Nature **444,** 323-329.

Kadota, Y., Sklenar, J., Derbyshire, P., Stransfeld, L., Asai, S., Ntoukakis, V., Jones, J.D., Shirasu, K., Menke, F., Jones, A., and Zipfel, C. (2014). Direct regulation of the NADPH oxidase RBOHD by the PRR-associated kinase BIK1 during plant immunity. Mol. Cell **54,** 43-55.

Kauschmann, A., Jessop, A., Koncz, C., Szekeres, M., Willmitzer, L., and Altmann, T. (1996). Genetic evidence for an essential role of brassinosteroids in plant development. The Plant Journal **9,** 701-713.

Kemmerling, B., Schwedt, A., Rodriguez, P., Mazzotta, S., Frank, M., Qamar, S.A., Mengiste, T., Betsuyaku, S., Parker, J.E., Müssig, C., Thomma, B.P., Albrecht, C., de Vries, S.C., Hirt, H., and Nürnberger, T. (2007). The BRI1-associated kinase 1, BAK1, has a brassinolide-independent role in plant cell-death control. Curr. Biol. **17,** 1116-1122.

Kim, H.S., Desveaux, D., Singer, A.U., Patel, P., Sondek, J., and Dangl, J.L. (2005). The Pseudomonas syringae effector AvrRpt2 cleaves its C-terminally acylated target, RIN4, from Arabidopsis membranes to block RPM1 activation. Proc. Natl. Acad. Sci. U. S. A. **102,** 6496-6501.

Kim, S.H., Gao, F., Bhattacharjee, S., Adiasor, J.A., Nam, J.C., and Gassmann, W. (2010). The Arabidopsis resistance-like gene SNC1 is activated by mutations in SRFR1 and contributes to resistance to the bacterial effector AvrRps4. PLoS Pathog. **6,** e1001172.

Kim, T.W., Guan, S., Sun, Y., Deng, Z., Tang, W., Shang, J.X., Sun, Y., Burlingame, A.L., and Wang, Z.Y. (2009). Brassinosteroid signal transduction from cell-surface receptor kinases to nuclear transcription factors. Nat. Cell Biol. **11,** 1254-1260.

Kinoshita, T., Cano-Delgado, A., Seto, H., Hiranuma, S., Fujioka, S., Yoshida, S., and Chory, J. (2005). Binding of brassinosteroids to the extracellular domain of plant receptor kinase BRI1. Nature **433,** 167-171.

Krol, E., Mentzel, T., Chinchilla, D., Boller, T., Felix, G., Kemmerling, B., Postel, S., Arents, M., Jeworutzki, E., Al-Rasheid, K.A., Becker, D., and Hedrich, R. (2010). Perception of the Arabidopsis danger signal peptide 1 involves the pattern recognition receptor AtPEPR1 and its close homologue AtPEPR2. J. Biol. Chem. **285,** 13471-13479.

Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T., and Felix, G. (2004). The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. Plant Cell **16,** 3496-3507.

Ladwig, F., Dahlke, R.I., Stuhrwohldt, N., Hartmann, J., Harter, K., and Sauter, M. (2015). Phytosulfokine Regulates Growth in Arabidopsis through a Response Module at the Plasma Membrane That Includes CYCLIC NUCLEOTIDE-GATED CHANNEL17, H+-ATPase, and BAK1. Plant Cell **27,** 1718-1729.

Lafon-Placette, C., and Kohler, C. (2015). Epigenetic mechanisms of postzygotic reproductive isolation in plants. Curr. Opin. Plant Biol. **23,** 39-44.

Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J. (1995). Systemic acquired resistance in Arabidopsis requires salicylic acid but not ethylene. Mol. Plant. Microbe Interact. **8,** 863-870.

Lenz, H.D., Haller, E., Melzer, E., Kober, K., Wurster, K., Stahl, M., Bassham, D.C., Vierstra, R.D., Parker, J.E., Bautor, J., Molina, A., Escudero, V., Shindo, T., van der Hoorn, R.A., Gust, A.A., and Nürnberger, T. (2011). Autophagy differentially controls plant basal immunity to biotrophic and necrotrophic pathogens. Plant J. **66,** 818-830.

Li, J. (2010). Multi-tasking of somatic embryogenesis receptor-like protein kinases. Curr. Opin. Plant Biol. **13,** 509-514.

Li, J., and Chory, J. (1997). A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell **90,** 929-938.

Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E., and Walker, J.C. (2002). BAK1, an Arabidopsis LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. Cell **110,** 213-222.

Li, L., Li, M., Yu, L., Zhou, Z., Liang, X., Liu, Z., Cai, G., Gao, L., Zhang, X., Wang, Y., Chen, S., and Zhou, J.M. (2014). The FLS2-associated kinase BIK1 directly phosphorylates the NADPH oxidase RbohD to control plant immunity. Cell host & microbe **15,** 329-338.

Lin, W., Lu, D., Gao, X., Jiang, S., Ma, X., Wang, Z., Mengiste, T., He, P., and Shan, L. (2013). Inverse modulation of plant immune and brassinosteroid signaling pathways by the receptor-like cytoplasmic kinase BIK1. Proc. Natl. Acad. Sci. U. S. A. **110,** 12114-12119.

Liu, Y., Schiff, M., Czymmek, K., Talloczy, Z., Levine, B., and Dinesh-Kumar, S.P. (2005). Autophagy regulates programmed cell death during the plant innate immune response. Cell **121,** 567-577.

Liu, Z., Wu, Y., Yang, F., Zhang, Y., Chen, S., Xie, Q., Tian, X., and Zhou, J.M. (2013). BIK1 interacts with PEPRs to mediate ethylene-induced immunity. Proc. Natl. Acad. Sci. U. S. A. **110,** 6205-6210.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25,** 402-408.

Loake, G., and Grant, M. (2007). Salicylic acid in plant defence--the players and protagonists. Curr. Opin. Plant Biol. **10,** 466-472.

Lockshin, R.A., and Williams, C.M. (1964). Programmed cell death—II. Endocrine potentiation of the breakdown of the intersegmental muscles of silkmoths. J. Insect Physiol. **10,** 643-649.

Lockshin, R.A., and Zakeri, Z. (2004). Apoptosis, autophagy, and more. Int. J. Biochem. Cell Biol. **36,** 2405-2419.

Lorrain, S., Vailleau, F., Balague, C., and Roby, D. (2003). Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants? Trends in plant science **8,** 263-271.

Lozano-Duran, R., and Zipfel, C. (2015). Trade-off between growth and immunity: role of brassinosteroids. Trends in plant science **20,** 12-19.

Lozano-Duran, R., Macho, A.P., Boutrot, F., Segonzac, C., Somssich, I.E., and Zipfel, C. (2013). The transcriptional regulator BZR1 mediates trade-off between plant innate immunity and growth. Elife **2,** e00983.

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L., and He, P. (2010). A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. Proc. Natl. Acad. Sci. U. S. A. **107,** 496-501.

Lu, D., Lin, W., Gao, X., Wu, S., Cheng, C., Avila, J., Heese, A., Devarenne, T.P., He, P., and Shan, L. (2011). Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity. Science **332,** 1439-1442.

Mackey, D., Holt, B.F., 3rd, Wiig, A., and Dangl, J.L. (2002). RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. Cell **108,** 743-754.

Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R., and Dangl, J.L. (2003). Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. Cell **112,** 379-389.

Malinovsky, F.G., Fangel, J.U., and Willats, W.G.T. (2014a). The role of the cell wall in plant immunity. Front Plant Sci **5**.

Malinovsky, F.G., Batoux, M., Schwessinger, B., Youn, J.H., Stransfeld, L., Win, J., Kim, S.K., and Zipfel, C. (2014b). Antagonistic regulation of growth and immunity by the Arabidopsis basic helix-loop-helix transcription factor homolog of brassinosteroid enhanced expression2 interacting with increased leaf inclination1 binding bHLH1. Plant Physiol. **164,** 1443-1455.

Matsubayashi, Y., and Sakagami, Y. (1996). Phytosulfokine, sulfated peptides that induce the proliferation of single mesophyll cells of Asparagus officinalis L. Proc. Natl. Acad. Sci. U. S. A. **93,** 7623-7627.

Matzinger, P. (2002). The danger model: a renewed sense of self. Science **296,** 301-305.

Mazzotta, S. (2012). Charakterisierung der Interaktion von BAK1 mit BIP89, einer Pathogen responsiven Rezeptor ähnlichen Kinase mit leuzinreichen Wiederholungen aus *Arabidopsis thaliana*. Dissertation.

McConn, M., Creelman, R.A., Bell, E., Mullet, J.E., and Browse, J. (1997). Jasmonate is essential for insect defense in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. **94,** 5473-5477.

Medzhitov, R., and Janeway, C.A., Jr. (1997). Innate immunity: the virtues of a nonclonal system of recognition. Cell **91,** 295-298.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., and Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. **104,** 19613-19618.

Moeder, W., and Yoshioka, K. (2008). Lesion mimic mutants: A classical, yet still fundamental approach to study programmed cell death. Plant signaling & behavior **3,** 764-767.

Monaghan, J., Matschi, S., Shorinola, O., Rovenich, H., Matei, A., Segonzac, C., Malinovsky, F.G., Rathjen, J.P., MacLean, D., Romeis, T., and Zipfel, C. (2014). The calcium-dependent protein kinase CPK28 buffers plant immunity and regulates BIK1 turnover. Cell host & microbe **16,** 605-615.

Mora-Garcia, S., Vert, G., Yin, Y., Cano-Delgado, A., Cheong, H., and Chory, J. (2004). Nuclear protein phosphatases with Kelch-repeat domains modulate the response to brassinosteroids in Arabidopsis. Genes Dev. **18,** 448-460.

Morel, J.B., and Dangl, J.L. (1997). The hypersensitive response and the induction of cell death in plants. Cell Death Differ. **4,** 671-683.

Mosher, S., Seybold, H., Rodriguez, P., Stahl, M., Davies, K.A., Dayaratne, S., Morillo, S.A., Wierzba, M., Favery, B., Keller, H., Tax, F.E., and Kemmerling, B. (2013). The tyrosine-sulfated peptide receptors PSKR1 and PSY1R modify the immunity of Arabidopsis to

biotrophic and necrotrophic pathogens in an antagonistic manner. Plant J. **73,** 469-482.

Mukhtar, M.S., Carvunis, A.R., Dreze, M., Epple, P., Steinbrenner, J., Moore, J., Tasan, M., Galli, M., Hao, T., Nishimura, M.T., Pevzner, S.J., Donovan, S.E., Ghamsari, L., Santhanam, B., Romero, V., Poulin, M.M., Gebreab, F., Gutierrez, B.J., Tam, S., Monachello, D., Boxem, M., Harbort, C.J., McDonald, N., Gai, L., Chen, H., He, Y., European Union Effectoromics, C., Vandenhaute, J., Roth, F.P., Hill, D.E., Ecker, J.R., Vidal, M., Beynon, J., Braun, P., and Dangl, J.L. (2011). Independently evolved virulence effectors converge onto hubs in a plant immune system network. Science **333,** 596-601.

Mur, L.A., Kenton, P., Atzorn, R., Miersch, O., and Wasternack, C. (2006). The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. Plant Physiol. **140,** 249-262.

Nam, K.H., and Li, J. (2002). BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. Cell **110,** 203-212.

Navarro, L., Bari, R., Achard, P., Lison, P., Nemri, A., Harberd, N.P., and Jones, J.D. (2008). DELLAs control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. Curr. Biol. **18,** 650-655.

Nomura, K., Debroy, S., Lee, Y.H., Pumplin, N., Jones, J., and He, S.Y. (2006). A bacterial virulence protein suppresses host innate immunity to cause plant disease. Science **313,** 220-223.

O'Brien, J.A., Daudi, A., Butt, V.S., and Bolwell, G.P. (2012). Reactive oxygen species and their role in plant defence and cell wall metabolism. Planta **236,** 765-779.

Osbourn, A.E. (1996). Preformed antimicrobial compounds and plant defense against fungal attack. Plant Cell **8,** 1821-1831.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell **10,** 2103-2113.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B., and Mundy, J. (2000). Arabidopsis map kinase 4 negatively regulates systemic acquired resistance. Cell **103,** 1111-1120.

Petutschnig, E.K., Jones, A.M., Serazetdinova, L., Lipka, U., and Lipka, V. (2010). The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in Arabidopsis thaliana and subject to chitin-induced phosphorylation. J. Biol. Chem. **285,** 28902-28911.

Pieterse, C.M., Leon-Reyes, A., Van der Ent, S., and Van Wees, S.C. (2009). Networking by small-molecule hormones in plant immunity. Nat. Chem. Biol. **5,** 308-316.

Poloni, A., and Schirawski, J. (2014). Red card for pathogens: phytoalexins in sorghum and maize. Molecules **19,** 9114-9133.

Postel, S., Küfner, I., Beuter, C., Mazzotta, S., Schwedt, A., Borlotti, A., Halter, T., Kemmerling, B., and Nürnberger, T. (2010). The multifunctional leucine-rich repeat receptor kinase BAK1 is implicated in Arabidopsis development and immunity. Eur. J. Cell Biol. **89,** 169-174.

Qiu, J.L., Fiil, B.K., Petersen, K., Nielsen, H.B., Botanga, C.J., Thorgrimsen, S., Palma, K., Suarez-Rodriguez, M.C., Sandbech-Clausen, S., Lichota, J., Brodersen, P., Grasser, K.D., Mattsson, O., Glazebrook, J., Mundy, J., and Petersen, M. (2008). Arabidopsis MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. EMBO J. **27,** 2214-2221.

Ranf, S., Gisch, N., Schaffer, M., Illig, T., Westphal, L., Knirel, Y.A., Sanchez-Carballo, P.M., Zahringer, U., Hückelhoven, R., Lee, J., and Scheel, D. (2015). A lectin S-domain

receptor kinase mediates lipopolysaccharide sensing in Arabidopsis thaliana. Nat. Immunol. **16,** 426-433.

Reape, T.J., Molony, E.M., and McCabe, P.F. (2008). Programmed cell death in plants: distinguishing between different modes. J Exp Bot **59,** 435-444.

Robatzek, S. (2007). Vesicle trafficking in plant immune responses. Cell. Microbiol. **9,** 1-8.

Robatzek, S., Chinchilla, D., and Boller, T. (2006). Ligand-induced endocytosis of the pattern recognition receptor FLS2 in Arabidopsis. Genes Dev. **20,** 537-542.

Rodriguez, M.C., Petersen, M., and Mundy, J. (2010). Mitogen-activated protein kinase signaling in plants. Annu. Rev. Plant Biol. **61,** 621-649.

Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., Malinovsky, F.G., Tor, M., de Vries, S., and Zipfel, C. (2011). The Arabidopsis leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. Plant Cell **23,** 2440-2455.

Schulze, B., Mentzel, T., Jehle, A.K., Mueller, K., Beeler, S., Boller, T., Felix, G., and Chinchilla, D. (2010). Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. J. Biol. Chem. **285,** 9444-9451.

Schwessinger, B., Roux, M., Kadota, Y., Ntoukakis, V., Sklenar, J., Jones, A., and Zipfel, C. (2011). Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. PLoS Genet **7,** e1002046.

Segonzac, C., Macho, A.P., Sanmartin, M., Ntoukakis, V., Sanchez-Serrano, J.J., and Zipfel, C. (2014). Negative control of BAK1 by protein phosphatase 2A during plant innate immunity. EMBO J. **33,** 2069-2079.

She, J., Han, Z., Kim, T.W., Wang, J., Cheng, W., Chang, J., Shi, S., Wang, J., Yang, M., Wang, Z.Y., and Chai, J. (2011). Structural insight into brassinosteroid perception by BRI1. Nature **474,** 472-476.

Shi, H., Shen, Q., Qi, Y., Yan, H., Nie, H., Chen, Y., Zhao, T., Katagiri, F., and Tang, D. (2013). BR-SIGNALING KINASE1 physically associates with FLAGELLIN SENSING2 and regulates plant innate immunity in Arabidopsis. Plant Cell **25,** 1143-1157.

Shinya, T., Yamaguchi, K., Desaki, Y., Yamada, K., Narisawa, T., Kobayashi, Y., Maeda, K., Suzuki, M., Tanimoto, T., Takeda, J., Nakashima, M., Funama, R., Narusaka, M., Narusaka, Y., Kaku, H., Kawasaki, T., and Shibuya, N. (2014). Selective regulation of the chitin-induced defense response by the Arabidopsis receptor-like cytoplasmic kinase PBL27. Plant J. **79,** 56-66.

Shirano, Y., Kachroo, P., Shah, J., and Klessig, D.F. (2002). A gain-of-function mutation in an Arabidopsis Toll Interleukin1 receptor-nucleotide binding site-leucine-rich repeat type R gene triggers defense responses and results in enhanced disease resistance. Plant Cell **14,** 3149-3162.

Spoel, S.H., Koornneef, A., Claessens, S.M., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Metraux, J.P., Brown, R., Kazan, K., Van Loon, L.C., Dong, X., and Pieterse, C.M. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell **15,** 760-770.

Sreeramulu, S., Mostizky, Y., Sunitha, S., Shani, E., Nahum, H., Salomon, D., Hayun, L.B., Gruetter, C., Rauh, D., Ori, N., and Sessa, G. (2013). BSKs are partially redundant positive regulators of brassinosteroid signaling in Arabidopsis. Plant J. **74,** 905-919.

Sun, T., Zhang, Q., Gao, M., and Zhang, Y. (2014). Regulation of SOBIR1 accumulation and activation of defense responses in *bir1-1* by specific components of ER quality control. Plant J. **77,** 748-756.

Sun, Y., Li, L., Macho, A.P., Han, Z., Hu, Z., Zipfel, C., Zhou, J.M., and Chai, J. (2013). Structural basis for flg22-induced activation of the Arabidopsis FLS2-BAK1 immune complex. Science **342,** 624-628.

Tang, W., Kim, T.W., Oses-Prieto, J.A., Sun, Y., Deng, Z., Zhu, S., Wang, R., Burlingame, A.L., and Wang, Z.Y. (2008). BSKs mediate signal transduction from the receptor kinase BRI1 in Arabidopsis. Science **321,** 557-560.

Thomma, B.P., Nelissen, I., Eggermont, K., and Broekaert, W.F. (1999). Deficiency in phytoalexin production causes enhanced susceptibility of Arabidopsis thaliana to the fungus Alternaria brassicicola. Plant J. **19,** 163-171.

Tintor, N., Ross, A., Kanehara, K., Yamada, K., Fan, L., Kemmerling, B., Nurnberger, T., Tsuda, K., and Saijo, Y. (2013). Layered pattern receptor signaling via ethylene and endogenous elicitor peptides during Arabidopsis immunity to bacterial infection. Proc. Natl. Acad. Sci. U. S. A. **110,** 6211-6216.

Ueda, H., Yamaguchi, Y., and Sano, H. (2006). Direct interaction between the tobacco mosaic virus helicase domain and the ATP-bound resistance protein, N factor during the hypersensitive response in tobacco plants. Plant Mol. Biol. **61,** 31-45.

Uren, A.G., O'Rourke, K., Aravind, L.A., Pisabarro, M.T., Seshagiri, S., Koonin, E.V., and Dixit, V.M. (2000). Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Mol. Cell **6,** 961-967.

van der Hoorn, R.A.L., and Kamoun, S. (2008). From Guard to Decoy: A new model for perception of plant pathogen effectors. Plant Cell **20,** 2009-2017.

van Doorn, W.G. (2011). Classes of programmed cell death in plants, compared to those in animals. J Exp Bot **62,** 4749-4761.

Vert, G., and Chory, J. (2006). Downstream nuclear events in brassinosteroid signalling. Nature **441,** 96-100.

Villaume, I., Thomas, D., and Legoy, M.D. (1990). Catalysis May Increase the Stability of an Enzyme - the Example of Horse Liver Alcohol-Dehydrogenase. Enzyme Microb. Technol. **12,** 506-509.

Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. (2003). An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J. **33,** 949-956.

Wang, X., and Chory, J. (2006). Brassinosteroids regulate dissociation of BKI1, a negative regulator of BRI1 signaling, from the plasma membrane. Science **313,** 1118-1122.

Wang, X., Kota, U., He, K., Blackburn, K., Li, J., Goshe, M.B., Huber, S.C., and Clouse, S.D. (2008). Sequential transphosphorylation of the BRI1/BAK1 receptor kinase complex impacts early events in brassinosteroid signaling. Dev. Cell **15,** 220-235.

Wang, Y., Li, Z., Liu, D., Xu, J., Wei, X., Yan, L., Yang, C., Lou, Z., and Shui, W. (2014). Assessment of BAK1 activity in different plant receptor-like kinase complexes by quantitative profiling of phosphorylation patterns. J. Proteomics **108,** 484-493.

Wang, Z., Meng, P., Zhang, X., Ren, D., and Yang, S. (2011). BON1 interacts with the protein kinases BIR1 and BAK1 in modulation of temperature-dependent plant growth and cell death in Arabidopsis. Plant J. **67,** 1081-1093.

Wang, Z.Y., Nakano, T., Gendron, J., He, J., Chen, M., Vafeados, D., Yang, Y., Fujioka, S., Yoshida, S., Asami, T., and Chory, J. (2002). Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. Dev. Cell **2,** 505-513.

Watanabe, N., and Lam, E. (2011). Arabidopsis metacaspase 2d is a positive mediator of cell death induced during biotic and abiotic stresses. Plant J. **66,** 969-982.

Wiermer, M., Feys, B.J., and Parker, J.E. (2005). Plant immunity: the EDS1 regulatory node. Curr. Opin. Plant Biol. **8,** 383-389.

Willmann, R., Lajunen, H.M., Erbs, G., Newman, M.A., Kolb, D., Tsuda, K., Katagiri, F., Fliegmann, J., Bono, J.J., Cullimore, J.V., Jehle, A.K., Gotz, F., Kulik, A., Molinaro, A., Lipka, V., Gust, A.A., and Nürnberger, T. (2011). Arabidopsis lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. Proc. Natl. Acad. Sci. U. S. A. **108,** 19824-19829.

Wolf, S., van der Does, D., Ladwig, F., Sticht, C., Kolbeck, A., Schurholz, A.K., Augustin, S., Keinath, N., Rausch, T., Greiner, S., Schumacher, K., Harter, K., Zipfel, C., and Hofte, H. (2014). A receptor-like protein mediates the response to pectin modification by activating brassinosteroid signaling. Proc. Natl. Acad. Sci. U. S. A. **111,** 15261-15266.

Yamaguchi, Y., Pearce, G., and Ryan, C.A. (2006). The cell surface leucine-rich repeat receptor for AtPep1, an endogenous peptide elicitor in Arabidopsis, is functional in transgenic tobacco cells. Proc. Natl. Acad. Sci. U. S. A. **103,** 10104-10109.

Yang, S., and Hua, J. (2004). A haplotype-specific Resistance gene regulated by BONZAI1 mediates temperature-dependent growth control in Arabidopsis. Plant Cell **16,** 1060-1071.

Yin, Y., Vafeados, D., Tao, Y., Yoshida, S., Asami, T., and Chory, J. (2005). A new class of transcription factors mediates brassinosteroid-regulated gene expression in Arabidopsis. Cell **120,** 249-259.

Yin, Y., Wang, Z.Y., Mora-Garcia, S., Li, J., Yoshida, S., Asami, T., and Chory, J. (2002). BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. Cell **109,** 181-191.

Zhang, J., Li, W., Xiang, T., Liu, Z., Laluk, K., Ding, X., Zou, Y., Gao, M., Zhang, X., Chen, S., Mengiste, T., Zhang, Y., and Zhou, J.M. (2010). Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a Pseudomonas syringae effector. Cell host & microbe **7,** 290-301.

Zhang, Q., Sun, T., and Zhang, Y. (2015). ER quality control components UGGT and STT3a are required for activation of defense responses in *bir1-1*. PLoS One **10,** e0120245.

Zhang, W., Fraiture, M., Kolb, D., Löffelhardt, B., Desaki, Y., Boutrot, F.F., Tor, M., Zipfel, C., Gust, A.A., and Brunner, F. (2013). Arabidopsis receptor-like protein30 and receptor-like kinase suppressor of BIR1-1/EVERSHED mediate innate immunity to necrotrophic fungi. Plant Cell **25,** 4227-4241.

Zhang, Y., Goritschnig, S., Dong, X., and Li, X. (2003). A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in suppressor of npr1-1, constitutive 1. Plant Cell **15,** 2636-2646.

Zhang, Z., Wu, Y., Gao, M., Zhang, J., Kong, Q., Liu, Y., Ba, H., Zhou, J., and Zhang, Y. (2012). Disruption of PAMP-induced MAP kinase cascade by a Pseudomonas syringae effector activates plant immunity mediated by the NB-LRR protein SUMM2. Cell host & microbe **11,** 253-263.

Zhou, N., Tootle, T.L., Tsui, F., Klessig, D.F., and Glazebrook, J. (1998). PAD4 functions upstream from salicylic acid to control defense responses in Arabidopsis. Plant Cell **10,** 1021-1030.

Zipfel, C. (2009). Early molecular events in PAMP-triggered immunity. Curr. Opin. Plant Biol. **12,** 414-420.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D., Felix, G., and Boller, T. (2004). Bacterial disease resistance in Arabidopsis through flagellin perception. Nature **428,** 764-767.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell **125,** 749-760.

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