The role of BRI1-ASSOCIATED KINASE 1 (BAK1) in the regulation of plant innate immunity: functional and genetic characterization of BAK1 overexpression in *Arabidopsis thaliana*

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Márta Kiss-Papp

Aus Budapest, Ungarn

Basel, 2014

Genehmigt von der Philosophisch- Naturwissenschaftlichen Fakultät auf Antrag von

Prof. Dr. Thomas Boller and Prof. Dr. Georg Felix

Basel, den 25 März 2014

Prof. Dr. Jörg Schibler

Dekan der Philosophisch-
Naturwissenschaftlichen Fakultät

Table of Contents

FREQUENT ABBREVIATIONS 1

SUMMARY 3

1 GENERAL INTRODUCTION 5

1.1 Pattern-triggered immunity (PTI) 7

1.1.1 MAMPs sensed by plants 7

1.1.2 Pattern recognition receptors (PRRs) perceive conserved molecular signatures to initiate broad range resistance 9

1.1.2.1 FLS2 is responsible for bacterial flagellin perception 11

1.1.2.2 Bacterial EF-Tu is sensed by EFR in Brassicaceae 12

1.1.2.3 The lysine motif (LysM) containing CERK1 is involved in chitin perception in Arabidopsis and rice 13

1.1.3 Signaling elements and physiological responses of PTI 14

1.1.3.1 Ion fluxes across the plasma membrane 14

1.1.3.2 Activation of calcium-dependent protein kinases (CDPKs) 14

1.1.3.3 Activation of mitogen-activated protein kinases (MAPKs) 15

1.1.3.4 Production of reactive oxygen species (ROS) 15

1.1.3.5 Plant hormones: important signaling components in immunity 16

1.1.3.6 Receptor endocytosis 18

1.1.3.7 Transcriptional reprogramming 18

1.1.3.8 Callose deposition 18

1.1.3.9 Inhibition of seedling growth 19

1.2 Effector-triggered immunity (ETI) 20

1.2.1 Microbial effector molecules suppress PTI 20

1.2.2 R proteins mediate effector recognition 21

1.3 BAK1 is a key signaling component in immunity as well as other biological processes 23

1.3.1 Molecular structures of BAK1 and its family members 24

1.3.2 BAK1 regulates brassinosteroid (BR)-dependent plant growth 25

1.3.3 BAK1 involvement in plant immunity 28
1.3.3.1 BAK1 – an important partner of many PRRs 28
1.3.3.2 Other SERKs seem to be functionally redundant with BAK1 in immunity 30
1.3.3.3 More players in the BAK1-dependent signaling pathways 30
1.3.3.4 BAK1 appears as an ideal target for bacterial effectors 31
1.3.3.5 BAK1 is required for pathogen resistance 32
1.3.3.6 BAK1 has a BR-independent role in cell death control 32
1.3.3.7 BAK1 at the crossroad of development and immunity 33
1.4 Aims of the thesis 34
2 MATERIAL AND METHODS 37
2.1 Plant material 37
2.1.1 *In vitro* conditions for *Arabidopsis thaliana* 37
2.1.2 *A. thaliana* "short day" conditions 37
2.1.3 "Long day" conditions for *A. thaliana* 37
2.2 Bacteria 37
2.2.1 *Escherichia coli* 37
2.2.2 Preparation of competent *E. coli* cells 38
2.2.3 Transformation of competent *E. coli* DH5α cells 38
2.2.4 *Agrobacterium tumefaciens* 38
2.2.5 Preparation of competent *A. tumefaciens* cells 38
2.2.6 Heat-shock transformation of chemical competent Agrobacteria cells 39
2.2.7 Glycerol stocks and storage of bacteria 39
2.3 Antibiotics 39
2.4 Solutions used for bioassays 39
2.4.1 β-Estradiol 39
2.4.2 Elicitors 40
2.4.3 Treatments for bioassays 40

2.5 Primers 40
2.6 Methods for molecular biology 41
2.6.1 Colony PCR 41
2.6.2 Analysis of nucleic acid by gel electrophoresis 41
2.6.3 Generation of constructs for plant transformation 41
2.6.4 Quantification of acid nucleic 45
2.6.5 Site directed mutagenesis 45
2.7 Stable transformation of *A. thaliana* plants 45
2.8 Analysis of transgene expression 46
2.9 Detection of MAPK activation 47
2.10 Analysis by quantitative real-time PCR (qRT-PCR) 47
2.11 Analysis of ethylene biosynthesis by gas chromatography 50
2.11.1 *A. thaliana* seedlings 50
2.11.2 *A. thaliana* leaf strips 50
2.12 Mesophyll cell death detection by trypan blue staining 51
2.13 Seedling growth inhibition assay 51
2.14 Pathogen growth assay 51
3 RESULTS 53
3.1 Chapter 1: BAK1 overexpression leads to a constitutive defense response in *Arabidopsis thaliana* 53
3.1.1 Abstract 53
3.1.2 Introduction 54
3.1.3 Results 58
3.1.3.1 Transgenic plants expressing BAK1 under a constitutive promoter display developmental defects, leaf necrosis and lethality 58
3.1.3.2 Early defense responses are activated by BAK1 overexpression in absence of a MAMP ligand 60

3.1.3.3 Defense marker genes are induced by BAK1 overexpression 63
3.1.3.4 The brassinosteroid pathway seems to be inhibited by overexpression of BAK1 64
3.1.3.5 Arabidopsis seedlings overexpressing BAK1 are impaired in growth and show cell death 65
3.1.3.6 BAK1 overexpression enhances resistance to Pseudomonas syringae pv tomato (Pto) DC3000 70
3.1.3.7 Elevated temperature does not alleviate the growth defects caused by BAK1 overexpression 73
3.1.3.8 BAK1 overexpression induces transcriptional upregulation of BAK1 interacting partners 73
3.1.3.9 Knock-out of SOBIR1 largely suppresses the BAK1 overexpression phenotype whereas lack of other selected defense-related genes does not. 75
3.1.3.10 BAK1 homologs are able to induce the activated-defense phenotype similar to BAK1 77
3.1.4 Discussion 80
3.1.4.1 BAK1 overexpression in Arabidopsis activates PTI 81
3.1.4.2 Possible role of truncated forms of BAK1 83
3.1.4.3 The BAK1 overexpression phenotype is independent of the known membrane-localized interaction partners of BAK1 84
3.1.4.4 Is the BAK1 overexpression phenotype a result of ETI activation? 86
3.1.4.5 Overexpression of SERK proteins induce the activated defense phenotype 87
3.1.4.6 Concluding remarks 88
3.1.5 Supporting information for Chapter 1: BAK1 overexpression leads to constitutive defense responses in *Arabidopsis thaliana* 91
3.2 Chapter 2: Structure-function analysis of BAK1 99
3.2.1 Abstract 99
3.2.2 Introduction 100
3.2.3 Results 102
3.2.3.1 BAK1 C-terminus is not determinant for the activated-defense phenotype 102
3.2.3.2 BAK1 kinase activity is not required to induce the constitutive ethylene phenotype 106
3.2.3.3 Overexpression of the plasma membrane anchored BAK1 ecto-domain causes phenotypes similar but not identical to BAK1 overexpression 109
3.2.4 Discussion 113

4 FINAL DISCUSSION 116

4.1 BAK1 overexpression may override mechanisms of negative regulation 117

4.2 Constitutive PTI might be based on aberrant activation of BAK1-interacting receptors 119

4.3 Strong kinase activity of BAK1 induces the downstream signaling cascade leading to constitutive PTI 120

4.4 BAK1 overexpression phenotype could be due to ETI activation 122

4.5 Are there other BAK1-dependent pathways additionally involved in the formation of the BAK1 overexpression phenotype? 126

4.6 Conclusion 128

5 OUTLOOK 128

5.1 Looking forward to BAK1 molecular signaling 128

5.2 Could pattern-triggered immunity be used to improve crop resistance? 130

ANNEX 132

LITERATURE 139

ACKNOWLEDGEMENTS 157

CURRICULUM VITAE 158

FREQUENT ABBREVIATIONS

At: *Arabidopsis thaliana*

avr: avirulence gene or protein

BAK1: BRASSINOSTEROIDE INSENSITIVE 1-ASSOCIATED KINASE1

BIK1: BOTRYTIS-INDUCED KINASE 1

BIR1: BAK1-INTERACTING RECEPTOR-LIKE KINASE 1

BR: brassinosteroid

BRI1: BRASSINOSTEROID INSENSITIVE 1

CaMV: Cauliflower mosaic virus

cDNA: complementary DNA

CDPK: calcium-dependent protein kinase

CERK1: CHITIN ELICITOR RECEPTOR KINASE 1

Col-0: Arabidopsis ecotype Columbia-0

DAMP: damage-associated molecular pattern

ddH_2O: double-distilled water

DNA: Deoxyribonucleic acid

dpi: day post-inoculation

EFR: ELONGATION FACTOR TU RECEPTOR

EF-Tu: elongation factor Tu

elf: EF-Tu peptide

EMS: ethyl methanesulphonate

ER: endoplasmic reticulum

ETI: effector-triggered immunity

EtOH: ethanol

flg22: flagellin 22

FLS2: FLAGELLIN SENSING 2

g: gramm

gDNA: genomic DNA

GFP: green fluorescent protein

HR: hypersensitive response

IP: immunoprecipitation

kDa: kilodalton

LRR: leucine-rich repeat

MAMP: microbe-associated molecular pattern

MAPK: mitogen-activated protein kinase

MEKK: mitogen-activated protein kinase kinase

NB: nucleotide binding site

PAMP: pathogen-associated molecular pattern

PEPR: PEP RECEPTOR

PR: pathogenesis-related

PRR: pattern recognition receptor

PTI: pattern-triggered immunity

R gene/protein: resistance gene/protein

RbohD: respiratory burst oxidase homologue D

RLK: receptor-like kinase

RLP: receptor-like protein

SA: salicylic acid

SAR: systemic acquired resistance

SERK: SOMATIC EMBRYOGENESIS RECEPTOR KINASE

SOBIR1: SUPPRESSOR OF *BIR1-1*

T-DNA: transfer-DNA

WT: wild type

SUMMARY

BAK1 (BRI1-ASSOCIATED KINASE 1) is an intensively studied member of the large leucine-rich repeat (LRR)-receptor-like kinase (RLK) family in Arabidopsis. It was initially identified as interacting partner of the brassinosteroid receptor BRI1 (BRASSINOSTEROID INSENSITIVE 1), which perceives the plant hormone brassinolide and thereby regulates a wide set of developmental and physiological processes in plants. In addition, BAK1 has been discovered to play an important role in one aspect of the active defense of plants against pathogens, the so-called pattern-triggered immunity (PTI). This involves perception of conserved microbe-associated molecular patterns (MAMPs) by so called pattern recognition receptors (PRRs). Some of these PRRs interact with BAK1 immediately after MAMP recognition, and this promotes receptor phosphorylation and initiates PTI.

In this work BAK1 was overexpressed in Arabidopsis in order to better understand its role in innate immunity (Chapter 1). Surprisingly, constitutive overexpression of BAK1 led to stunted plant stature, leaf necrosis and premature death of the plant. Using an inducible system to express BAK1, it was revealed that this phenotype was probably due to the constitutive activation of defense responses triggered by the accumulation of BAK1. As a consequence, these plants displayed an enhanced resistance to the bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000. Likewise, overexpression of BAK1 homologs, SERK1 and SERK4, also induced constitutive activation of defense responses, supporting the idea of a redundant function for SERK proteins in innate immunity. Mutation of a defense related gene *SOBIR1* (*SUPPRESSOR OF BIR1-1)* almost entirely reverted the BAK1 overexpression phenotype. The SOBIR1 protein kinase appears to be involved in PTI as well as in a second aspect of the plant's active defense, effector-triggered immunity (ETI).

Since overexpression of BAK1 always produced putative truncated forms of the BAK1 protein, the possible connection between the accumulation of these protein fragments and the overexpression phenotype was studied in more detail (Chapter 2). Apparently, the extracellular domain anchored to the plasma membrane as well as the activity of the kinase domain both contribute to the BAK1 overexpression phenotypes.

Our study demonstrates that BAK1 over-accumulation causes a constitutive defense phenotype likely due to constitutive PTI activation. However we can not exclude that in addition ETI gets constitutively activated in these conditions. Moreover, the presence of the BAK1 extracellular domain as well as its kinase function appears to be crucial for its ability to induce defense responses.

1 GENERAL INTRODUCTION

Green plants are photosynthetic organisms able to capture and preserve light energy by converting inorganic, atmospheric CO_2 into organic carbohydrates. In contrast, many other organisms rely on the availability of these organic carbohydrates making plants very attractive not only as symbionts but also as simple food or energy sources for herbivores and pathogenic microbes. To defend themselves against such attacks, plants evolved an innate immune system which is known to rely on different complementary and redundant mechanisms. It is organized in layers to face a broad range of enemies including microbes, insects and herbivores (Thordal-Christensen, 2003; Jones and Dangl, 2006; Mithofer and Boland, 2008).

The first, very unspecific line of plant immunity to block invaders consists in pre-formed constitutive barriers, i.e. a “passive” host defense (**Figure 1.1**). This includes both mechanical and chemical barriers (Thordal-Christensen, 2003). The mechanical barriers are formed by the waxy cuticle, the plant epidermis and the complex cell wall, which surrounds each cell. In addition, plants developed chemical barriers including peptides (i.e. plant defensins), proteins (i.e. protease inhibitors) or secondary metabolites (i.e. phytoanticipins, glucosinolates, saponines), which are constitutively produced and confer a large-spectrum resistance against invaders (Broekaert et al., 1995; Joshi et al., 1999; Halkier and Gershenzon, 2006).

Microbes able to bypass this first layer of defense can be perceived by broadly conserved molecular signatures, collectively known as microbe-associated molecular patterns (MAMPs), which are specifically recognized by pattern recognition receptors (PRRs) of the host organism and induce active defense responses leading to pattern-triggered immunity (PTI) (Boller, 1995; Jones and Dangl, 2006; Boller and Felix, 2009) (**Figure 1.1**). This basal immune system confers resistance to a broad range of microbes. Nevertheless some pathogenic microbes are still successful by secreting or injecting effector molecules that suppress PTI. Plants then evolved a strategy based on plant resistance (R) proteins to recognize these effectors and trigger an even stronger immune reaction called effector-triggered immunity (ETI), which is often associated with a form of programmed cell death called hypersensitive response (HR) (Thordal-Christensen, 2003; Jones and Dangl, 2006). In contrast to PTI, ETI

confers a highly specific resistance to individual pathogenic species (**Figure 1.1**). This layered immune system suggests a co-evolutionary dynamic between host plants and pathogenic microbes, in which plants develop perception systems with increasing specificity to detect the pathogens presence in order to mount an active defense (Boller and He, 2009; Lehti-Shiu et al., 2009; Dodds and Rathjen, 2010). Considering this, one could imagine that there is a constant arms race between plants and their pathogens.

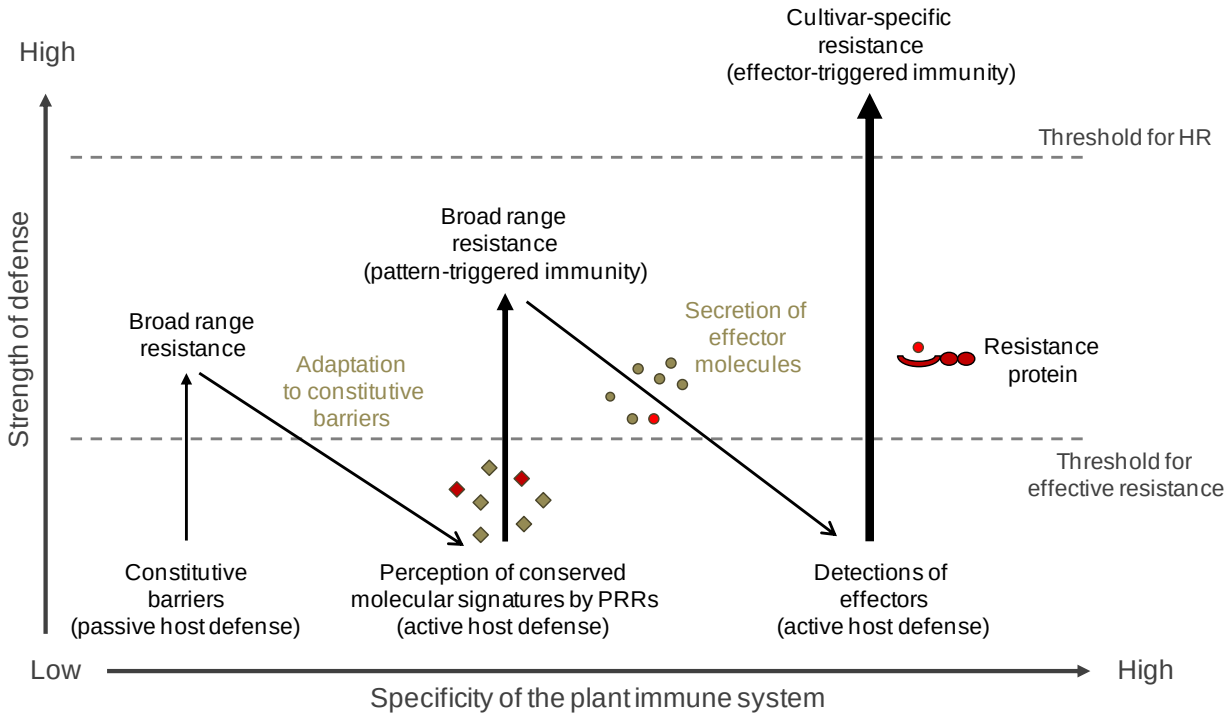


Figure 1.1: Plant disease resistance is composed of a multilayered surveillance system. Passive host defense, consisting of pre-formed, constitutive barriers confers effective resistance against a broad range of microbes. Microbes bypassing this first layer of defense are recognized via conserved microbe/pathogen-associated molecular patterns (MAMPs/PAMPs, represented as diamonds) and elicit active defense reactions leading to a broad range resistance called pattern-triggered immunity (PTI). Successful pathogens can interfere with this non-specific immune response by secreting effector molecules (represented as circles), which can be than recognized by host resistance proteins and trigger a cultivar-specific immune response called effector-triggered immunity (ETI). ETI is often accompanied by the programmed death of cells, called hypersensitive response (HR), at the site of the infection. The scheme depicted here is an extension of the “zigzag model” of Jones and Dangl (2006) and represents the correlation between the gradually increasing pathogen recognition-specificity of the host detection

system, where the amplitude of the defense reactions is determined by the level required for effective immunity.

1.1 Pattern-triggered immunity (PTI)

Once microbial invaders successfully overcame the preformed barriers, the plants perceive their presence by sensing chemically diverse molecules of microbial origin, previously collectively called pathogen-associated molecular patterns (PAMPs) (Boller and Felix, 2009). Because these molecules are also characteristic for non-pathogenic microbes, e.g. symbiotic rhizobacteria, it has been proposed and widely accepted to name them microbe-associated molecular patterns (MAMPs) instead of PAMPs (Radutoiu et al., 2003; He et al., 2006; Mackey and McFall, 2006; Boller and Felix, 2009). Perception of MAMPs by the plant triggers PTI. Beside MAMPs diverse molecules of distinct origin have been identified that trigger PTI as well. There are herbivore-associated molecular patterns (HAMPs) as well as damage- or danger-associated molecular patterns (DAMPs). Whereas MAMPs and HAMPs share the feature of being foreign ("non-self") to the plant, DAMPs are molecules of plant origin ("self") which are supposed to be released in situations of damage or danger to activate PTI locally or systemically (Pearce et al., 1991; Huffaker et al., 2006). Since MAMPs and the molecular machinery behind the MAMP perception system are central to this thesis, I further focus mainly on MAMPs.

1.1.1 MAMPs sensed by plants

Typically, MAMPs are highly conserved and crucial for the microbial lifestyle, thus mutation or deletion of a MAMP in order to avoid recognition will have deleterious effects on the microbial survival. For example, plants sense fungal microbes through perception of fragments of chitin, the main structural component of the fungal cell wall (Felix et al., 1993; Shibuya et al., 1993). Other examples for fungal MAMPs are ergosterol, a component of fungal cell membranes (Granado et al., 1995); xylanase, an enzyme able to degrade hemicelluloses, one of the major components of plant cell walls (Hanania and Avni, 1997); or β-glucans, which are cell wall components of certain fungi, oomycetes and bacteria (Klarzynski et al., 2000).

Bacteria can be sensed through perception of bacterial flagellin, the main protein subunit of bacterial flagellum, which is the motility organ of bacteria and essential for the overall pathogenicity of bacterial plant pathogens (Felix et al., 1999). Indeed, some bacteria with mutated flagellin are unable to build a functional flagellum and are thus usually immobile and less pathogenic (Naito et al., 2008). The sensitivity to flagellin was first demonstrated in tomato cell cultures treated with peptidic preparations from *Pseudomonas syringae* pathovar (pv) *tomato* (Felix et al., 1999). Another well studied example of bacterial MAMPs is the bacterial elongation factor Tu (EF-Tu), the most abundant bacterial protein, essential for protein translation (Kunze et al., 2004; Zipfel et al., 2006). EF-Tu was found as the active compound of crude bacterial extracts perceived by Arabidopsis plants blind to flg22 (Kunze et al., 2004). Other examples for bacterial MAMPs are the cold-shock protein, an RNA-binding protein inducible by cold-shock (Felix and Boller, 2003), or peptidoglycan (PGN), an important component of the bacterial cell envelope (Erbs et al., 2008). These examples well illustrate that MAMPs are required for microbial fitness, and highly conserved in a broad range of microbes, but absent from the host, and therefore ideal targets for basal immune recognition.

In general MAMPs are active at subnanomolar concentrations, and are recognized through specific epitopes. This is the case for the highly conserved 22 amino acid section of flagellin (flg22) and the 18 amino acid sequence of EF-Tu (elf18), both localized in the N-terminal part of the corresponding protein (Felix et al., 1999; Kunze et al., 2004). Recently a second flagellin epitope defined as flgII-28was identified which is different from flg22 (Cai et al., 2011). It was shown that flg22 was able to elicit defense responses in various plant species, including Arabidopsis, tobacco and potato, revealing an evolutionarily conserved recognition system for bacterial flagellin (Felix et al., 1999). By contrast, flgII-28 is only active in Solanaceae species (Cai et al., 2011). Pretreatment of Arabidopsis plants with flg22 increased the resistance against bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 (*Pto* DC3000) (Zipfel et al., 2004). Interestingly elf18 induces a set of signaling events and defense responses highly similar to that induced by flg22 (Zipfel et al., 2006). However, in contrast to flg22, responsiveness to elf18 is restricted to Arabidopsis and other Brassicaceae, indicating that the

perception system responsible for the detection of this MAMP is evolutionarily younger (Kunze et al., 2004).

These examples demonstrate that the chemical composition of MAMPs is very diverse, ranging from carbohydrates, over lipids to proteins. The key feature of all these compounds is the elicitation of PTI upon detection.

1.1.2 Pattern recognition receptors (PRRs) perceive conserved molecular signatures to initiate broad range resistance

MAMPs, HAMPs and DAMPs are perceived by plants possessing the corresponding PRRs. Several PRR-ligand pairs have been well characterized, which are illustrated in **Figure 1.2**. Many of the identified PRRs are receptor-like kinases (RLKs). Well known examples are FLS2 (FLAGELLIN SENSING 2), EFR (ELONGATION FACTOR TU (EF-Tu) RECEPTOR), CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1), PEPR1 and 2 (PEP RECEPTOR 1 and 2) and WAK1 (WALL-ASSOCIATED KINASE 1). These PRRs are localized in the plasma membrane with different motif-repetitions (e.g. leucine-rich repeat (LRR) or lysine motif (LysM)) responsible for ligand binding in their extracellular domains (also called ecto-domain), and an intracellular Serine/Threonine (Ser/Thr) protein kinase domain. Most kinases contain a conserved aspartate (D) residue, which is essential for catalytic activity, within the catalytical loop, which is responsible for phosphorylation and phosphotransfer efficiency (Johnson et al., 1996; Adams, 2003). In Ser/Thr kinases this catalytic aspartate is often preceded by an arginine (R) amino acid therefore they are called “RD” kinases. Interestingly most of the RLK PRRs, such as FLS2 and EFR, are non-RD kinases lacking the arginine preceding the catalytic aspartate and thus generally fail to auto-phosphorylate the activation loop (Dardick et al., 2012). Therefore non-RD kinases are thought to require other mechanisms or additional proteins for their regulation (Krupa et al., 2004; Dardick and Ronald, 2006; Dardick et al., 2012).

Beside the RLK-type of PRRs, some PRRs have a typical receptor-like protein (RLP) structure, which contains a ligand-binding ecto-domain and a trans-membrane domain but lacks the intracellular kinase domain. The CEBiP (CHITIN ELICITOR-BINDING PROTEIN), LYM1/3 (LYSM DOMAIN GPI-ANCHORED PROTEIN 1 and 3) and EIX1/2 receptors (*ETHYLENE-INDUCING*

XYLANASE RECEPTOR 1 and 2) belong to this group (Shimizu et al., 2010; Bar et al., 2011; Willmann et al., 2011).

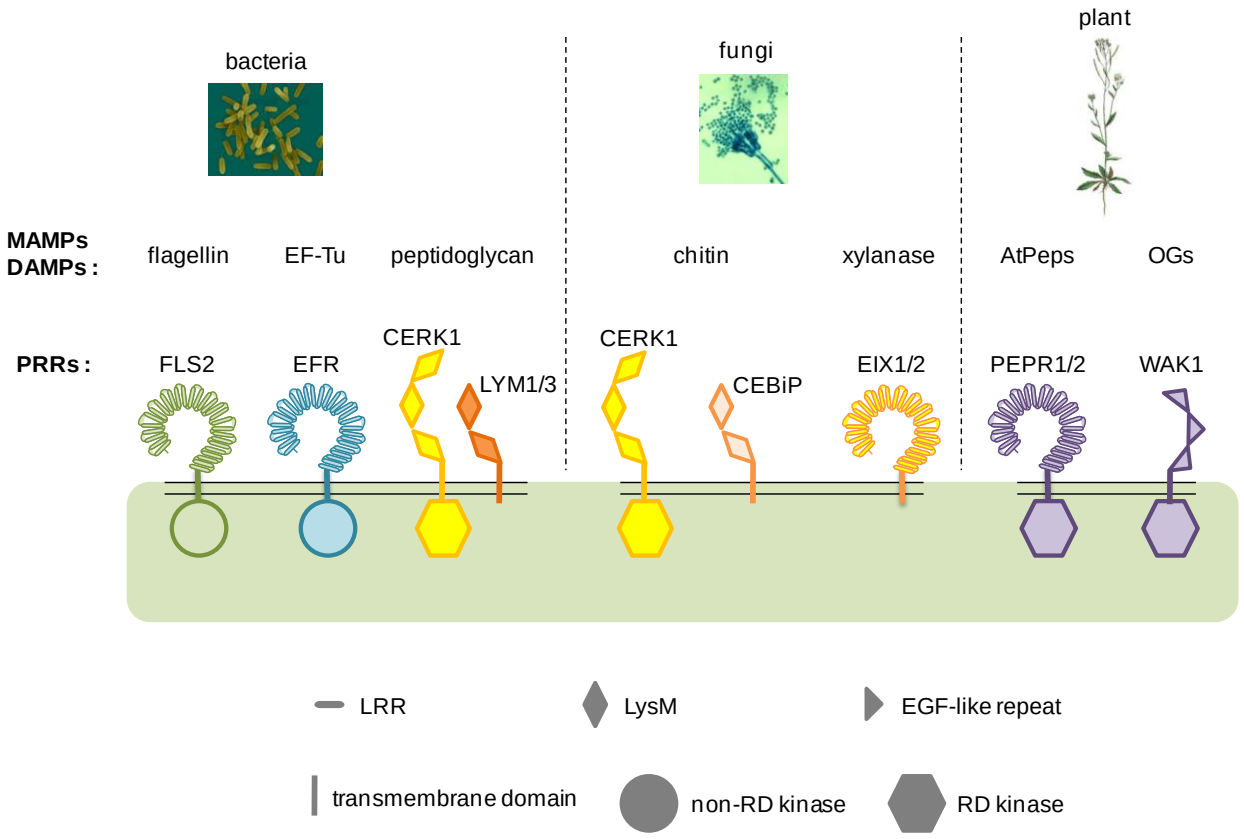


Figure 1.2: Schematic representation of characterized ligand-receptor pairs involved in pattern recognition in plants. PRR: pattern recognition receptor; **MAMP**; microbe-associated molecular pattern; **DAMP**: damage-associated molecular pattern; **FLS2** (FLAGELLIN SENSING 2) was characterized as the receptor for bacterial flagellin (the recognized synthetic epitope is flg22) in *A. thaliana* (Chinchilla et al., 2006), *N. benthamiana* (Hann and Rathjen, 2007), tomato (Robatzek et al., 2007) and rice (Takai et al., 2008); **EFR** (ELONGATION FACTOR TU (EF-Tu) RECEPTOR) was found to be responsible to EF-Tu (or synthetic epitope elf18) recognition in Brassicaceae (Zipfel et al., 2006); **CERK1** (CHITIN ELICITOR RECEPTOR KINASE 1) recognizes chitin in *A. thaliana* (Miya et al., 2007), **LYM1/3** (LYSM DOMAIN GPI-ANCHORED PROTEIN 1 and 3) together with CERK1 mediates recognition of bacterial peptidoglycan (PGN) in *A. thaliana* (Willmann et al., 2011); **CEBiP** (CHITIN ELICITOR-BINDING PROTEIN) in association with CERK1 is responsible for chitin perception in rice (Shimizu et al., 2010); **EIX1/2** (ETHYLENE-INDUCING XYLANASE RECEPTOR 1 and 2*)* are identified xylanase receptors in tomato where EIX2 is the signaling competent receptor and EIX1 is a decoy receptor (Bar et al., 2011); **PEPR1/2** (PEP RECEPTOR1 and 2) are responsible for the recognition of self-derived molecules, *At*Peps, in *A. thaliana* (Yamaguchi et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010); **WAK1** (WALL-ASSOCIATED KINASE 1) binds plant cell wall-derived oligogalacturonides (OGs) (Brutus et al., 2010).

1.1.2.1 FLS2 is responsible for bacterial flagellin perception

Soon after the discovery of a flagellin perception system in Arabidopsis (Felix et al., 1999), it was observed that flg22 treatment induces inhibition of Arabidopsis seedling growth (Gomez-Gomez et al., 1999). In an Arabidopsis mutant screen using the insensitivity of seedlings to flg22 as read-out, FLS2 was identified as the flagellin receptor (Gomez-Gomez and Boller, 2000). Thereafter heterologous expression of *Arabidopsis thaliana* FLS2 (*At*FLS2) in tomato cells and binding studies with ^{125}I-labeled peptides in Arabidopsis cells confirmed FLS2 as the bona fide receptor for flg22 (Chinchilla et al., 2006; Robatzek et al., 2006). Up to date direct evidence of the capability of FLS2 orthologs to perceive flg22 was demonstrated in Arabidopsis, tobacco, tomato, and rice (Chinchilla et al., 2006; Hann and Rathjen, 2007; Robatzek et al., 2007; Takai et al., 2008) (**Figure 1.2**). Interestingly, the other active flagellin epitope, flgII-28, is not recognized by FLS2 and its receptor remains to be identified (Cai et al., 2011; Clarke et al., 2013). The fact that Arabidopsis *fls2* mutants were more susceptible to *Pto* DC3000 than wild type plants provided direct evidence that flagellin perception is crucial for disease resistance against bacteria (Zipfel et al., 2004).

FLS2 belongs to the LRR-RLK XII subfamily of Arabidopsis RLKs (Shiu and Bleecker, 2003). This family has ten members, but FLS2 has no true homologue in the Arabidopsis genome. Orthologs of *At*FLS2 with highly conserved architecture were found in all analyzed genomes of higher plants further supporting the primary importance of this PRR in plant immunity (Boller and Felix, 2009). Interestingly, also in mammals, bacterial flagellin is perceived by an LRR-containing membrane localized receptor, called TLR5 (TOLL-LIKE RECEPTOR 5) (Hayashi et al., 2001). However the epitope perceived by TLR5 is different (Smith et al., 2003).

The extracellular ligand-binding domain of FLS2 contains 28 LRR repetitions arranged in tandem flanked by LRR N-terminal (LRRNT) and C-terminal (LRRCT) domains with characteristic double-cysteine motifs (Boller and Felix, 2009). Recently, functional and binding studies of chimeric receptors obtained by a domain swapping approach between the LRR domain of tomato and Arabidopsis FLS2 revealed two equally important interaction sites distributed within the FLS2 ecto-domain important for flg22 binding (Mueller et al., 2012a). The

extracellular domain is followed by a membrane-spanning region characteristic to RLKs and an intracellular domain including a non-RD kinase and a C-terminus (Shiu and Bleecker, 2001a; Dardick and Ronald, 2006) .

Surprisingly it has been reported that *At*FLS2 also mediates the recognition of *Xoo* Ax21-derived peptides, such as the axY^{S}22, a synthetic sulfated 17 amino acids peptide claimed to be recognized by the rice PRR XA21 to mediate immunity (Lee et al., 2009; Danna et al., 2011). Even more surprisingly, *At*FLS2 has also been described to be responsible for the recognition of the endogenous CLAVATA3 peptide (CLV3p) to induce a so-called "stem-cell-triggered immunity" (Lee et al., 2011). The CLV3p is known to be involved in the regulation of the development of the shoot and floral meristems mediated through CLAVATA1 and 2 receptors (Fletcher et al., 1999; Rojo et al., 2002). Neither axY^{S}22 nor CLV3p have any similarity to flg22. Why did these peptides interact with FLS2? Recent studies demonstrated that pure, freshly synthesized axY^{S}22 and CLV3p peptides applied even at extremely high concentrations, did not activate the FLS2 receptor. This indicated that the results mentioned were due to a contamination of the peptide preparations by flg22, confirming the high specificity of FLS2 for its ligand flg22 (Danna et al., 2012; Mueller et al., 2012a; Mueller et al., 2012b).

1.1.2.2 Bacterial EF-Tu is sensed by EFR in Brassicaceae

EFR is responsible for the perception of EF-Tu and has been identified in a targeted T-DNA insertion mutant screen on Arabidopsis seedlings (Zipfel et al., 2006) (**Figure 1.2**). Its presence is restricted to the plant family of Brassicaceae. Heterologous expression of EFR in *Nicotiana benthamiana* plants naturally lacking the EF-Tu perception system provided additional evidence that EFR is responsible for EF-Tu perception. This study also demonstrated that signaling cascades downstream of PRRs are similar in *N. benthamiana* and *A. thaliana* model systems (Zipfel et al., 2006). The *EFR* mutants of Arabidopsis showed higher susceptibility to an infection by *Agrobacterium tumefaciens*, confirming its importance in PTI (Zipfel et al., 2006). In addition, it has been shown that tobacco and tomato plants expressing the *EFR* gene are less susceptible to bacterial pathogens revealing the biological importance of EF-Tu perception system in disease resistance (Lacombe et al., 2010). Hence there is a

possibility to use heterologous expression of EFR or similar PRRs as a strategy to improve the natural disease resistance of crop plants (Segonzac and Zipfel, 2011).

The EF-Tu receptor belongs to the LRR-RLK XII family, similar to FLS2, but it contains 21 LRRs instead of 28 (Shiu and Bleecker, 2003). The Arabidopsis genome contains six homologs of EFR with closely related structural features in their intracellular parts (Shiu and Bleecker, 2003), however these homologs have no affinity for EF-Tu derivatives (Albert et al., 2010). EFR also possesses a non-RD Ser/Thr kinase domain in its intracellular part (Dardick and Ronald, 2006). Functional analysis and affinity binding studies of EFR-derived constructs demonstrated that the absence of EFR kinase domain does not influence the ligand-binding functions of the receptor; furthermore EFR-FLS2 chimeras revealed that different and non-contiguous parts of the EFR ecto-domain are required for functional ligand binding (Albert et al., 2010).

1.1.2.3 The lysine motif (LysM) containing CERK1 is involved in chitin perception in Arabidopsis and rice

It was known previously that LysM domains are important for chitin binding in animals (Zhang et al., 2007b). In plants, the first evidence of a role of LysM-domain containing proteins in chitin perception came from high-affinity binding studies in rice (Kaku et al., 2006). The identified protein, called CEBiP (CHITIN ELICITOR-BINDING PROTEIN), encodes an RLP with an extracellular LysM domain, a trans-membrane domain and a short cytoplasmic tail (**Figure 1.2**). Moreover, CERK1, a receptor kinase, was identified in Arabidopsis by a screen on LysM domain-containing protein mutants unresponsive to chitin (Miya et al., 2007). CERK1 encodes an RLK with LysM motifs in its ecto-domain (**Figure 1.2**). Interestingly, in contrast to FLS2 and EFR, which belong to the non-RD kinase family, CERK1 contains an RD kinase in its intracellular domain. In Arabidopsis, CERK1 has been shown to bind chitin and to be indispensable for chitin-induced defense responses (Miya et al., 2007; Wan et al., 2008; Petutschnig et al., 2010). Recently co-IP studies showed that chitin induces the dimerisation of CERK1 ecto-domains which revealed to be indispensable for chitin signaling (Liu et al., 2012). Although CERK1 is also required for chitin responsiveness in rice and associates with CEBiP, it is unclear if it has a role in chitin binding in this system (Shimizu et al., 2010).

1.1.3 Signaling elements and physiological responses of PTI

As soon as microbial pathogens patterns or endogenous danger signals are sensed, the plant responds with an ordered sequence of signal transduction and defense responses. These responses comprise signaling events, which e. g. transmit the information from the outside of the cell to the inside, as well as later defense responses like the production of anti-microbial compounds. Accordingly to the kinetics of these responses, they are ranging from seconds to days. Below, they are ordered on a temporal scale, as in a recent review (Boller and Felix, 2009).

1.1.3.1 Ion fluxes across the plasma membrane

Very rapidly, within the first minutes upon elicitor perception, ion fluxes are observed including Ca^{2+} influx, Cl^- efflux and K^+/H^+ exchange (**Figure 1.3**) (Boller, 1995; Nurnberger et al., 2004; Jeworutzki et al., 2010). H^+ influxes induce the alkalinization of extracellular media of suspension cells, which can be easily detected and frequently used as bioassay for MAMP perception (Felix et al., 1991a). Among these ion fluxes cytosolic accumulation of Ca^{2+} is considered to possess a high biological importance since Ca^{2+} acts as second messenger in many signaling pathways (Bush, 1995).

1.1.3.2 Activation of calcium-dependent protein kinases (CDPKs)

Importantly, cytosolic Ca^{2+} can activate CDPKs (Trewavas and Malho, 1998; Ludwig et al., 2005; Lecourieux et al., 2006). Recently, in transient Arabidopsis leaf protoplast assays, closely related *CDPK* genes *CPK5/6* and *CPK4/11* were identified to monitor cytoplasmic Ca^{2+} during PTI and activate transcription of some defense genes (Boudsocq et al., 2010). Indeed qRT-PCR analysis of the expression of early flg22-responsive genes in Arabidopsis protoplasts showed that while the activation of *PHI-1* (*PHOSPHATE INDUCED 1*) depends on CDPKs, the activation of *FRK1* (*FLG22-INDUCED RECEPTOR KINASE 1*) is controlled by the activation of mitogen-activated protein kinases (MAPKs, see below). The expression of *NHL10* (*NDR1/HIN1 LIKE 1*) is

synergistically regulated by CDPKs and MAPKs. Thus, CDPKs seem to function in parallel of MAPKs to regulate distinct and convergent genetic programs (Boudsocq et al., 2010). Additionally, the quadruple CDPK mutant showed severely impaired flg22-induced oxidative burst, suggesting a role for these CDPKs in the regulation of ROS (see below), potentially through direct phosphorylation of NADPH (NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE) oxidase (Kobayashi et al., 2007; Boudsocq et al., 2010).

1.1.3.3 Activation of mitogen-activated protein kinases (MAPKs)

Similar to CDPKs, MAPKs are central signaling components in PTI (Nuhse et al., 2000; Asai et al., 2002). MAPKs are fairly diverse but are an universal feature of eukaryotic cells. MAPK cascades typically include MAP kinase kinase kinases (MKKKs), which phosphorylate MAP kinase kinases (MKKs), which phosphorylate MAPKs (**Figure 1.3**). In Arabidopsis, two MAPKs are activated during PTI responses by double phosphorylation: MPK3 and MPK6 (Asai et al., 2002). Activation of these cascades leads to sub-cellular relocalization and/or phosphorylation of downstream substrates including transcription factors, such as for example VIP1 (VIRE2-INTERACTING PROTEIN 1) (Djamei et al., 2007) or WRKY33 (WRKY DNA-BINDING PROTEIN 33) (Mao et al., 2011) to induce transcriptional reprogramming. Interestingly it has been reported that the *Pseudomonas syringae* effector HopAI1 dephosphorylates MPK6 and MPK3 by its phosphothreonine lyase activity and therefore blocks PTI signaling (Zhang et al., 2007a). The fact that several bacterial effectors target MAPK cascades (e.g. HopAI1, HopPtoD2) can be taken as an indication for the biological importance of MAPK signaling in plant-pathogen interactions (Espinosa et al., 2003; Zhang et al., 2007a; Cui et al., 2010; Wang et al., 2010; Zhang et al., 2012).

1.1.3.4 Production of reactive oxygen species (ROS)

Production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) or superoxide (O_2^-) is induced upon microbe perception (**Figure 1.3**). These ROS are active anti-microbial compounds and serve also as substrate for oxidative cross-linking to reinforce the

plant cell wall (Lamb and Dixon, 1997). The oxidative burst is a consequence of the activation of the membrane localized NADPH oxidase also called RESPIRATORY BURST OXIDASE (RBO) (Lamb and Dixon, 1997). The Arabidopsis genome encodes 10 *RBO* gene homologues (*AtRBOHs*); *AtRbohD* was shown to be the main enzyme responsible for the production of apoplastic ROS (Kroj et al., 2003; Torres and Dangl, 2005). Plants mutated in this gene are impaired in MAMP-induced ROS production (Nuhse et al., 2007). Additionally, ROS are also important signals for mediating transcriptional reprogramming including activation of defense genes, and they may have regulatory functions in association with other signaling molecules such as salicylic acid (SA) for example in the hypersensitive response (Levine et al., 1996; Torres et al., 2005). However their mode of action remains poorly understood.

1.1.3.5 Plant hormones: important signaling components in immunity

Plant hormones were first recognized to be determinants in growth and development but later on it became clear that they also play an important role in plant-pathogen interactions. The best-characterized defense hormones are salicylic acid (SA), jasmonic acid (JA) and ethylene (Delaney et al., 1994; Glazebrook, 2005).

SA is a major regulator of plant innate immunity and plays important roles both in PTI and ETI (Delaney et al., 1994; Wildermuth et al., 2001; Tsuda et al., 2008). Indeed, the SA-mediated signaling pathway is involved in the expression of PR proteins and HR (Greenberg and Yao, 2004; Glazebrook, 2005). In addition to its role in local defense responses, SA accumulation is required for the establishment of defense activation in non-infected distal leaves during systemic acquired resistance (SAR) (Gaffney et al., 1993). However SA does not seem to be the translocated signal responsible to induce SAR (Vernooij et al., 1994; Smith-Becker et al., 1998). In summary, SA signaling is an important factor in disease resistance, especially for the restriction of growth of biotrophic and hemibiotrophic pathogens (Greenberg and Yao, 2004; Glazebrook, 2005).

Conversely, ethylene and JA are believed to play a major role in resistance to necrotrophic microorganisms, but also to insects and other herbivores (Glazebrook, 2005; Howe and Jander, 2008). This is supported by the fact that ethylene and JA signaling mutants

were shown to be more susceptible to the necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola* while these mutants did not show altered resistance to biotrophic pathogen *Pto* DC3000 (Thomma et al., 1998; Ferrari et al., 2003; Glazebrook, 2005). Strong ethylene accumulation is induced within 10 minutes by MAMPs (**Figure 1.3**), indicating a role for ethylene in PTI (Felix et al., 1991b; Boller, 1995; Zipfel et al., 2004). Indeed, it was reported that flg22-induced ROS production as well as flg22-triggered stomatal closure (see below) was impaired in ethylene insensitive mutants (Mersmann et al., 2010). Interestingly these mutants showed reduced expression levels of FLS2 compared to wild type plants. Additionally, it was shown that *ein2* ethylene-signaling mutants are impaired in all flg22-induced responses, apparently due to reduction of *FLS2* expression, which was find to be directly controlled by EIN2-dependent EIN3 and EIN3-like transcription factors. (Boutrot et al., 2010). These data confirm the functional importance of ethylene in PTI. While ethylene and JA act synergistically, the ethylene/JA and SA defense pathways interact most of the time antagonistically (Glazebrook et al., 2003; Glazebrook, 2005; Mur et al., 2006; Leon-Reyes et al., 2009). This hormonal cross-communication has a major regulatory role in successful plant defense (Pieterse et al., 2009). Some pathogens try to perturb this cross-talk by producing molecules mimicking plant hormones. For example, coronatine is a JA-mimicking phytotoxin produced by *Pto* DC3000, which is able to suppress SA-dependent defenses and to induce stomatal opening in Arabidopsis plants (Collmer et al., 2002; Brooks et al., 2005; Melotto et al., 2006).

In addition to SA, JA and ethylene, other plant hormones were identified as actors in plant immunity beside of their known regulatory role in growth, development and abiotic stress (Pieterse et al., 2009). These are abscisic acid (ABA), auxins, gibberellins, cytokinins and brassinosteroids (BRs). For example, exogenous application of BRs on tomato and rice induces disease resistance against various microbes (Nakashita et al., 2003). Moreover, the closure of stomata upon *Pseudomonas syringae* infection requires a functional ABA signaling pathway (Melotto et al., 2006). In summary it appears that the interaction of the distinct hormone pathways is an important factor to fine-tune immune responses depending on the invading pathogen.

1.1.3.6 Receptor endocytosis

Interestingly, it has been demonstrated with a green fluorescent protein (GFP) labeled FLS2 stably expressed in Arabidopsis plants that flg22 treatment induces within 10-20 minutes FLS2 delocalization from the plasma membrane to intracellular vesicles (**Figure 1.3**) (Robatzek et al., 2006). Since prolonged activation of defense responses can impose negative effects on plant fitness (Lorrain et al., 2003; Tian et al., 2003; Korves and Bergelson, 2004; Liew et al., 2005), FLS2 endocytosis is possibly meant to remove and degrade the activated protein to attenuate the signal. Whether or not this event also has particular signaling functions is still unknown.

1.1.3.7 Transcriptional reprogramming

Application of flg22 treatment on Arabidopsis cell cultures and seedlings induces important transcriptional changes (**Figure 1.3**) (Navarro et al., 2004; Zipfel et al., 2004). Already after 30 minutes of flg22 treatment about 1100 genes (≈ 5% of the Arabidopsis genome) are differentially regulated (Zipfel et al., 2004). The induced gene expression pattern is almost identical for flg22-, elf18-, and chitin-mediated signaling suggesting a conserved regulation of genetic reprogramming in PTI (Ramonell et al., 2002; Zipfel et al., 2006; Libault et al., 2007). Among the genes, which are up-regulated upon MAMP perception, are those encoding enzymes responsible for the synthesis of anti-microbial compounds (e.g. camalexin (Glawischnig, 2007)) and, notably, for proteins involved in signal perception and transduction such as PRRs, transcription factors, kinases and phosphatases (Navarro et al., 2004; Zipfel et al., 2004; Moscatiello et al., 2006; Zipfel et al., 2006). Up-regulation of PRR expression upon MAMP treatment suggests a positive feedback control for early transcriptional reprogramming to increase the perception abilities of the host plant (Zipfel et al., 2006).

1.1.3.8 Callose deposition

Plant cell wall reinforcement through the synthesis of callose and lignin, and localized formation of particular structures like papillae, which can contain callose, serve to physically block invaders (Schmelzer, 2002). Indeed, Arabidopsis leaf tissue treated with flg22, fixed and

stained with aniline blue displays the accumulation of fluorescent spots, which are thought to be callose deposits (**Figure 1.3**) (Gomez-Gomez et al., 1999). It was proposed that the timing and intensity of pathogen-induced callose is dependent on abiotic growth conditions and the controlling pathways are different according to the applied MAMP (Luna et al., 2011). However the contribution of callose deposition in disease resistance has not been proven yet.

1.1.3.9 Inhibition of seedling growth

Arabidopsis seedlings show inhibition of their growth in response to MAMP treatment (**Figure 1.3**) (Gomez-Gomez et al., 1999; Zipfel et al., 2006). This reaction could indicate a possible physiological switch from a growth to a defense program. Similarly, mutations leading to constitutive activation of plant defense produce plants with stunted growth and overall reduced biomass production (Bowling et al., 1994; Rate et al., 1999).

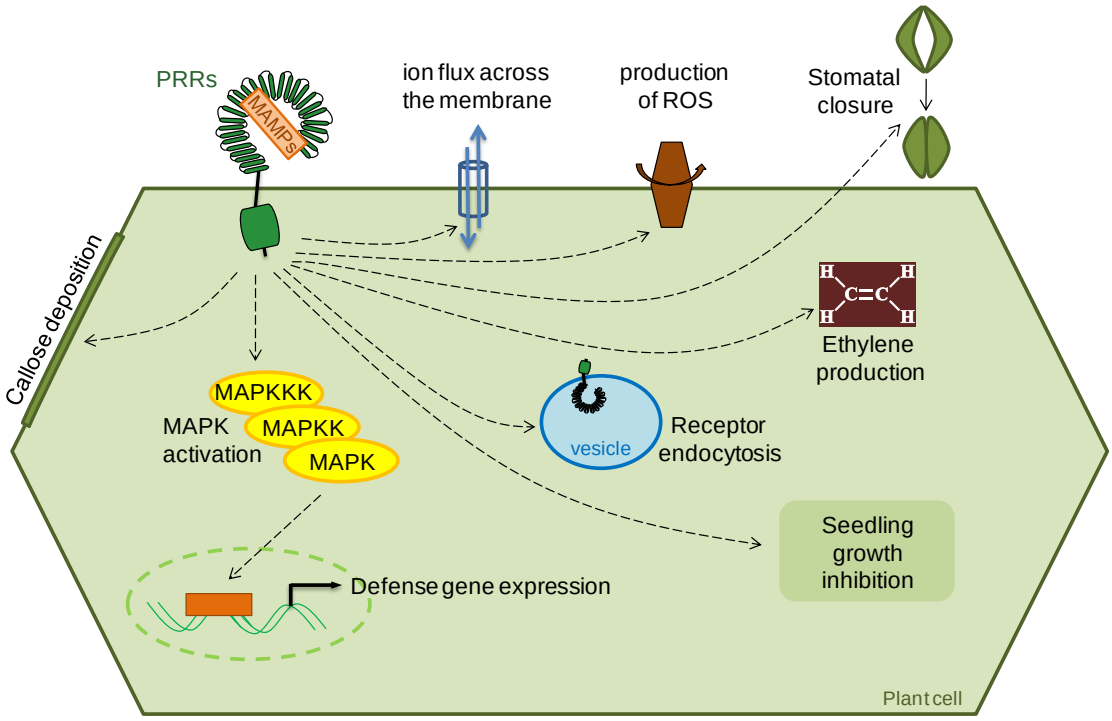


Figure 1.3: MAMP-induced defense responses. In response to microbe detection, several cellular responses are induced which are indicators for defense activation. These responses are conserved among the different perception systems. Early responses are ion fluxes across the plasma membrane,

MAPK activation, induction of defense gene expression, receptor endocytosis, production of reactive oxygene species (ROS) and production of ethylene. Later responses include callose deposition in the cell wall, closure of stomata cells and inhibition of seedling growth.

1.2 Effector-triggered immunity (ETI)

In contrast to PTI, which is triggered by common microbial features, ETI is more specific (Jones and Dangl, 2006). Microbes manipulate the immune system of their hosts through delivery of effector molecules to the plant cell (Staskawicz et al., 1984; Chisholm et al., 2006; De Wit et al., 2009). The major objectives of these effectors are to interfere with the plant immune system and promote pathogen proliferation. In turn, plants can monitor via resistance (R) proteins the presence or actions of effectors and override suppression of PTI by reinforcement of immune responses, leading to ETI (Van der Biezen and Jones, 1998; Bogdanove, 2002; Gohre and Robatzek, 2008; Boller and He, 2009) (**Figure1.1**).

1.2.1 Microbial effector molecules suppress PTI

Pathogenic as well as symbiotic bacteria evolved the ability to overcome PTI by delivering effector molecules into the plant cytoplasm through a special structure called type III secretion system (TTSS) (Viprey et al., 1998; He et al., 2004). The delivery of effectors to the plant cytoplasm through different mechanisms is also used by fungi and oomycetes to defeat plant immunity (Ellis et al., 2007; Kamoun, 2007).

Effectors secreted into the plant cell have essential roles in pathogenesis and to promote virulence (Alfano and Collmer, 2004; Nomura et al., 2005). The repertoire of individual effectors is highly variable among closely related bacterial strains, and effectors themselves can act redundantly (Kvitko et al., 2009). More than 30 effectors are delivered through the TTSS by *Pto* DC3000 with highly divergent structures and functions identified, for example as E3 ubiquitin ligase or cysteine protease, however many of them have yet unknown functions (Petnicki-Ocwieja et al., 2002; Kim et al., 2005a; Abramovitch et al., 2006). One of the main roles of effector molecules is to interfere with PTI leading to effector-triggered susceptibility (Jones and Dangl, 2006). For example, it has been reported that a *Pto* DC3000 bacterial effector AvrPtoB targets the flagellin and chitin receptor complexes to block PTI (Gohre et al., 2008;

Shan et al., 2008; Gimenez-Ibanez et al., 2009b; Zhang et al., 2010). Another example of effector inhibiting PTI signaling is the *Pto* DC3000 effector HopAI1, which interferes with the mitogen-activated protein kinase cascade to suppress PTI (Zhang et al., 2007a; Zhang et al., 2012).

1.2.2 R proteins mediate effector recognition

Effectors in general are characteristic of one given well-adapted pathogen therefore they are good targets for perception of specific pathogens by the host plant; however some effectors, for example the bacterial AvrPtoB (Jackson et al., 2002; Kim et al., 2002; Janjusevic et al., 2006; Lin and Martin, 2007) or the fungal Ecp6 (Extracellular protein 6) (Bolton et al., 2008; de Jonge and Thomma, 2009) display wide distribution and thus are reminiscent to MAMPs. Effectors are recognized directly or indirectly by R-proteins, intracellular host immune receptors, to trigger ETI (Flor, 1971; Jones and Dangl, 2006). The major class of R proteins contain a central nucleotide binding (NB) site and a C-terminal LRR domain (Meyers et al., 2003). Interestingly, NB and LRR domains are also present in CATERPILLER (or NOD-like) receptors involved in the recognition of generic MAMPs in animal innate immune system (Inohara and Nunez, 2003). Despite this structural resemblance, in Arabidopsis NB-LRR proteins are thought to participate only in effector, but not MAMP, recognition and are divided into three major classes regarding their N-terminal protein-protein interaction domains. The first common class possesses a Toll-interleukin-1-like (TIR) domain and is called TIR-NB-LRRs and the second a coiled-coil (CC) domain determining CC-NB-LRRs whereas others have no conserved N-terminal region (Meyers et al., 2003). Extensive investigation of these NB-LRRs in the last thirty years revealed that depending on their structures R proteins function in distinct disease resistance pathways (Aarts et al., 1998). At least two of these pathways were well described. The first one is *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY*)- and *PAD4* (*PHYTOALEXIN DEFICIANT 4*)-dependent and involves TIR-NB-LRRs, and the second is *NDR1* (*NONRACE-SPECIFIC DISEASE RESISTANCE*)-dependent and involves CC-NB-LRRs (Century et al., 1995; Parker et al., 1996; Glazebrook et al., 1997).

There are two major pathogen recognition modes ensured by NB-LRRs in host plants. One is the direct physical interaction between an effector molecule and an R protein where the LRR domain determines the specificity of the interaction (Van der Biezen and Jones, 1998). The other, known as the “guard hypothesis”, is based on an indirect recognition (Jones and Dangl, 2006). This model postulates that R proteins “survey” or “guard” specific host targets (or “guardees”) and activate defense if the guardee is perturbed by an effector. One possibility for this indirect recognition is that the R protein is constitutively associated to its guardee and activates defense when this association is modified by effectors. In Arabidopsis one of the best studied examples is RIN4 (RPM1 INTERACTING PROTEIN 4), which constitutively binds to the CC-NB-LRR immune receptors RPM1 (RESISTANCE TO P. SYRINGAE PV MACULICOLA 1) and RPS2 (RESISTANCE TO P. SYRINGAE 2) (Mackey et al., 2002; Mackey et al., 2003). Modification of RIN4 induced by three structurally unrelated *Pto* DC3000 effectors, namely AvrRpm1, AvrB and AvrRpt2, consequently activates RPM1 and RPS2. RIN4 degradation by AvrRpt2 de-represses RPS2, while phosphorylation of RIN4 by AvrRpm1 and AvrB activates RPM1 (Mackey et al., 2002; Axtell and Staskawicz, 2003). This example well demonstrates the robustness of the host immune system since one effector target (e.g. RIN4) can be guarded by more than one R protein, and one R protein (e.g. RPM1) can recognize the activity of more than one effector molecule. Interestingly a recent study reported that FLS2 may physically associate with RPM1 and RPS2 suggesting a signaling interaction between ETI and PTI (Qi et al., 2011). Another variant for indirect recognition is the co-called ”decoy” model (van der Hoorn and Kamoun, 2008). In this model, a duplication of the plant target gene or an independent evolution of a target mimic takes place, which then allows the recognition of the effector protein. One example is the Pto kinase, which confers resistance against *P. syringae* strains carrying AvrPto (Xiang et al., 2008). Pto is closely related to the kinase domain of PRRs targeted by AvrPto and AvrPtoB effectors (Gimenez-Ibanez et al., 2009b). In tomato it has been shown that Pto constitutively interacts with an NB-LRR, called Prf (*Pseudomonas* resistance and fenthion sensitivity) (Salmeron et al., 1996; Mucyn et al., 2006). This could indicate that Prf “guards” Pto and detects modification and/or complex formation of Pto with AvrPto/AvrPtoB (or maybe other effectors) and subsequently activates defense. In a third variant of the indirect

recognition, first the effector protein is bound to its target and as a consequence the R protein binds to its guardee (Caplan et al., 2008). While a large amount of information is available on effector recognition by NB-LRRs and the corresponding signaling pathway, little is known about how the effector recognition leads to NB-LRR activation.

Interestingly there is a big overlap between PTI and ETI signaling elements and physiological responses, indicating that plants use convergent signaling mechanisms during defense (Tao et al., 2003; Navarro et al., 2004; Denoux et al., 2008; Boller and Felix, 2009). However kinetics of these signaling events can be variable depending on the recognized elicitor molecule (Nuhse et al., 2000; Asai et al., 2002; Garcia-Brugger et al., 2006; Denoux et al., 2008). In general ETI gives rise to qualitatively stronger and faster defense reactions and often involves the hypersensitive response (HR) (Greenberg and Yao, 2004) (**Figure 1.1**). Finally, activation of local defense results in the induction of systemic acquired resistance (SAR), which confers immunity to not infected distal tissues (Conrath, 2006).

Plants have evolved large and redundant panoply of immune receptors, i. e. PRRs and R proteins, to recognize common and specific microbial features. Not only the nature of these receptors and ligands but also how microbial signals are converted into integrated defense responses leading to PTI and ETI remain primary important questions in plant research.

1.3 BAK1 is a key signaling component in immunity as well as other biological processes

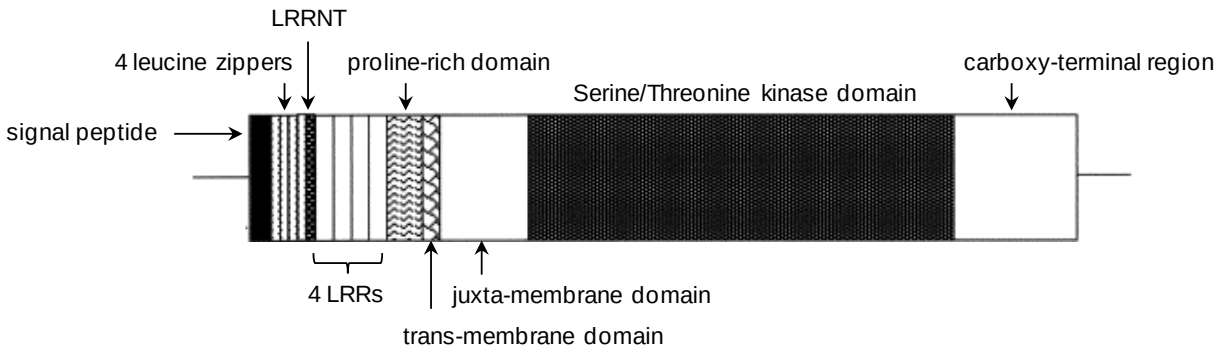
BAK1 is a member of the LRR-RLK II subfamily and within this subfamily belongs to the five member SERK (SOMATIC EMBRYOGENESIS RECEPTOR KINASE) group, therefore it is also called SERK3 (Shiu and Bleecker, 2003). SERKs were defined in Arabidopsis by their sequence homology with the *Daucus carota* (carrot) somatic embryogenesis receptor kinase (DcSERK) protein (Schmidt et al., 1997; Hecht et al., 2001). Arabidopsis SERKs share more than 86 % homology at the amino acid level and orthologs exist not only in all angiosperms but also in the moss *Physcomitrella patens*, suggesting that BAK1 has evolutionarily conserved functions in plant signaling (Boller and Felix, 2009).

1.3.1 Molecular structures of BAK1 and its family members

As shown in **Figure 1.4A** the ecto-domain of BAK1 is composed of a hydrophobic signal peptide followed by a leucine-zipper domain and a short four repeat LRR domain with two conserved glycosylation sites (Hecht et al., 2001; Boller and Felix, 2009). Within the SERK family, in SERK1 and SERK2, the LRR domain is flanked by the LRRNT and LRRCT motifs, typical for plant LRRs, including characteristic double-cysteine pairs (Hecht et al., 2001; Boller and Felix, 2009). These double-cystein motifs may be involved in processing and stability, as it was described for FLS2 (Dunning et al., 2007). In BAK1 (SERK3) and its closest homolog SERK4 (or BKK1 for BAK1-LIKE KINASE 1) and also in SERK5, the LRRCT motif is deleted (Boller and Felix, 2009; Delphine Chinchilla personal communication). The LRR domain is followed by a SERK-specific SPP (Serine-Proline-Proline) proline-rich domain, which was suggested to act as a hinge to provide flexibility to the extracellular domain (Hecht et al., 2001; Boller and Felix, 2009). A single-pass trans-membrane domain and a juxtamembrane domain are preceding an intracellular Ser/Thr kinase domain (Hecht et al., 2001; Boller and Felix, 2009). Phosphorylation and dephosphorylation of residues in the juxtamembrane domain in plant RLKs has been shown to be necessary for downstream signaling and kinase regulation (Johnson and Ingram, 2005; Wang et al., 2005b; Yoshida and Parniske, 2005; Chen et al., 2010). While FLS2 kinase domain is a non-RD kinase, the kinase domains of the SERKs contain a characteristic RD motif in their catalytic loops and display strong kinase activities, except SERK5, which has an inactive kinase due to a mutation in its kinase domain (Li et al., 2002; Dardick and Ronald, 2006). Interestingly the C-terminal tail and especially the last four amino acids (SGPR) are highly conserved within the LRR-RLK II subfamily, indicating a functional importance for this C-terminal domain (Boller and Felix, 2009) (**Figure 1.4B**). This may explain why the C-terminally tagged versions of BAK1 failed to complement *bak1* null mutants for flg22 responsiveness (Delphine Chinchilla unpublished data; Ntoukakis et al., 2011). Furthermore, SERK1 and SERK2 share just before the terminal SGPR motif seven additional amino acids, which are also conserved in SERK orthologs of other higher plants. Interestingly, the amino acids preceding the SGPR motif are different in BAK1 and SERK4

compared to SERK1/SERK2, indicating that BAK1 and SERK4 are the result of independent evolutionary events in Brassicaceae and could have specific functions (Boller and Felix, 2009).

A



B

```
SERK1          RMLEGDGLAEKWDEWQKVEILREEIDLSPN--PNSDWILDSTYNL-HAVELSGPR
SERK2          RMLEGDGLAEKWDEWQKVEVLRQEVELSSH--PTSDWILDSTDNL-HAMELSGPR
SERK3/BAK1     RMLEGDGLAERWEEWQKEEMFRQDFNYPTHHPAVSGWIIGDSTSQIENEYPSGPR
SERK4/BKK1     RMLEGDGLAERWEEWQKEEMPIHDFNYQAYPHAGTDWLIPYSNSLIENDYPSGPR
SERK5          RMLEGDGLAERWEEWQKEEMPIHDFNYQAYPHAGTDWLIPYSNSLIENDYPSGPR
consensus      **********:*:**** *:  .:.:  .   . :.*::  : .  .    ****
```

Figure 1.4: Structure of BAK1 (BRASSINOSTEROID RECEPTOR 1–ASSOCIATED KINASE 1) and its homologues from Arabidopsis. A: Schematic representation of BAK1. LRR: leucine-rich repeat. **B:** Amino acid sequence alignment of the carboxy-terminal region of Arabidopsis SERK1 to SERK5. Sequences were taken from TAIR (The Arabidopsis Information Resource) and the alignment was done with the T-Coffee multiple sequence alignment tool: http://www.tcoffee.org (Di Tommaso et al., 2011). In the consensus sequence an asterisk (*****) represents conserved amino acids in all sequences, a colon (**:**) indicates a position composed of amino acids with similar physicochemical properties, a dot (**.**) represents a position where semi-conserved substitutions are observed.

1.3.2 BAK1 regulates brassinosteroid (BR)-dependent plant growth

BAK1 was originally identified as an interacting partner of the BR receptor, BRI1 (BRASSINOSTEROID INSENSITIVE 1) (Li et al., 2002; Nam and Li, 2002). BRs are plant hormones naturally produced during various developmental processes, such as seed germination, flowering or senescence and in response to biotic and abiotic stress (Clouse, 1996; Clouse and

Sasse, 1998). Deficiency in BR perception and responses results in altered developmental phenotypes such as dwarfed stature, decreased rate of seed germination, reduced male fertility and delayed leaf senescence (Clouse et al., 1996; Szekeres et al., 1996). BRs are mainly perceived by BRI1, although two paralogs BRL1 (BRI1-LIKE 1) and BRL3 (BRI1-LIKE 3) exist in Arabidopsis (Li and Chory, 1997; Wang et al., 2001; Cano-Delgado et al., 2004). BRI1 is a LRR-RLK with a structure reminiscent of the flagellin receptor FLS2; but in contrast to FLS2, BRI1 is an RD kinase displaying strong kinase activity (Dardick and Ronald, 2006). In absence of BR, BRI1 is found as a homodimer in the plasma membrane, and its cytoplasmic domain interacts with BKI1 (BRI1 KINASE INHIBITOR 1), which prevents the association between BRI1 and its interacting partner BAK1 (Wang and Chory, 2006). Perception of BRs by BRI1 results in trans-phosphorylation events, BRI1 dimer stabilization, recruitment of BAK1 into a heteromeric complex, and activation of BR signaling (Wang et al., 2005b; Wang et al., 2005a) (**Figure 1.5**). It was proposed that upon ligand binding by BRI1 there is a reciprocal and sequential phosphorylation process between the BR receptor and BAK1. In this model, BR binding induces a basal level of phosphorylation of BRI1 (Wang et al., 2008). This first phosphorylation event occurs independently of the presence of BAK1 and results in the phosphorylation and release of BKI1 from BRI1 (Wang and Chory, 2006; Wang et al., 2008; Jaillais et al., 2011). It was proposed that in the next step BRI1 oligomerises with BAK1 via a kinase-to-kinase and extracellular domain-to extracellular domain double lock mechanism (Li, 2011). Consequently BRI1 activates BAK1 by trans-phosphorylation on residues in the catalytical loop (Wang et al., 2008). The active BAK1 then phosphorylates BRI1 on several residues within the juxtamembrane and the C-terminal domains (Wang et al., 2008). Full activation of BRI1 allows the activation of other components downstream of BRI1, such as cytoplasmic BSKs (BRI1-SIGNALING KINASES) (Tang et al., 2008) (**Figure 1.5**). BSKs activate a protein phosphatase BSU1 (BRI1 SUPPRESSOR 1), which inhibits the activity of BIN2 (BRASSINOSTEROID INSENSITIVE 2) (Kim et al., 2009). Inactivation of BIN2 induces the accumulation of two unphosphorylated transcription factors BZR1 (BRASSINAZOL-RESISTANT 1) and BES1 (BRI1-EMS-SUPPRESSOR 1), which directly mediate the expression of BR responsive genes in the nucleus (He et al., 2002; Wang et al., 2002; Yin et al., 2002; He et al., 2005; Sun et al., 2010).

Besids BAK1, other SERKs, namely SERK1, SERK2 and SERK4/BKK1, but not SERK5, are interacting partners of BRI1 in a BR-dependent manner (Kinoshita et al., 2005; Karlova et al., 2006; He et al., 2007; Gou et al., 2012; Santiago et al., 2013). Furthermore, it was shown by BRI1 and BAK1/SERK1 LRR domain crystal structure analysis that BAK1 and SERK1 LRR domains are involved in ligand perception and in the activation of BR pathway (Santiago et al., 2013; Sun et al., 2013a).

A functional redundancy of SERKs was suggested by the observation that *bak1* null mutants show only a weak *bri1*-like phenotype (Nam and Li, 2002). Indeed a recent study indicated that *serk1 bak1 bkk1* triple mutant displayed a typical null *bri1* mutant phenotype (Gou et al., 2012). This study showed that in the triple mutant the phosphorylation level of BRI1 was unresponsive to exogenous BR treatment suggesting that in contrast to the model of Wang and collaborators (2008), BAK1 and its homologs are indispensable to initiate BRI1-mediated BR signaling. Additionally, it was also reported that BRI1 protein stability is reduced in *bak1 bkk1* mutant at low BR concentration after brassinazol (a specific inhibitor of BR biosynthesis) treatment, which may indicate that SERKs prevent BRI1 from degradation (Wang et al., 2008). Interestingly, it has been shown that BAK1 is involved in BRI1 endocytosis, but in contrast to FLS2 endocytosis (Robatzek et al., 2006), the BRI1-SERKs complexes seem to undergo ligand-independent recycling, and BRI1 signaling activity remains detectable after internalization (Russinova et al., 2004; Geldner et al., 2007). Collectively these results demonstrate the crucial role of BAK1 together with its homologs in BRI1-mediated signal transduction pathway.

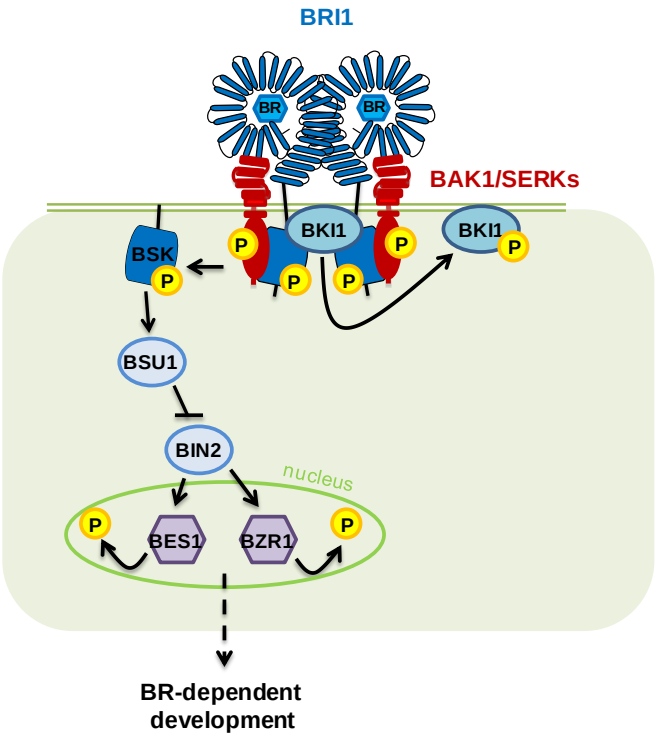


Figure 1.5: Involvement of BAK1 and its homologs in BR-dependent plant development. BRI1-mediated BR binding induces a heteromeric complex formation between the BR receptor (BRI1) and BAK1/SERKs and the trans-phosphorylation of the interacting partners (Li et al., 2002; Nam and Li, 2002; Wang et al., 2005b). Activation of BRI1 allows the phosphorylation and release of BKI1 (BRI1 KINASE INHIBITOR 1), and the phosphorylation of BSK (BRI1-SIGNALING KINASES) (Wang and Chory, 2006; Tang et al., 2008; Jaillais et al., 2011). BSK activates BSU (BRI1 SUPPRESSOR 1), which inhibits the activity of BIN2 (BRASSINOSTEROID INSENSITIVE 2) (Kim et al., 2009). Inactivation of BIN2 allows the accumulation of two transcription factors, BES1 (BRI1-EMS-SUPPRESSOR 1) and BZR1 (BRASSINAZOL-RESISTANT 1) in their unphosphorylated forms, which results in their nuclear transfer where they directly regulate the expression of BR-responsive genes (Wang et al., 2002; Yin et al., 2002; He et al., 2005; Sun et al., 2010).

1.3.3 BAK1 involvement in plant immunity

1.3.3.1 BAK1 – an important partner of many PRRs

Interestingly, in addition to its role in plant development through the enhancement of BR signaling, BAK1 was shown to be required for the signaling activity of some but not all PRRs involved in plant immunity (Chinchilla et al., 2009) (**Figure 1.5**). T-DNA insertion *bak1* mutants

are impaired in responsiveness to several MAMPs, including bacterial flg22, elf18, but not fungal chitin (Chinchilla et al., 2007; Heese et al., 2007; Gimenez-Ibanez et al., 2009a). In fact BAK1 was shown to rapidly heteromerize *in vivo* with FLS2 and EFR in a ligand-dependent manner but the chitin receptor CERK1 appeared to work independently of BAK1 (Chinchilla et al., 2007; Heese et al., 2007; Schulze et al., 2010; Roux et al., 2011) (**Figure 1.5**).

Upon ligand perception, the flagellin receptor FLS2 forms almost instantaneously (<1 second) a heteromeric complex with BAK1. The rapid interaction between FLS2 and BAK1 indicates that they may exist in close proximity in the plasma membrane (Chinchilla et al., 2007; Schulze et al., 2010). As shown by the crystal structure of FLS2 and BAK1 ecto-domains complexed with flg22, BAK1 acts as a co-receptor by recognition of FLS2-bound flg22 (Sun et al., 2013b). After the formation of the FLS2-BAK1 receptor complex the interacting partners are rapidly phosphorylated (<15 seconds) in an flg22-dependent manner (Schulze et al., 2010). Moreover application of the kinase inhibitor K-252a before flg22 treatment interfered with FLS2 and BAK1 phosphorylation but not with the heteromeric complex formation indicating that dimerization is phosphorylation independent (Schulze et al., 2010). Furthermore, other BAK1-interacting PRRs, namely EFR and PEPR1 were shown to be very rapidly phosphorylated in response to efl18 and *At*Pep1, respectively, but not chitin, further confirming that BAK1 is not required for the regulation of CERK1-dependent signaling (Schulze et al., 2010).

Congruently with these results, *bak1* null mutants are compromised in defense responses induced by *At*Pep1 (Krol et al., 2010). BAK1 was shown to interact in a ligand-independent manner with PEPR1/2 in yeast-two-hybrid assays, and simultaneous phosphorylation of BAK1 and PEPR1 was observed upon *At*Pep1 treatment indicating that *At*Pep perception mediated by PEPR1 receptor is also regulated by BAK1 (Postel et al., 2010; Schulze et al., 2010) (**Figure 1.5**). Interestingly, PEPR1 is an RD kinase, indicating that despite signaling similarities, differences may exist in early regulatory events between MAMP-induced and DAMP-induced signaling pathways (Dardick et al., 2012). BAK1 and BKK1 (see below) are the determinant elements in FLS2-, EFR-, and PEPR1/2-mediated signaling since the *bak1-5 bkk1* double mutants are almost insensitive to flg22, elf18 and *At*Pep1 treatments (Roux et al., 2011; Schwessinger et al., 2011).

BAK1 is also thought to positively regulate Ve1-mediated signaling (Ve1 is a putative PRR for the fungal Ave1 peptide) responsible for tomato *Verticillium* resistance (Fradin et al., 2009; Fradin et al., 2011; Schwessinger and Ronald, 2012). Additionally, *BAK1*-silenced *N. benthamiana* plants were less sensitive to bacterial cold shock protein csp22 and *Phytophtora infestans* elicitor INF1 suggesting that BAK1 may form ligand-dependent complexes with several other PRRs (Heese et al., 2007; Chaparro-Garcia et al., 2011).

In contrast, in xylanase-triggered signaling in tomato, BAK1 seems to have a negative regulatory role through association, *in vitro* and *in vivo*, with the decoy receptor EIX1 but not with EIX2, which is the signaling-competent receptor (Bar et al., 2010).

1.3.3.2 Other SERKs seem to be functionally redundant with BAK1 in immunity

As in BR signaling, a functional redundancy was proposed for SERK homologs in defense signaling, since *bak1* null mutant plants exhibited residual sensitivity to flg22 (Chinchilla et al., 2007; Heese et al., 2007). It was demonstrated, by co-immunoprecipitation and mass spectrometry analysis, that beside of BAK1 other SERKs are also recruited into FLS2- and EFR-complexes in a ligand-dependent manner (Roux et al., 2011). Heterologous expression of *At*SERKs in *N. benthamiana* revealed that while FLS2 interacts preferentially with BAK1, EFR seems to be less selective for a particular interacting partner the these four SERK proteins, indicating an unequal importance for SERKs in FLS2 and EFR receptor complexes (Roux et al., 2011).

1.3.3.3 More players in the BAK1-dependent signaling pathways

A new signaling element, a receptor-like cytoplasmic kinase (RLCK) BIK1 (BOTRYTIS-INDUCED KINASE 1), has been identified in the complex of FLS2 (Lu et al., 2010b). BIK1, which was previously shown to be up-regulated upon necrotrophic fungi *Botrytis cinerea* and flg22 treatments, and its paralogs PBS1 (AVRPPHB SUSCEPTIBLE 1), PBL1 (PBS-LIKE 1) and PBL2 have been found to constitutively associate with FLS2 (Veronese et al., 2006; Lu et al., 2010b; Zhang et al., 2010). It was established that BIK1 is phosphorylated approximately 2 minutes with a

peak at 10 minutes after flagellin perception, thus, after BAK1-FLS2 complex formation and the initial trans-phosphorylation events (Lu et al., 2010b). According to the authors' model, following flagellin perception BAK1 phosphorylates BIK1; then BIK1 subsequently phosphorylates FLS2 and BAK1; and finally, BIK1 is released from the FLS2-BAK1 complex (Lu et al., 2010b; Zhang et al., 2010) (**Figure 1.5**). Interestingly BIK1 is also found in constitutive association with EFR and CERK1 and gets phosphorylated upon ligand perception, indicating that BIK1 may represent a convergent signaling element between BAK1-dependent and BAK1-independent PRR complexes (Lu et al., 2010b; Zhang et al., 2010). An important role for BIK1 and PBL1 in plant immunity is supported by the findings that they are required for ethylene induced defenses, flg22-induced ROS production, stomatal defense and flg22-mediated resistance to *Pto* DC3000 (Lu et al., 2010b; Zhang et al., 2010; Laluk et al., 2011; Liu et al., 2013; Li et al., 2014).

1.3.3.4 BAK1 appears as an ideal target for bacterial effectors

Consistent with its important function in plant immunity, BAK1 is a logical target for bacterial effectors. It was reported initially that two sequence-distinct *P. syringae* effectors, AvrPto and AvrPtoB, can suppress signaling induced by flg22 as well as by other MAMPs up-stream of the MAPK cascade (de Torres et al., 2006; He et al., 2006) (**Figure 1.5**). Subsequently, it was shown that AvrPto, when overexpressed in Arabidopsis protoplasts, acts as a kinase inhibitor for FLS2 and EFR, and also prevents flg22 induced BIK1 phosphorylation and BIK1 dissociation from FLS2 (Xiang et al., 2008; Zhang et al., 2010). Because constitutive overexpression of AvrPto in Arabidopsis plants leads to a BR-insensitive phenotype it was investigated whether BAK1 is a target for AvrPto and AvrPtoB (Shan et al., 2008). In the same study it has been shown, by co-immunoprecipitation analysis in Arabidopsis protoplasts, that these two effectors interact with BAK1 and FLS2 and interfere with their heteromerisation. Moreover other groups showed that AvrPtoB targets FLS2 and CERK1 for degradation by ubiquitination (Gohre et al., 2008; Gimenez-Ibanez et al., 2009b). Finally it was recently reported by protoplast- and plant-based co-IP and BiFC (bimolecular fluorescence complementation) assays that FLS2 but not BAK1 is targeted by AvrPto (Xiang et al., 2011).

Although some of these results seem to be controversial, we can say that these two effector proteins interfere with the flagellin receptor complex to block PTI.

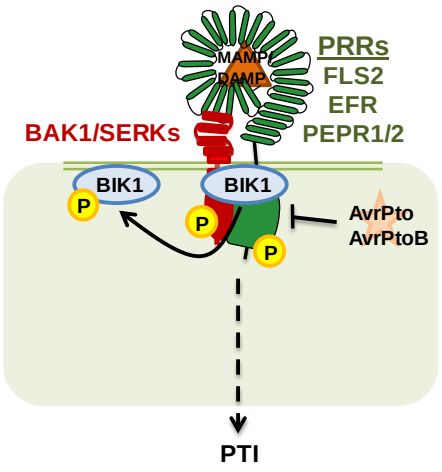


Figure 1.5: BAK1 is a central element of PTI (pattern-triggered immunity) through association with multiple PRRs (pattern recognition receptors). Upon MAMP (microbe-associated molecular pattern) or DAMP (damage-associated molecular pattern) recognition BAK1 interacts with several PRRs such as FLS2 (FLAGELLIN SENSING 2), EFR (EF-TU RECEPTOR) and PEPR1/2 (PEP RECEPTOR 1/2). After heteromerisation, the interacting partners get phosphorylated and allow the phosphorylation of downstream signaling elements in the cytoplasm such as BIK1 (BOTRYTIS-INDUCED KINASE1). As a central element of PTI the receptor complex is targeted by AvrPto and AvrPtoB, two bacterial effectors.

1.3.3.5 BAK1 is required for pathogen resistance

BAK1-silenced *N. benthamiana* plants were shown to be more susceptible to *P. syringae* and to *Hyaloperonospora arabidopsidis* (Heese et al., 2007). These effects were difficult to study in Arabidopsis *bak1* null mutants, but they were confirmed in the Arabidopsis *bak1-5* semi-dominant mutant allele, which is only impaired in defense but not in other pathways such as the BR or cell death pathways normally regulated by BAK1 (Kemmerling et al., 2007; Roux et al., 2011; Schwessinger et al., 2011). Indeed, as mentioned above, it has been demonstrated that the *bak1-5* mutant is strongly impaired in FLS2- and EFR-dependent PTI signaling including ROS production, MAPK activation and defense gene expression in response to flg22 and elf18 (Schwessinger et al., 2011). As a consequence the *bak1-5* mutant displayed hyper-susceptibility to *Pto* DC3000, which confirmed the positive role of BAK1 in disease resistance.

1.3.3.6 BAK1 has a BR-independent role in cell death control

Knock out mutants of BAK1 (*bak1-3* and *bak1-4*) displayed spreading necrosis upon infection with biotrophic bacterial pathogens. As a consequence, *bak1* mutants showed

increased susceptibility to necrotrophic fungi such as *Alternaria brassicicola* or *Botrytis cinerea* (Kemmerling et al., 2007). This effect was found to be BR-independent since exogenous application of BR did not rescue the altered cell death phenotype while it did rescue *bak1-3* growth defect. Furthermore, mutants impaired in BR perception, such as *bri1-5* (Noguchi et al., 1999) or in BR biosynthesis, such as *cbb1* (Mussig et al., 2002) did not exhibit perturbed cell death control upon pathogen infection (Kemmerling et al., 2007). BAK1 involvement together with BKK1 (SERK4) in cell death control was further supported by the light dependent stress responses and lethality observed in the *bak1 bkk1* double mutant seedlings in sterile conditions (He et al., 2007; He et al., 2008). Collectively these results indicate that BAK1 together with BKK1 (SERK4) is a negative regulator of cell death control (**Figure 1.6**).

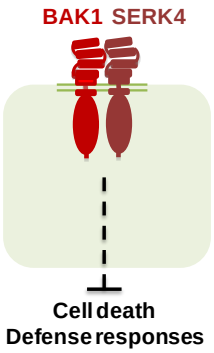


Figure 1.6: BAK1 seems to play a role in cell death control. Lack of BAK1 and its closest homolog SERK4 (SOMATIC EMBRYOGENESIS RECEPTOR KINASE 4) leads to cell death and seedling lethality.

1.3.3.7 BAK1 at the crossroad of development and immunity

A tradeoff may exist between immunity and growth, as indicated by the fact that flg22 treatment induces seedling growth inhibition (Gomez-Gomez et al., 1999). Is this due to the involvement of BAK1 both in BR-signaling for growth and in MAMP-signaling for defense? Two recent studies revealed unidirectional antagonism between BR and PTI pathways. On the one hand it has been shown that activation of BRI1 by exogenous application of BR or by genetic modification inhibited PTI responses mediated by several PRRs; on the other hand there was no direct effect of active PTI signaling on the BR pathway (Belkhadir et al., 2011; Albrecht et al., 2012). However co-immunoprecipitation analysis revealed that the amount of FLS2 recruited in the receptor complex upon flg22 treatment was equal in presence and in absence of BRs and

additionally, FLS2 and BIK1 phosphorylation was also not altered in these conditions indicating that BAK1 is not a rate-limiting factor between the two pathways (Albrecht et al., 2012).

Despite the common feature, that BR-dependent development, immunity and cell death control pathways all require BAK1, there are important kinetic and mechanistic differences. As an example, the assembly and the phosphorylation kinetics of FLS2-BAK1 (seconds) and BRI1-BAK1 (20-90 minutes) are highly different (Li et al., 2002; Nam and Li, 2002; Chinchilla et al., 2007; Schulze et al., 2010). Moreover, it is still unknown whether the regulatory activity of BAK1 in cell death control requires or not a ligand-binding receptor (Kemmerling et al., 2007). Another important difference is that, while FLS2 and EFR are non-RD kinases and for heteromerisation with BAK1 the kinase activity of the interacting partners is not needed, BRI1 is an RD kinase and for its heteromerisation with BAK1 the kinase activity is required (Dardick and Ronald, 2006; Schulze et al., 2010; Clouse, 2011; Schwessinger et al., 2011). In addition, C-terminally tagged versions of BAK1 are impaired in PTI functions but not in BR-signaling (Ntoukakis et al., 2011). How BAK1 functional specificity is determined in distinct biological processes, which are often active simultaneously in the same cell, is still elusive. The identification and characterization of BAK1 phosphosites and new *BAK1* mutant alleles, such as *bak1-5*, point into the direction that differential phosphorylation of BAK1 might be decisive to trigger appropriate responses to a given environmental stimulus (Wang et al., 2008; Oh et al., 2010; Schwessinger et al., 2011). Further investigations of BAK1-dependent pathways are required to fully understand the role of BAK1 in plant immunity and development.

1.4 Aims of the thesis

The plant LRR-RLK BAK1 has been identified as an interacting partner of ligand-binding LRR-RLKs, in particular the hormone receptor BRI1 and the immune receptor FLS2 (Li et al., 2002; Nam and Li, 2002; Chinchilla et al., 2007; Heese et al., 2007). Through physical association with its interacting partners, BAK1 emerged as a positive regulator of BR-dependent plant development and PRR-mediated innate immunity (Li et al., 2002; Nam and Li, 2002; Chinchilla et al., 2007; Heese et al., 2007). Additionally, BAK1 was proposed to control BR-independent cell death pathways (He et al., 2007; Kemmerling et al., 2007). Interestingly, it has been

demonstrated that flg22 inhibits growth of Arabidopsis seedlings (Gomez-Gomez et al., 1999). This is reminiscent to the phenotype presented by *bri1* knockout mutants (Li and Chory, 1997). This observation raised the question whether BAK1 recruitment by FLS2 during PTI activation prevents or removes BAK1 from the BRI1-BAK1 complex and thus inhibits BRI1 signaling. Thus, our first idea was to increase the BAK1 abundance, which might suppress the flg22-mediated seedling growth inhibition by relieving the possible suppression of BRI1 signaling. Accordingly, *BAK1* was overexpressed under the control of a strong constitutive promoter. Surprisingly, the generated transgenic *BAK1* expressing plants already showed growth impairments and necrosis without any elicitor treatment and finally died before setting seeds (Delphine Chinchilla, unpublished results). To study this astonishing effect of *BAK1* overexpression more deeply, an inducible expression system was established in order to check the presence, properties and kinetics of the defense responses including cell death triggered by *BAK1* expression.

Additionally, two homologs of BAK1, SERK1 and SERK4, were overexpressed using the same inducible system, to determine if they can have similar effects as BAK1. Since plants defective in *BAK1* showed only reduced sensitivity to flg22 and other MAMPs (Chinchilla et al., 2007), it was proposed that functional redundancy may exist between BAK1 and the other members of the SERK protein family in MAMP signaling as it was also proposed for the BR pathway.

To study genes potentially involved in the *BAK1* overexpression phenotype the same construct was transformed into different mutant backgrounds of Arabidopsis and analyzed. First, the influence of mutations of BAK1-interacting partners (e.g. PRRs) on the BAK1 overexpression phenotype was checked. Furthermore, since BAK1 is a central regulator of PTI- and BR-signaling and highly conserved within the plant kingdom, it might be a good target for pathogen effectors and thus could be involved in ETI-signaling (Shan et al., 2008). Therefore we also wanted to study the effect of ETI mutants such as mutants of *R* genes, and regulators of *R* genes or mutants defective in SA signaling on the BAK1 overexpression phenotype.

In the second part of my thesis we were interested to find out which part of BAK1 is required for the BAK1 overexpression phenotype. To answer this question, defense responses

were analyzed comparatively in *A. thaliana* plants overexpressing full-length BAK1 and BAK1-derived constructs.

I hope that with this study I can contribute to the better understanding of the role of BAK1 in immunity and its impact on development. This knowledge may be helpful in the future to improve disease resistance in important crop plants without negative effects on plant development and yield.

2 MATERIAL AND METHODS

2.1 Plant material

2.1.1 *In vitro* conditions for *Arabidopsis thaliana*

Seeds were sterilized twice with 70% EtOH (ethanol) and once with 100% EtOH for one minute. Seeds were sown on solid Murashige and Skoog salt medium (Sigma), 1% sucrose and 0.8% agar at pH 5.7 and vernalized during two days at 4°C in the dark before transferring them to continuous light at 20°C for germination.

2.1.2 *A. thaliana* "short day" conditions

Seeds were sterilized as described above (2.1.1), sown directly on soil and then vernalized for two days at 4°C in the dark. Pots were placed in the following short day conditions: ten hours light at 21°C / 14 hours dark at 18°C with 60% humidity. Plants were grown as one plant per pot. Plants grown in these conditions for six weeks were used for bioassays.

2.1.3 "Long day" conditions for *A. thaliana*

Seeds were sterilized as described above (2.1.1), then vernalized for two days in the dark at 4°C and then placed in 16 hours light at 21°C / 8 hours dark at 18°C with 55% humidity. Alternatively adult plants were transferred from short day conditions in long day conditions. Plants were grown as one plant per pot. Plants grown in these conditions were used for floral dip transformation, crossing and seed production.

2.2 Bacteria

2.2.1 *Escherichia coli*

E. coli DH5α was used for cloning plasmids. These bacteria were cultivated on LB (Luria-Bertani) medium (tryptone 1%, yeast extract 0.5%, NaCl 1% and if solid medium agar 15 g/l) supplemented with appropriate antibiotics overnight at 37°C.

2.2.2 Preparation of competent *E. coli* cells

Competent *E. coli* cells were prepared according to the "The Inoue Method for Preparation and Transformation of Competent *E. coli*: "Ultra Competent" Cells" described in the Maniatis laboratory manual (Maniatis, T., Fritsch, E.F. and Sambrook, J., 1982).

2.2.3 Transformation of competent *E. coli* DH5α cells

Competent cells (stored at -80°C) were defrosted on ice. 5 µl (maximum) of the DNA suspension were added to 50 µl of competent cells and incubated on ice for 30 minutes. The mixture was heat-shocked at 42°C for 90 seconds and placed back on ice for three minutes. 1 ml LB medium without antibiotic(s) was added and bacteria were incubated for 1 hour at 37°C. Thereafter, the suspension was centrifuged at 3000 rpm for 5 minutes (bench centrifuge) and 950 µl of supernatant were removed. The pellet was resuspended gently in the remaining 100 µl of supernatant and plated on LB medium containing the appropriate antibiotic(s). Plates were incubated overnight at 37°C.

2.2.4 *Agrobacterium tumefaciens*

A. tumefaciens GV3101 was used for stable transformation of *A. thaliana* plants and for transient transformation of *N. benthamiana* leaves. This strain contains a Ti (tumor inducing) plasmid whose oncogenes were deleted but still has the virulence (*vir*) genes responsible for mediating transduction of T-DNA to the plant cell genome. This strain is resistant to Rifampicin. *A. tumefaciens* was cultivated on YEB (Yeast Extract Beef) medium (beef extract 0.5%, yeast extract 0.1%, trypton 0.5%, sucrose 0.5%, and if solid medium agar 15g/l) with appropriate antibiotics at 28°C.

2.2.5 Preparation of competent *A. tumefaciens* cells

One colony from a fresh plate was pre-cultured in 5 ml YEB medium supplemented with Rifampicin for two days. Fifty µl of this pre-culture were used to inoculate 50 ml YEB medium supplemented with Rifampicin and incubated at 28°C until OD_{600} = 0.5-1. The bacterial culture was transferred in pre-chilled 50 ml tube and centrifuged at 3000 x *g* for 10 minutes. The pellet was dried and resuspended in 1 ml pre-chilled $CaCl_2$ 20 mM. Competent *A. tumefaciens* cells were stored at -80°C.

2.2.6 Heat-shock transformation of chemical competent Agrobacteria cells

Hundred µl of competent cells were mixed with 300 ng DNA and incubated on ice for 30 minutes. The mixture was snap-frozen in liquid nitrogen and then incubated five minutes at 37°C. 1 ml of YEB medium without antibiotics was added and bacteria were incubated two hours at 28°C. The bacterial suspension was centrifuged at 3000 rpm for 5 minutes and then the pellet was resuspended in 100 µl of supernatant. The bacteria were plated on YEB medium containing appropriate antibiotics and incubated two days at 28°C.

2.2.7 Glycerol stocks and storage of bacteria

One ml of bacterial culture (*E. coli* or *A. tumefaciens*) was mixed with 1 ml of 50 % glycerol and snap-frozen in liquid nitrogen. Glycerol stocks were kept at -80°C for further usage.

2.3 Antibiotics

Antibiotics were used for selection of bacteria or transgenic plant material. Stock solutions were kept at -20°C.

Antibiotic	Dissolved in	Stock concentration	Final concentration
Gentamicin	H_2O	25 mg/ml	25 µg/ml
Rifampicin	DMSO	50 mg/ml	100 µg/ml
Kanamycin	H_2O	50 mg/ml	50 µg/ml
Spectinomycin	H_2O	100 mg/ml	100 µg/ml
Hygromycin B	H_2O	25 mg/ml	25 µg/ml

2.4 Solutions used for bioassays

2.4.1 β-Estradiol

β-Estradiol (Sigma, E2758; powder stored at 4°C) was dissolved in EtOH 100% to a stock concentration of 10 mM and was stored at -20°C. β-Estradiol was used as inducer for the *XVE* promoter.

2.4.2 Elicitors

Peptides of flg22 (QRLSTGSRINSAKDDAAGLQIA) from *Pseudomonas aeruginosa* (Felix et al., 1999), and elf18 (ac-SKEKFERTKPHVNVGTIG) from *E. coli* (Kunze et al., 2004) and chitin from crab shells (SIGMA) were used as elicitors in bioassays. Flg22 and elf18 were obtained from EZBiolab and were dissolved in ddH_2O to stock solutions of 10 mM. "In use" flg22 and elf18 dilutions were made in BSA 0.1% and NaCl 0.1 M. Chitin (Sigma) was diluted in water to a stock solution of 1 mg/ml.

2.4.3 Treatments for bioassays

Estradiol treatment: Addition of 2 µl β-Estradiol of a stock solution (100 µM) and 2 µl BSA 0.1% NaCl 0.1 M per 200 µl ddH_2O.

Flg22 treatment: Addition of 2 µl flg22 of a stock solution (100 µM) and 2 µl ethanol (100%) per 200 µl ddH_2O.

Double treatment: Addition of 2 µl β-Estradiol of a stock solution (100 µM) and 2 µl flg22 of a stock solution (100 µM) per 200 µl ddH_2O.

Elf18 treatment: Addition of 2 µl elf18 of a stock solution (100 µM) and 2 µl ethanol (100%) per 200 µl ddH_2O.

Control treatment: Addition of 2 µl ethanol (100%) and 2 µl BSA 0.1% NaCl 0.1 M per 200 µl ddH_2O.

2.5 Primers

Primers were obtained from Microsynth AG (Balgach, Switzerland) without special modifications and diluted in ddH_2O to 100 µM. For cloning, colony PCR, site directed mutagenesis and sequencing reactions primers were used at 5 µM concentration and for qRT-PCR analysis at 100 µM concentration. Beacon Designer 2.0 (http://www.premierbiosoft.com/molecular_beacons/index.html) was used to design primers for qRT-PCR analysis with target T_m= 60°C +/- 1°C; primer length range from 18 to 25 bp (base

pairs) and amplicon length from 100 to 250 bp. The sequence of all primers used can be found in **Tables 2.1** and **2.2**.

2.6 Methods for molecular biology

2.6.1 Colony PCR

Colonies were picked with a pipette tip and resuspended in 50 µl of ddH_2O. 1 µl of the suspension was used for PCR reaction. One vector specific primer and one construct specific primer were chosen to amplify a DNA fragment between 400 bp and 1 kb.

PCR reagents		**PCR program**			
DNA	1 µl	denaturing	94°C / 6’	94°C / 20’’	
buffer FirePol	1 µl	hybridization		55°C / 30’’	
dNTP (10 µM)	0.2 µl	elongation		72°C / 1’	72°C / 5’
primer A (5 µM)	0.2 µl	number of cycles	1	30	1
primer B (5 µM)	0.2 µl				
DNA polymerase *	0.1 µl				
ddH_2O	7.3 µl				

*: FirePol DNA Polymerase (Solis Biodyme, Tartu, Estonia; 0.5 U/µl); ’: minute; ’’: second.

2.6.2 Analysis of nucleic acid by gel electrophoresis

Nucleic acids were placed on a 1% agarose gel containing 0.1 µg/ml EtBr (Ethidium bromide). After electrophoresis (at 100 mV for 20 minutes) in TAE buffer (Tris-HCl 50 mM, pH 8.0, acetic acid 20 mM, EDTA 0.5 mM) amplified DNA fragments, genomic DNA or total RNA were detected under ultraviolet light and sizes were established by comparison with a commercial gene ruler (GeneRuler^{TM} 1kb DNA Ladder, Fermentas, #SM0311).

2.6.3 Generation of constructs for plant transformation

Target genes or specific pars of target genes were amplified by PCR from gDNA (genomic DNA) to be cloned using Gateway® Technology (Invitrogen). Specific primers were

designed and amplification conditions were established according to the manufacturer's instructions.

PCR reagents	1st PCR reaction for Gateway® cloning	2nd PCR reaction for Gateway® cloning
gDNA	≈ 120 ng	10 µl from the 1st PCR reaction
5x Phusion GC Buffer (Finnzymes)	10 µl	8 µl
dNTP (10 µM)	1 µl	1 µl
primer A (5 µM)	1 µl	4 µl
primer B (5 µM)	1 µl	4 µl
Phusion™ High-Fidelity DNA Polymerase (Finnzymes)	0.5 µl	0.5 µl
ddH_2O to	50 µl	40 µl

primers A/B: in the 1st PCR reaction these are specific primers for the gene of interest fused to *att*B sites (**Table 2.1**); in the 2nd PCR reaction they correspond to "Att adaptater fwd" / "Att adaptater rev".

PCR programs	First PCR reaction for Gateway® cloning (temperature / time)		Second PCR reaction for Gateway® cloning (temperature / time)		
denaturing	95°C / 2'	94°C / 15''	95°C / 1'	94°C / 15''	94°C / 15''
hybridization		58°C / 30''		45°C / 30''	58°C / 30''
elongation		72°C / *		72°C / *	72°C / *
number of cycles	1	10	1	5	15

*: time dependent on the amplicon length; ': minute; '': second.

To purify the PCR product it was mixed by vortexing with three volumes of TE buffer (10 mM Tris HCl + 1 mM EDTA) pH 8.0 and two volumes of PEG (30% PEG (Polyethylene glycol), 30 mM $MgCl_2$) and centrifuged (Centrifuge 3510R, Eppendorf) at 20800 x *g* for 30 minutes at 20°C. The supernatant was removed carefully and the pellet was resuspended in 10 µl of TE pH 8.0.

The purified *att*B-PCR product (gene of interest) was introduced by BP recombination reaction to the entry vector *pDONR*^{TM}207 (Invitrogen). Competent *E. coli* DH5α cells were transformed with this reaction and positive colonies were identified by colony PCR. After extraction of the recombined entry clone by miniprep (NucleoSpin® Plasmid QuickPure, Macherey-Nagel) the insert was sequenced (Genetic Analyzer 3500, Applied Biosystems). Sequencing data were analyzed with SeqMan^{TM}II (DNAStar, http://www.dnastar.com/) software.

In the next step an LR recombination reaction was performed to transfer the insert in the destination vector which was subsequently transformed in competent *E. coli* DH5α cells as previously described (2.2.3). Positive colonies were identified by colony PCR.

BP recombination reaction		**LR recombination reaction**	
*att*B-PCR product	3.5 µl	Entry clone	1 µl (≈100-300 ng)
pDONR207^{TM}	0.5 µl (≈150 ng/µl)	Destination vector (pMDC32^{TM}, pMDC7^{TM})	1 µl (≈150 ng/µl)
BP Clonase^{TM} enzyme mix	1 µl	LR Clonase^{TM} enzyme mix	0.5 µl
Final volume	5 µl	Final volume	2.5 µl
Incubate reaction at 25 °C for one hour			
Addition of 0.5 µl Proteinase K (Invitrogen)			
Incubate reaction at 37 °C for ten minutes			
Transformation of competent *E. coli* DH5α cells			

Destination vectors containing the gene of interest were extracted from DH5α cells by miniprep and transformed into competent Agrobacteria cells as previously described (2.2.6).

For generation of transgenic plants, *pMDC7*^{TM}, *pMDC32*^{TM} (**Figure 2.1**), pMDC83^{TM} and pK7FWG2^{TM} were used as destination vectors to express BAK1, BAK1-derived constructs, SERK1,

SERK4 and FLS2 under the control of the inducible *XVE* promoter (Zuo et al., 2000) or the constitutive double or single *35S* CaMV promoter (Odell et al., 1985).

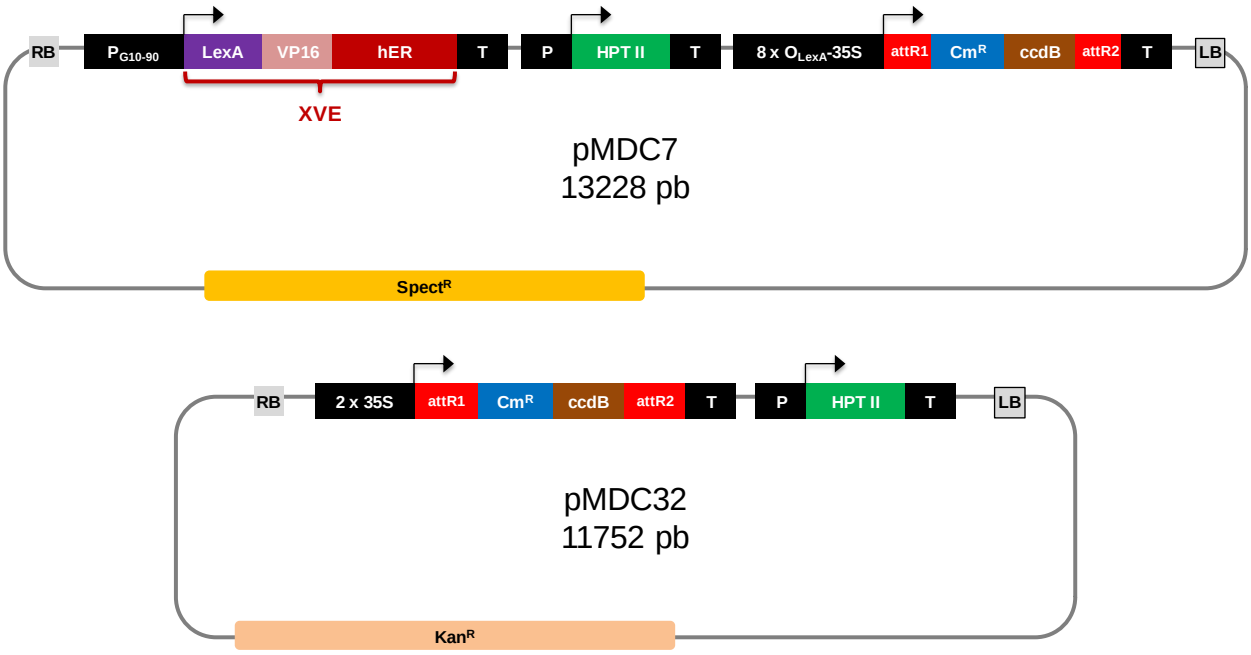


Figure 2.1: Schematic representation of *pMDC7*™ vector with inducible XVE system and *pMDC32*™ vector. RB: T-DNA right border; ***P*$_{G10-90}$:** synthetic promoter controlling XVE; ***XVE*:** coding sequence of a chimeric transcription factor; ***Lex*A:** DNA-binding domain of bacterial repressor LexA; **VP16:** transcriptional activation domain of VP16 protein from herpes simplex virus; **hER:** interaction domain of estradiol from human estrogen receptor; **T:** terminateur; **P:** promoter; ***HPT II*:** coding sequence of hygromycin phosphotransferase II plant-selectable marker gene; ***8 x O*$_{LexA}$*-35S*:** eight copies of LexA operator sequence fused to *35S* promoter; ***att*R1, *att*R2:** Gateway® recombination sites; ***Cm*^{R}:** Chloramphenicol resistance gene; ***ccd*B:** allows negative selection of the destination vectors in *E. coli*; **LB:** T-DNA left border; ***Spect*^{R}:** Spectinomycin resistance gene; ***2 x 35S*:** two copies of *35S* promoter; ***Kan*^{R}:** Kanamycin resistance gene; **bp:** base pair. Arrows indicate the direction of transcription.

The inducible XVE system is composed of several transcriptional units. The first is the chimeric *XVE* transcription factor (*Lex*A, VP16, hER) under the control of a constitutive promoter *P*$_{G10-90}$. The second transcriptional unit corresponds to eight copies of *Lex*A bacterial operator fused to *35S* promoter (*8 x O*$_{LexA}$*-35S*) which controls the expression of the insert recombined between the *att*R Gateway® sites. These two transcriptional units are separated by the coding sequence of hygromycin phosphotransferase II (HPT II). When estradiol binds to the *XVE* transcription factor, transcription of the insert is activated.

2.6.4 Quantification of acid nucleic

For quantification and verification of purity of acid nucleic (DNA, RNA) a NanoDrop 2000 (Thermo-Scientific) was used according to the manufacturer's instructions.

2.6.5 Site directed mutagenesis

For single amino acid mutation *in vitro* site directed mutagenesis was performed using as template *pDONR^{TM}207* constructs. A pair of complementary mutagenic primers was designed with the desired mutation in the middle of the primer and with a T_m (melting temperature) equal or higher than 78°C to amplify the entire plasmid by PCR using Phusion^{TM} High-Fidelity DNA Polymerase (Finnzymes). The following formula was used to calculate the T_m of primers:

T_m =81.5 + 0.41(%GC) – 675/*N* – % mismatch (*N*: primer length in bases)

The methylated template plasmid extracted from *E. coli* was digested using *Dpn* I (Stratagene) enzyme treatment (1 hour at 37°C) while the mutated plasmid synthesized *in vitro*, therefore unmethylated, remained undigested. The nicked vector containing the desired mutation was then transformed into competent *E. coli* DH5α cells for multiplication. The entire insert was verified by sequencing. Sequencing data were analyzed by SeqMan^{TM}II (DNAStar, http://www.dnastar.com/) software.

2.7 Stable transformation of *A. thaliana* plants

Stable transformants of Arabidopsis plants were obtained by floral dip procedure (Clough and Bent, 1998). Agrobacteria containing the DNA construct of interest were cultured in YEB medium overnight at 28°C and then centrifuged (Megafuge 1.0R, Heraeus Instruments) at 3345 x *g* for 10 minutes at 23°C. The pellet was resuspended gently in 5% sucrose solution and 0.02% Silvett L-77 (Lehle Seeds, USA) to a density of OD_{600} = 0.8. Inflorescences of flowering plants were submerged for 15 seconds in the bacterial solution. Plants are placed into long day conditions under cover to provide a high humidity environment for one day and then without cover until seed ripening. T1 transformants were selected *in vitro* on MS medium plates supplemented with Hygromycin B.

2.8 Analysis of transgene expression

Fifty mg of plant material (seedlings or leaves) were frozen in liquid nitrogen, ground with glass beads (d=2 mm, Roth) in a mixing device (Silamat® S6, Ivoclar Vivadent®) and resuspended in 100 µl of cold extraction buffer (50 mM Tris-HCl pH 8, 50 mM NaCl) and 1 µl protease inhibitor cocktail (Sigma-Aldrich, P9599). After denaturing in SDS-loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% Bromophenol blue, 20% Glycerol, 5% β-mercaptoethanol) at 95°C for 5 minutes, equal amounts of proteins were separated on a 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and analyzed by Western blot. Prestained Protein Marker, Broad Range (BioLabs, P7708L) was used to determine the size of the proteins revealed on the membrane.

Primary antibodies	
Rabbit anti-BAK1 (dilution 1/300)	Polyclonal antibody (Schulze et al., 2010)
Rabbit anti-FLS2 (dilution 1/300)	Polyclonal antibody (Chinchilla et al., 2006)
Rabbit anti-phospho-p44/42 MAPK (Erk1/2) (dilution 1/1000)	Monoclonal antibody; Cell Signaling Technology
Mouse anti-GFP (dilution 1/1000)	Monoclonal antibody; Roch Applied Science
Secondary antibody	
Goat anti-rabbit IgG alkaline phosphatase conjugate (dilution 1/30000)	Polyclonal antibody; Sigma-Aldrich A3687
Goat anti-mouse IgG alkaline phosphatase conjugate (dilution 1/30000)	Polyclonal antibody; Sigma-Aldrich A3562

A chemo-luminescent substrate, CDP-Star (Roch Applied Science, 11759051001), was used to detect rapidly alkaline phosphatase (secondary antibody) labeled molecules. This technique is extremely sensitive as a result of low background luminescence coupled with high intensity and prolonged light output from the enzyme catalysis. Image acquisition for luminescence detection was performed using ChemiDoc videoscopy (BioRad).

2.9 Detection of MAPK activation

Arabidopsis seedlings germinated for six days on MS-agar plates *in vitro* were transferred to 200 µl ddH_2O as five seedlings per well in 24 well plates and were placed back into *in vitro* conditions overnight. Seedlings were treated with 1 µM estradiol or equal volume of ethanol (negative control) for three, four, six, eight, ten and 27 hours. MAPK activation was analyzed by Western blot using anti-phospho-p44/42 MAPK (Erk1/2) antibodies (Cell Signaling Technology) which recognize phosphorylated MAPKs.

2.10 Analysis by quantitative real-time PCR (qRT-PCR)

Six-day-old *A. thaliana* seedlings germinated *in vitro* were transferred to 200 µl ddH_2O as 15 seedlings per well in 24 wells plates. On the next day seedlings were treated, frozen in liquid nitrogen and ground with autoclaved mortar and pestle. Total RNA was extracted from 80 mg of plant material by NucleoSpin® RNA Plant kit (Macherey-Nagel). Quality of RNAs was checked by gel electrophoresis. An additional DNase treatment was performed on 5 µg of RNA (dissolved in 30 µl RNase free water) for each sample using the following conditions:

DNase treatment	
Five µg RNA	30 µl
DNase buffer (Fermentas)	5 µl
DNase I (Fermentas)	5 µl
RNasin® Ribonuclease Inhibitor (Promega)	0.5 µl
H_2O RNase free (Promega)	9.5 µl

The reactions were centrifuged for five seconds and incubated at 37°C for 30 minutes. Five µl of EDTA (Fermentas) was then added to each of the reactions, and they were incubated at 65°C for ten minutes. In a next step, nucleic acids were precipitated using 1/10 volume of 3 M sodium acetate pH 4.8 and 2.5 volumes ethanol 100% overnight at -20°C. Samples were centrifuged (Centrifuge 3510R, Eppendorf) at 20,800 x *g* for 30 minutes at 4°C and the pellets were washed with 200 µl EtOH 70% by vortexing. After 10 minutes centrifugation at 20,800 x *g*

at 4°C, supernatants were discarded and the pellets were dried by a speed vacuum device. RNAs were resuspended in 20 µl of RNase free water.

One microgram DNase treated RNAs were reverse transcribed to cDNA using AMV reverse transcriptase. In a first step RNA, secondary structures were resolved for two minutes at 75°C. Subsequently the RT reaction was conducted as described below:

RT reaction		**RT program**
One µg RNA	8.4 µl	20°C / 10’ 42°C / 15’ 56°C / 2’ 95°C / 5’
AMV Reverse Transcriptase 5X Reaction Buffer (Promega)	5 µl	
$MgCl_2$ (25 mM)	5 µl	
oligo dT (25 µM)	2.5 µl	
dNTP (25 mM)	2.5 µl	
AMV Reverse Transcriptase (Promega)	0.8 µl (30 units)	
RNasin® Ribonuclease Inhibitor (Promega)	0.8 µl	

’: minutes

After stopping the RT reactions by heating at 95°C for 5 minutes, 25 µl of ddH_2O were added to each reaction.

To verify the absence of gDNA contamination, a PCR reaction was performed on synthesized cDNAs and on Col-0 gDNA. Primers (RPL4for and RPL4rev) were chosen to bind on a housekeeping gene and cross an intron. The amplification product was resolved using agarose gel electrophoresis (II.6.2) to assess size differences.

PCR reagents		**PCR program**			
buffer FirePol	1.5 µl	Denaturing	94°C / 5’	94°C / 1’	

dNTP (10 µM)	1.8 µl	Hybridization		57°C / 1’	
RPL4for (5 µM)	1.5 µl	Elongation		72°C / 1’	72°C / 5’
RPL4rev (5 µM)	1.5 µl	number of cycles	1	35	1
DNA polymerase *	0.08 µl				
DNA	0.5 µl				
ddH_2O	8.12 µl				

*: FirePol DNA Polymerase (Solis Biodyme, Tartu, Estonia; 0.5 U/µl); ’: minute.

All cDNA samples were diluted 100 times to perform qRT-PCR reactions. In this technique, the amplified DNA is quantified in real time as the reaction progresses. For standard curves, cDNAs from all samples were pooled and further diluted 50, 100, 200 times as described:

Standard curve dilutions	50	100	200
H_2O	686 µl	300 µl	200 µl
cDNA	14 µl (1.75 µl of each cDNA)	300 µl (from the “50” dilution)	200 µl (from the “100” dilution)
final volume	700 µl	600 µl	400 µl

For one qRT-PCR reaction, 5 µl cDNA, 7.3 µl ddH_2O, 12.5 µl Power SYBR® Green PCR Master Mix (Applied Biosystems) and 0.1 µl (0.4 µM) of each primer (Table 2) were used. For all samples three technical replicates were prepared.

qRT-PCR program	temperature / time	number of cycles
holding stage	50°C / 2’	1
holding stage	95°C / 10’	1
cycling stage	95°C / 15’’ 60°C / 1’	40

melt curve stage	95°C / 15'' 60°C / 1' 95°C / 30'' 60°C / 15''	1

': minute; '': second.

The experiments were performed with an ABI 7500 Real-Time PCR instrument (Applied Biosystems) and data were analyzed by 7500 Software v2.0.5. Data obtained for the selected genes were normalized to the housekeeping gene *EIF4a* (Boudsocq et al., 2010). Final results are the mean of at least three biological replicates.

2.11 Analysis of ethylene biosynthesis by gas chromatography

2.11.1 *A. thaliana* seedlings

Whole six-day-old seedlings germinated *in vitro* were placed in 7 ml glass tubes in 100 µl ddH_2O as five germinations (≈5 mg fresh weight) per tube. Open tubes with plant material were placed overnight into *in vitro* light conditions and treated as indicated in the figure legends. Assay tubes were closed with rubber septa and ethylene accumulating in the free air space was measured by gas chromatography (GC-14A Shimadzu) after three hours of incubation.

2.11.2 *A. thaliana* leaf strips

Leaf strips about 1 mm thick were cut from four-week-old *A. thaliana* plants, grown in short day conditions. Two leaf strips (≈15 mg) per tube were placed in 7 ml glass assay tubes in 500 µl ddH_2O. Either estradiol with 1 µM final concentration or ethanol was added in tubes. Open tubes with prepared plant material were placed overnight in "short day" conditions. The next morning flg22 treatment to 1 µM final concentration or the appropriate control solution was added in adequate tubes. Four technical replicates were performed per treatment. Ethylene accumulation was measured in the same way as described above for seedlings by gas chromatography (GC-14A Shimadzu) after three hours incubation.

2.12 Mesophyll cell death detection by trypan blue staining

Seedlings grown for six days *in vitro* were transferred to liquid MS medium (two seedlings per well) containing estradiol, elf18 or control solution (described above). For each treatment twelve seedlings were stained. After three days incubation with the different treatments under continuous light, seedlings were placed in 15 ml Falcon tubes and covered with 200 µl of Trypan blue staining solution (one volume ddH_2O, trypan blue 0.067%, one volume lactic acid 90%, one volume glycerol 99.5%, six volumes EtOH 100% and one volume phenol 90%). Tubes were placed in boiling water for one minute and cooled down for two hours. Staining solution was replaced by 1 ml of clearing solution (chloral hydrate 2.5 g/ml) and samples were placed on a shaker to distain tissues. Clearing solution was changed regularly until the seedlings stopped to release blue color. After transferring them to 60% glycerol, seedlings were examined by light microscopy (microscope: Axioplan, ZEISS; camera: Olympus DP70).

2.13 Seedling growth inhibition assay

Six-day-old *A. thaliana* seedlings germinated *in vitro* were transferred in liquid MS medium (one seedling per well) supplemented either with estradiol, flg22, flg22 plus estradiol or a control solution. For each treatment six technical replicates were performed. After six days images were taken and the seedling fresh weight and main root length was measured.

2.14 Pathogen growth assay

Seedlings grown for six days *in vitro* were transferred in 15 ml of water supplemented either with estradiol, flg22 or control solution. After 24 hours the seedlings were inoculated with *Pseudomonas syringae* pv *tomato* DC3000 (OD_{600} = 0.01). Seedlings were incubated with the bacteria for 1.5 hours at 22°C. Seedlings were then washed 3 times with ddH_2O and transferred back to fresh tubes containing the appropriate supplements until measurement. Before the measurements seedlings were surface sterilized with 70% ethanol, and 30 seedlings were ground in 100 µl ddH_2O per replicate for the first time point or 10 seedlings per replicate were used for time point 48 h. From the ground suspensions, serial dilutions were prepared and 10 µl were plated on YEB-plates supplemented with antibiotics. The number of colonies was determined after 4 hours for time point 0 dpi (day post-inoculation) and 2 days later for time

point 2 dpi. Number of colonies obtained was represented as cfu (colony forming units) per 10 seedlings.

3 RESULTS

3.1 Chapter 1: BAK1 overexpression leads to a constitutive defense response in *Arabidopsis thaliana*

3.1.1 Abstract

Plants employ membrane localized pattern recognition receptors (PRRs) to sense widely conserved microbe-associated molecular patterns (MAMPs) in their surrounding environment. In Arabidopsis, detection of MAMPs like flagellin or elongation factor Tu by specific PRRs called FLS2 and EFR, respectively, initiates pattern-triggered immunity (PTI). BRI1-ASSOCIATED KINASE 1 (BAK1) positively regulates PTI by interacting with FLS2 and EFR. In addition, BAK1 is an enhancer of brassinosteroid (BR)-dependent development through interaction with the BR receptor BRI1, and a negative regulator of cell death control. Here, we overexpressed BAK1 to better understand its role in innate immunity. We demonstrate that up-regulation of BAK1 leads to MAMP-independent growth impairment, necrosis and premature plant death. By using an estradiol-inducible promoter (XVE) we further observed that already 6 hours after induction of BAK1 expression, defense responses like ethylene production or MAP kinase phosphorylation were elicited leading to an increased resistance against pathogenic *Pseudomonas syringae* pv *tomato* (*Pto*) DC3000. Likewise, overexpression of the BAK1 homologs SERK1 and SERK4 also triggered constitutive defense responses.

Interestingly, overexpression of BAK1 in a *sobir1* (a mutant affected in the LRR-RLK *SUPPRESSOR OF BAK1-INTERACTING RECEPTOR-LIKE KINASE 1-1*) knock-out mutant background did not produce the aberrant growth and developmental phenotype. SOBIR1 emerges as a new regulator for PRRs from the receptor like proteins (RLP) family. Thus, our results indicate that high levels of BAK1 expression constitutively activate PTI. Nevertheless we cannot exclude that other BAK1-dependent pathways are involved in the observed phenotype.

3.1.2 Introduction

Plants are constantly challenged by biotic and abiotic stimuli coming from their environment. For a quick and efficient response, they need to sense and translate these extracellular signals into intracellular responses. Especially for biotic stimuli they use a wide set of receptor-like kinases (RLKs) that possess diverse ligand binding specificities (Shiu and Bleecker, 2001a).

Arabidopsis BAK1 (BRASSINOSTEROID INSENSITIVE 1 (BRI1)-ASSOCIATED KINASE 1) is a leucine-rich repeat (LRR)-RLK, which plays an important regulatory role in plant development and disease resistance. For this it interacts with other LRR-RLKs like the brassinosteroid receptor BRI1 (BRASSINOSTEROID INSENSITIVE 1), an important regulator of plant development (Li et al., 2002; Nam and Li, 2002; Kinoshita et al., 2005), as well as the pattern recognition receptors (PRRs) FLS2 (FLAGELLIN SENSING2) and EFR (EF-TU RECEPTOR), that are central for plant innate immunity (Chinchilla et al., 2009). The latter PRRs are able to recognize extracellular non-self molecular signatures present in microbes called microbe-associated molecular patterns (MAMPs) to activate intracellular signaling cascades leading to a set of defense responses known as pattern-triggered immunity (PTI) (Nurnberger et al., 2004; Chisholm et al., 2006; Zipfel, 2008; Boller and Felix, 2009). PTI responses include e. g. mitogen-activated protein kinase (MAPK) activation, defense gene expression, ethylene production, and seedling growth inhibition.

In Arabidopsis, FLS2 is able to bind a highly conserved N-terminal peptide within bacterial flagellin (flg22), while EFR binds an N-terminal peptide within EF-Tu (elf18) (Chinchilla et al., 2006; Zipfel et al., 2006). Co-immunoprecipitation (Co-IP) studies demonstrated that flg22 binding induces an extremely rapid heteromeric complex formation between the ligand-activated FLS2 and BAK1 (Chinchilla et al., 2007; Heese et al., 2007). Complex formation of FLS2 with BAK1 was complete almost instantaneously (≤ 1 second) after flg22 treatment and was followed by rapid trans-phosphorylation (within 30 seconds) of the interacting partners suggesting that these two events represent the molecular mechanisms for receptor activation (Schulze et al., 2010). Interestingly, an X-ray chystallographic study recently showed that FLS2-

bound flg22 recognition by BAK1 is required to form a signaling-active complex (Sun et al., 2013b).

A receptor-like cytoplasmic kinase (RLCK) BOTRYTIS-INDUCED KINASE 1 (BIK1), has been found to constitutively associate with FLS2 (Lu et al., 2010b; Zhang et al., 2010). Within 10 minutes, activation of FLS2 led to phosphorylation of BIK1 (Lu et al., 2010b; Lu et al., 2010a). BIK1 has previously been identified as an important component of Arabidopsis for resistance to necrotrophic fungi and as a negative regulator of basal defense responses to virulent bacterial strains of *Pseudomonas syringae* pv *tomato* (Veronese et al., 2006). After trans-phosphorylation events between the interacting partners, BIK1 is likely released from the FLS2-BAK1 complex (Lu et al., 2010b). Interestingly, it was shown that in addition to BIK1, one of its homologs, PBL1 (AVRPPHB SUSCEPTIBLE 1 (PBS)-LIKE 1), is transiently phosphorylated as well upon flg22 treatment, suggesting that PBL1 is also an actor of flg22-triggered signaling (Zhang et al., 2010). Collectively these events may initiate the downstream signaling cascade to activate defense responses and finally lead to PTI.

In addition to microbes, PRRs can recognize endogenous plant-derived molecules called danger- or damage-associated molecular patterns (DAMPs) that are thought to appear in response to the damage caused by pathogen attack. An example of DAMPs in Arabidopsis is a group of peptides known as *At*Peps, which are perceived by two other LRR-RLKs, PEP RECEPTOR 1 and 2 (PEPR1 and PEPR2) (Yamaguchi et al., 2006; Huffaker and Ryan, 2007; Ryan et al., 2007; Krol et al., 2010). Like FLS2 and EFR, PEPR1 also associates with BAK1 in a ligand-dependent manner and this complex triggers similar defense responses as FLS2-BAK1 and EFR-BAK1 complexes do and seems also to contribute to plant immunity (Huffaker and Ryan, 2007; Krol et al., 2010; Schulze et al., 2010; Huffaker et al., 2011; Roux et al., 2011; Bartels et al., 2013; Flury et al., 2013).

BAK1 belongs to the five members SOMATIC EMBRYOGENESIS-RELATED KINASE (SERK) family, therefore it is also named SERK3 (Hecht et al., 2001). Additionally to BAK1, BKK1 (BAK1-LIKE KINASE 1), also called SERK4, has been associated with plant immunity, and both seem to work at least partially redundantly (Roux et al., 2011). Beside its important function in PTI, BAK1 was also shown to play a role in plant cell death control. BAK1 deficient mutants, *bak1-3* and

bak1-4 (Col-0 background), develop spreading necrosis upon biotrophic bacterial infection and show enhanced susceptibility to necrotrophic pathogens (Kemmerling et al., 2007).

In order to suppress PTI, successful pathogenic bacteria developed a set of virulence molecules, which can be secreted inside the plant cell through the type III secretion system (T3SS) (Cunnac et al., 2009; Lindeberg et al., 2009). These virulence molecules, also called effectors, can specifically inhibit important signaling elements of PTI (Boller and He, 2009). In response to effectors, plants developed resistance (R) proteins, which can recognize directly (gene-for-gene theory) (Flor, 1971) or indirectly (guard hypothesis) (Van der Biezen and Jones, 1998) effectors and activate defense responses leading to effector-triggered immunity (ETI) (Jones and Dangl, 2006). ETI is usually associated with the development of localized hypersensitive cell death response (HR) at the infection site, a sort of cell suicide, to limit pathogen invasion and increase resistance against biotrophic pathogens (Jones and Dangl, 2006).

It has been found that BAK1 interacts *in vivo* with BIR1 (BAK1-INTERACTING RECEPTOR-LIKE KINASE 1), another LRR-RLK (Gao et al., 2009). The knock-out *bir1-1* mutant shows constitutive activation of defense responses, high salicylic acid content and extensive cell death, which results in strongly increased resistance against the virulent oomycete pathogen *Hyaloperonospora parasitica* Noco2. The *bir1-1* phenotype can be largely suppressed by the *sobir1-1* (*suppressor of bir1-1*) mutation (Gao et al., 2009). *SOBIR1* encodes an LRR-RLK whose overexpression induces cell death and activation of defense responses. Interestingly, in a recent study it was described that SOBIR1 is a central signaling element of plant resistance against fungal infection (Liebrand et al., 2013). The authors showed that SOBIR1 interacts in a ligand independent manner in tomato and in Arabidopsis with two receptor-like proteins (RLPs) Cf-4 and Ve1 and mediates resistance against *Cladosporium fulvum* and *Verticillium dahlia*, respectively (Liebrand et al., 2013). Since BIR1 interacts with BAK1 *in vivo* it has been suggested that BIR1 or the BIR1-BAK1 complex could be guarded by two or several R proteins (Gao et al., 2009). In absence of BIR1, these R protein-mediated pathways (e.g. *SOBIR1*-dependent) may be activated and trigger plant immunity. This hypothesis was further supported by the identification of a calcium-dependent phospholipid binding protein BON1 (BONZAI1) as an

interacting partner of BIR1 and BAK1, both *in vitro* and *in vivo* (Wang et al., 2011). BON1 was proposed to be a negative regulator of cell death and defense responses in a temperature-dependent manner via modulation of expression of *R* genes such as the *SUPPRESSOR OF NPR1-1 CONSTITUTIVE 1* (*SNC1*) and the *LESION CELL DEATH* genes (*LCD*s) (Jambunathan et al., 2001; Yang and Hua, 2004). Because loss-of-function of *R* genes (*SNC1* and *LCD*s) partially rescued the cell death and the activated-defense phenotype of *bon1-1* and *bir1-1* mutants, the hypothetical BAK1-BIR1-BON1 complex was proposed to be guarded by several R proteins to control plant survival and defense responses (Wang et al., 2011).

These previous studies show that BAK1 is an important component of development and plant immunity. To study the role of BAK1 in innate immunity in more detail we generated plants overexpressing BAK1. These plants developed a stunted plant stature and showed constitutive activation of immune responses, including growth retardation and leaf necrosis. This phenotype seems to be independent of the presence of BAK1 interacting PRRs (FLS2, EFR and PEPRs) and BRI1. To further investigate the physiological and molecular basis of this phenotype we overexpressed BAK1 in an estradiol inducible manner in Arabidopsis wild type background. We discovered that estradiol-induced BAK1 overexpression induced ethylene production, MAPK activation, defense gene expression, seedling growth inhibition and also cell death. These results indicate that BAK1 over-accumulation is sufficient to constitutively activate defense responses and cell death in the absence of any elicitor/microbe. Consequently, BAK1 overexpression significantly increased the plant's resistance against *Pto* DC3000. Similar to BAK1 overexpression, overexpression of its homologues, namely SERK1 and 4 (SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 and 4), could trigger plant defense responses independently of the presence of elicitors, indicating a redundant role for SERK proteins in the activated-defense phenotype.

Further we analyzed the dependency of the activated-defense phenotype on functional SA signaling and the presence of SOBIR1 or BIK1/PBL1. We found that the *BAK1* overexpression phenotype was partially rescued in the *sobir1-13* mutant and possibly in the *bik1 pbl1* double mutant background.

3.1.3 Results

3.1.3.1 Transgenic plants expressing BAK1 under a constitutive promoter display developmental defects, leaf necrosis and lethality

To better understand the role of BAK1 in plant immunity the coding sequence of the *BAK1* gene was amplified from genomic DNA and cloned into the constitutive expression vector pMDC32 harboring a dual *CaMV 35S* promoter (Curtis and Grossniklaus, 2003). This construct (hereafter referred to as *2x35S-BAK1*) was used to stably transform Col-0 wild-type plants by floral dip (Clough and Bent, 1998). Surprisingly, several plants from the T1 generation grown in short day conditions developed leaf necrosis three weeks after germination. After six weeks a panoply of growth phenotypes were observed ranging from wild type-like plants with low BAK1 expression levels to severely stunted, necrotic plants accumulating high amounts of BAK1 (**Figure 3.1.1A**, D. Chinchilla unpublished results). After nine weeks several plants, which formerly did not show cell death, developed necrosis, and finally 75% of the selection died without producing seeds. Western blot analysis of six-week-old plants showed that the observed phenotype correlated with BAK1 expression levels (**Figure 3.1.1A and B**). Intriguingly, besides over-accumulation of a full-length BAK1 protein the accumulation of two other bands of lower molecular weight were detected. Since these two other bands were recognized by an antibody raised against a C-terminal peptide sequence of BAK1 (Schulze et al., 2010) we assumed that these are truncated forms of BAK1. Taken together, the observed growth phenotype was linked to the accumulation of BAK1 and putative BAK1 truncated versions.

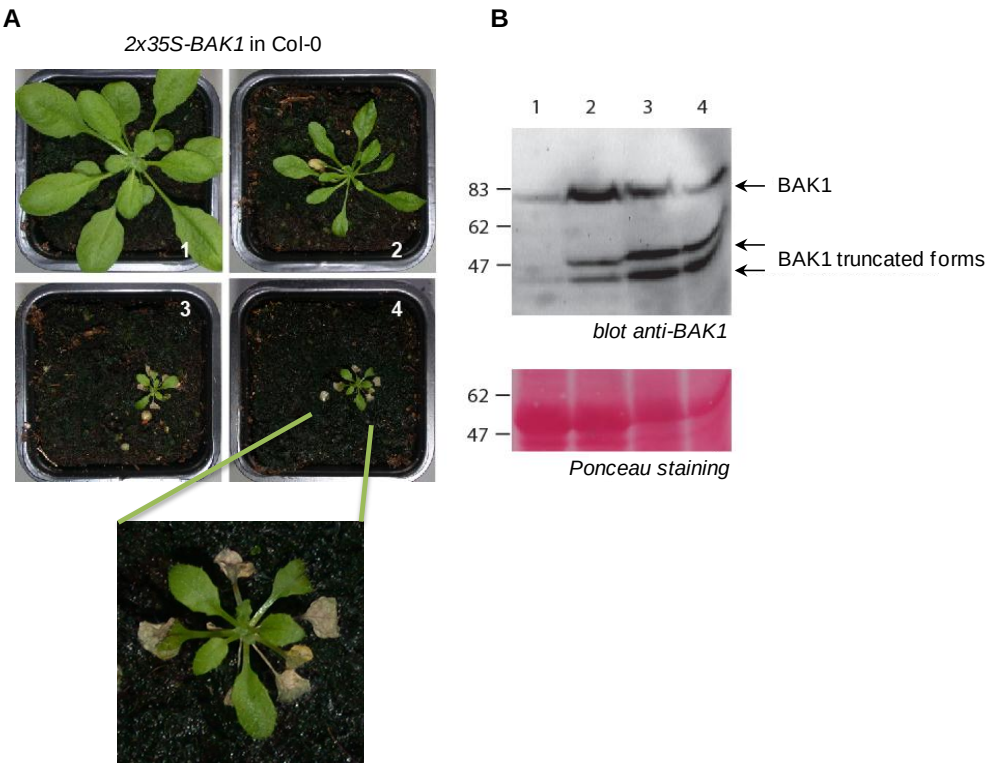


Figure 3.1.1: Overexpression of BAK1 impedes plant development and causes necrosis and lethality in Arabidopsis Col-0. A: Phenotype exhibited by T1 plants overexpressing BAK1grown for six weeks in short day conditions. **B:** Total proteins were extracted from leaf material and analyzed by Western blot using antibodies raised against the C-terminal part of BAK1 (Schulze et al., 2010) (upper panel). Ponceau staining was performed to verify the amount of total proteins present on the membrane (lower panel). Asterisks highlight the putative truncated forms of BAK1.

The strong stunting phenotype of *2x35S-BAK1* plants complicated the investigation of the different PTI-associated defense responses due to potential pleiotropic effects caused by the aberrant growth phenotype. Therefore we chose a system in which we could control the accumulation of BAK1, namely the estradiol-inducible XVE system, which is inducible at many developmental stages and apparently does not stress the plants (Zuo et al., 2000). Upon transformation of *BAK1* under the control of the inducible *XVE* promoter (hereafter referred to as *XVE-BAK1*) in Col-0 wild-type plants, we obtained 13 independent lines in the T1 generation, all of which grew like to wild type plants in the absence of estradiol (**Figure S3.1.1A**). Western blot analysis done on T2 seedlings showed that BAK1 and its truncated forms over-accumulated in these lines upon overnight β-estradiol treatment (**Figure S3.1.1B**).

3.1.3.2 Early defense responses are activated by BAK1 overexpression in absence of a MAMP ligand

We found that constitutive BAK1 overexpression is associated with the arrest of plant growth and massive cell death independent of the presence of elicitors (**Figure 3.1.1**). Similar effects are characteristic of constitutive defense mutants such as the *lmm* (*lesion mimic mutants*), *cpr1* (*constitutive expresser of PR genes 1*) or *acd6* (*accelerated cell death 6*) mutants (Bowling et al., 1994; Rate et al., 1999; Lorrain et al., 2003). This prompted us to investigate if BAK1 accumulation triggers defense responses in *XVE-BAK1* plants.

First we examined the activation of MAPKs, which is one of the earliest responses triggered by a MAMP treatment. It was described that MPK3 and MPK6 are rapidly (after 5 minutes) and transiently activated in Arabidopsis cell cultures and adult plants after MAMP perception (Nuhse et al., 2000; Asai et al., 2002). MAPK activation was studied in six-day-old *XVE-BAK1* and wild type Col-0 seedlings treated with or without estradiol for various times ranging from 3 h to 27 h, using Western blotting with antibodies which recognize the phosphorylated (active) form of MAPKs (**Figure 3.1.2 and S3.1.2**). Two bands corresponding to activated MPK6 and MPK3 were detected four hours after estradiol treatment. These bands reached their highest intensity from six to ten hours, and remained detectable until 27 hours after addition of estradiol. This response correlated with overexpression of BAK1 and its truncated forms (**Figure 3.1.2**). However in contrast to the MAPK activation, which declined after ten hours, BAK1 expression levels stayed high even at 27 hours after treatment. Mock-treated *XVE-BAK1* seedlings did not show MAPK activation or BAK1 overexpression (**Figure S3.1.2**).

It was described previously that in Col-0 seedlings MPK6 and MPK3 are activated already 2 minutes after flg22 treatment (Suarez-Rodriguez et al., 2007). This activation is stable until 30 minutes and then declines at 60 minutes. Our results showed that overexpression of BAK1 and/or its truncated forms induced a prolonged MAPK activation in an elicitor-independent manner.

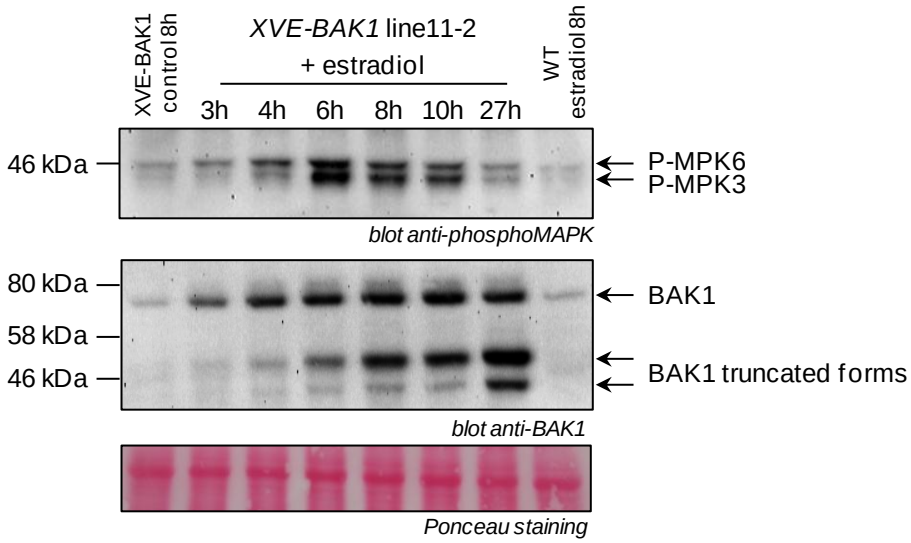


Figure 3.1.2: Overexpression of BAK1 in seedlings induces MAPK activation in absence of MAMPs. Western blot analysis was performed on total protein extracts from six-day-old *XVE-BAK1* and WT seedlings treated with 1 µM estradiol or solvent (control). MAPK activation and BAK1 overexpression were detected with antibodies recognizing the phosphorylated forms of MAPKs (upper panel) or anti-BAK1 (middle panel) respectively. Ponceau staining was done on the membrane to verify equal loading of proteins (lower panel). This experiment was performed four times with similar results.

Another early response to MAMP treatment is the increased production of the stress-related hormone ethylene. To examine the effect of BAK1 overexpression on ethylene production, we used again six day old seedlings. *XVE-BAK1* and wild type Col-0 seedlings were treated with estradiol for a time frame ranging from 3 h to 27 h (same as for MAPK assay). In addition, a flg22 treatment for three hours was performed to determine if the tissue was still able to produce a detectable ethylene response after the time of pretreatment. Increased accumulation of ethylene in presence of estradiol was observed in *XVE-BAK1* seedlings at the earliest after six hours of estradiol treatment (**Figure 3.1.3**). In contrast, no induction of ethylene accumulation was observed in wild-type seedlings treated with estradiol over this time frame (**Figure 3.1.3**). Ethylene values were higher in estradiol treated transgenic samples than when solely treated with flg22 showing that the observed defense response is even stronger than the flg22 response at saturating concentrations of MAMP ligand (here used at 1 µM). This MAMP-independent ethylene phenotype remained stable over the first ten hours of treatment. Surprisingly this response disappeared at 27 hours in presence of estradiol

treatment, whereas plants were still able to respond to flg22 (excluding the possibility of an exhausted ethylene production system or dead tissue). Ethylene production correlated with over-accumulation of BAK1 and/or truncated versions reminiscent to the observation made in the MAPK activation experiment (**Figure 3.1.2**). Integrating these first results we chose to investigate further defense responses and concentrated on the time point of six hours after addition of estradiol because that was the first time point for which a clear induction of both, MAPK and ethylene was observed.

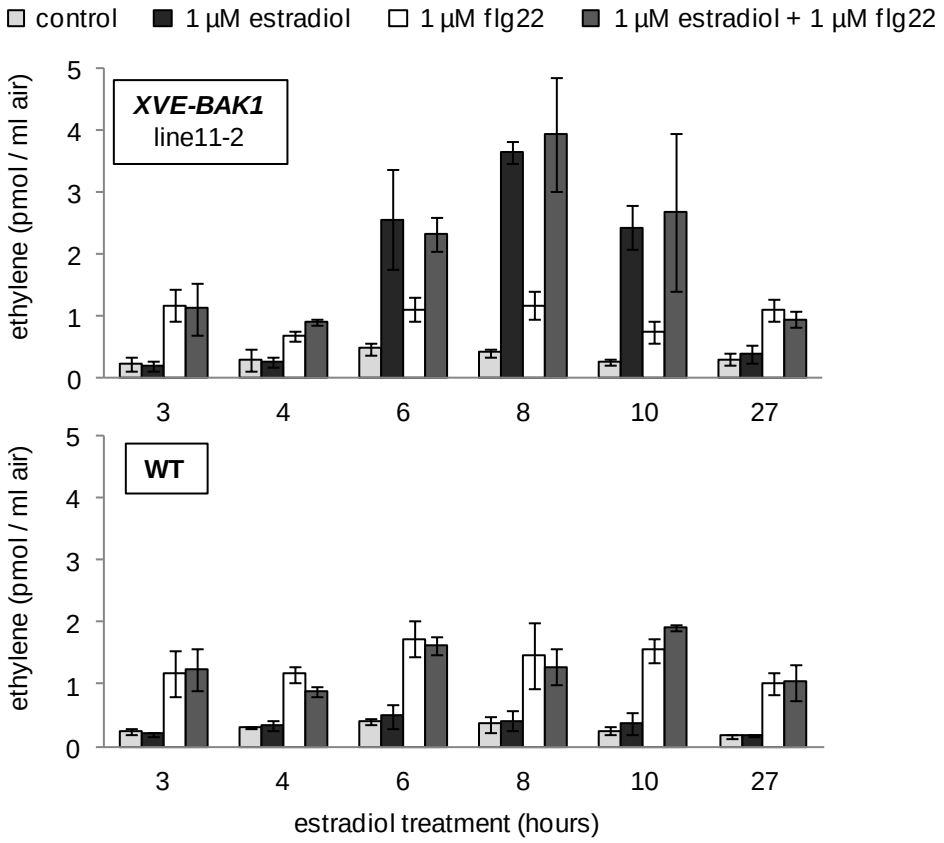


Figure 3.1.3: BAK1 overexpression induces increased ethylene accumulation in absence of MAMPs. Six-day-old *XVE-BAK1* (upper graph) and Col-0 wild type (lower graph) seedlings were treated with solvent (control) or with 1µM estradiol for different times (as indicated in the figure) and/or 1µM flg22 for three hours. In all cases ethylene production was measured three hours after closing tubes. Error bars correspond to standard deviation with n=4. Similar results were obtained in four independent biological replicates.

3.1.3.3 Defense marker genes are induced by BAK1 overexpression

Perception of elicitors by PRRs induces transcriptional changes in the nucleus within 30 minutes (Asai et al., 2002; Zipfel et al., 2004; Boudsocq et al., 2010). In the previous experiments we showed that different early PTI responses (ethylene production and MAPK activation) were activated by BAK1 overexpression in absence of elicitors and this activation occurred after six hours of estradiol treatment. Here we tested if the overexpression of BAK1 and/or its truncated forms could induce up-regulation of MAMP responsive genes in a MAMP-independent manner. Therefore total RNAs were extracted from *XVE-BAK1* and wild type seedlings treated with or without 1 µM estradiol for six hours. A control treatment with 1 µM flg22 (solvent + flg22 for one hour) was performed on both genotypes as positive control for induction of marker genes. The data were normalized to the constitutively expressed *EIF4α* gene (Boudsocq et al., 2010). In addition of *BAK1* itself, we selected early flg22-responsive genes, *FRK1* (*FLG22-INDUCED RECEPTOR KINASE 1*), *PHI-1* (*PHOSPHATE INDUCED 1*) and *NHL10* (*NDR1/HIN1-LIKE 10*) to test the accumulation of their transcripts (Asai et al., 2002; Boudsocq et al., 2010). As expected, *BAK1* transcripts accumulated massively upon estradiol treatment in *XVE-BAK1*, but not in wild type seedlings (**Figure 3.1.4**). Expression of *FRK1* and *NHL10* was significantly induced by estradiol treatment in absence of any elicitor in XVE-BAK1 seedlings. The induction of *NHL10* expression in estradiol treated seedlings was significantly stronger than induction by flg22 while in case of *FRK1* flg22 treatment induced a stronger up-regulation than estradiol treatment. This difference could be explained for example by differential expression dynamics of these two genes after BAK1 overexpression in six-day-old germinations. Induction by flg22 of these two marker genes was comparable between wild type and *XVE-BAK1* seedlings in absence of estradiol. *PHI-1* expression levels did not show significant changes in response to flg22 treatment or estradiol treatment, but this is likely due to the variation between biological replicates as reflected by large error bars. Taken together, the observed induction of at least two flg22-induced genes in response to BAK1 overexpression further supports the idea of a constitutive defense activation triggered by the accumulation of BAK1 and/or its truncated forms.

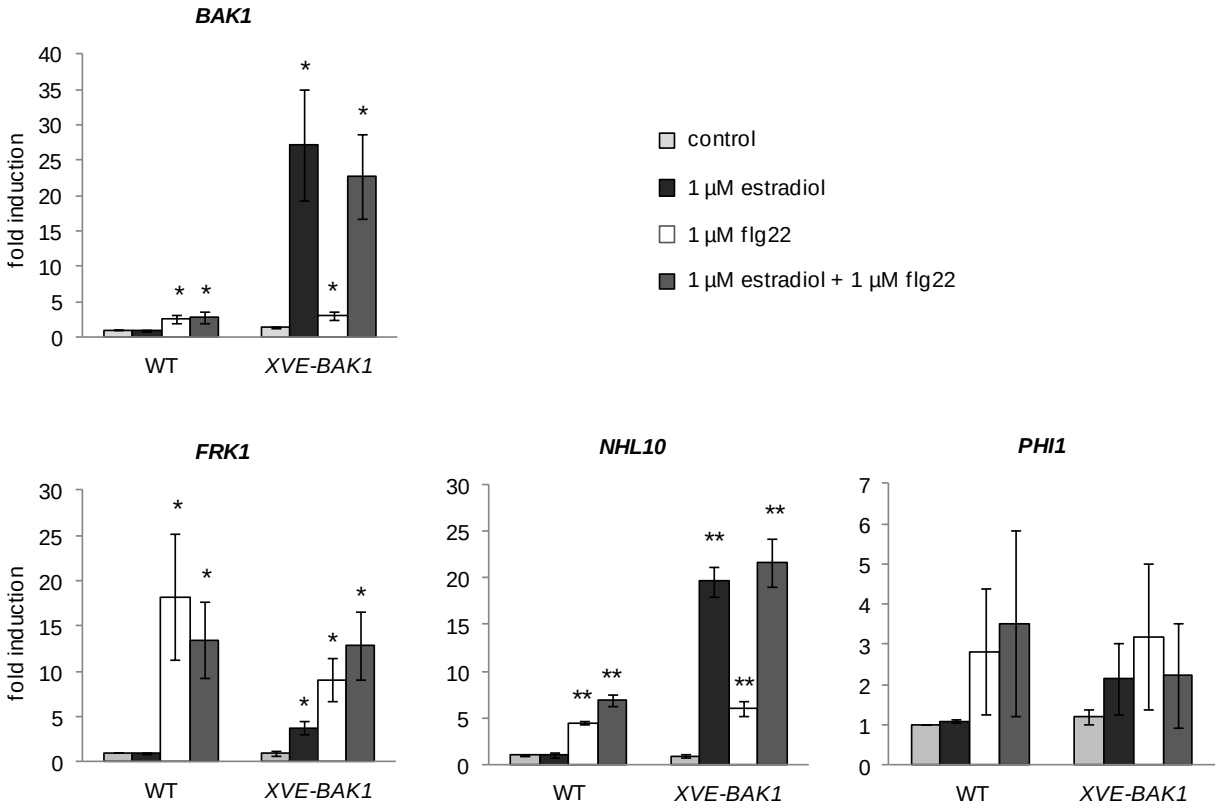


Figure 3.1.4: Some defense-response marker genes are up-regulated by BAK1 overexpression. Seedlings were treated with or without 1 µM estradiol for six hours and/or 1µM flg22 for one hour. RNAs were extracted from six-day-old *XVE-BAK1* (line11-2) and WT (Col-0) seedlings and reverse transcribed. Transcript accumulation was measured by qRT-PCR analysis. For each treatment three technical replicates were performed. Transcript levels were normalized to the *EIF4a* gene and are presented as relative to WT control. Graphs represent the mean of three biological replicates. "*" represents statistically significant difference compared to control treatment. ***:** p value<0.05; ****:** p value<0.01. ANOVA (Newman-Kleus post test) test was used to analyze the data.

3.1.3.4 The brassinosteroid pathway seems to be inhibited by overexpression of BAK1

Beside the constitutive activation of defense responses by BAK1 overexpression the observed developmental defects displayed by the *2x35S-BAK1* plants might also be based on a misregulation within the brassinosteroid signaling pathway, since BAK1 interacts also with the brassinosteroid receptor BRI1. Thus, we wanted to determine the effect of BAK1 overexpression on the brassinosteroid-dependent developmental pathway. Therefore we investigated the expression level of the brassinosteroid receptor *BRI1* and also *CPD* (*CONSTITUTIVE PHOTOMORPHOGENIC DWARF*), encoding a cytochrome P450, which

transcripts are negatively controlled by brassinosteroids (Mathur et al., 1998). In BAK1 overexpressors, *CPD* expression was down-regulated after estradiol treatment, which could hint at an activation of the brassinosteroid pathway (**Figure 3.1.5**). However we also found that *CPD* expression is repressed after 1 hour of flg22 treatment. A similar pattern was observed for *BRI1* expression, which was repressed by BAK1 overexpression and also by flg22 (**Figure 3.1.5**). Thus these results confirm that gene regulation exerted by BAK1 overexpression follows the same pattern as the one observed after flg22 induction even for markers of developmental pathways.

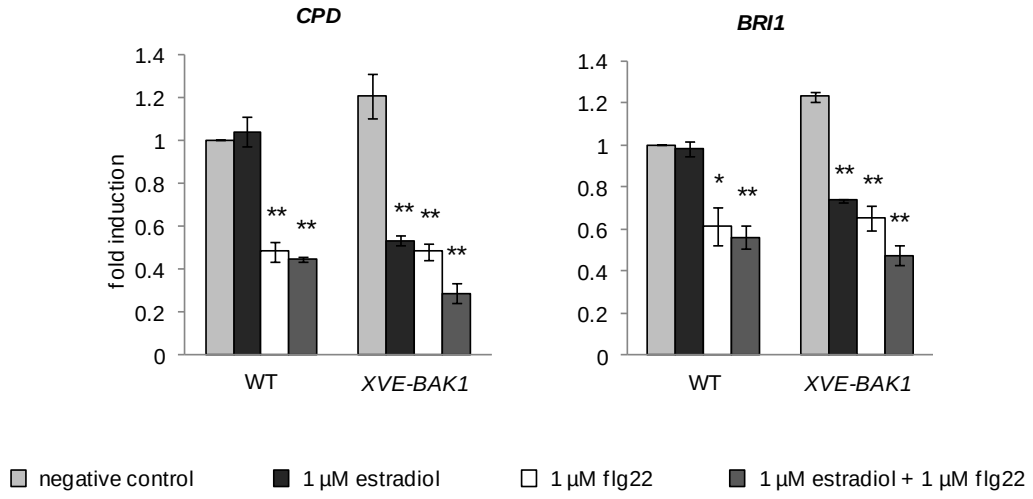


Figure 3.1.5: Brassinosteroid-related genes are repressed by BAK1 overexpression. Same samples and conditions were used as in Figure 3.1.4.

3.1.3.5 Arabidopsis seedlings overexpressing BAK1 are impaired in growth and show cell death

Treatment with bacterial flg22 results in a strong and dose dependent reduction of growth in young Arabidopsis seedlings (Gomez-Gomez et al., 1999). This growth inhibition effect was characterized by size reduction of roots, leaves and cotyledons. The growth impairment induced by flg22 was not associated with necrosis since even after prolonged treatment with flg22 the seedlings remained green. Here we were interested to see how the seedling growth is influenced by BAK1 overexpression. Therefore six-day-old seedlings were grown for an additional six days in the presence of control, estradiol, flg22 or double (flg22 + estradiol) treatments. *XVE-BAK1* seedlings treated with the control treatment developed in a

comparable manner to wild type seedlings, and in the presence of flg22 both showed an inhibition of growth characteristic for flg22 treatment. *XVE-BAK1* seedlings treated with estradiol or double treatment showed growth inhibition compared to control seedlings but, interestingly, this inhibitory effect was stronger/different from the one provoked by flg22 (**Figure 3.1.6A and B**). Indeed estradiol-treated seedlings were more strongly inhibited in the growth of roots, cotyledons and leaves than those treated with flg22 (**Figure 3.1.6B**). Additionally, while leaves of flg22 treated seedlings stayed green, estradiol treated seedlings showed yellow-brown coloration on leaves indicating that cell death was occurring (**Figure 3.1.6A**). Western blot analysis showed that overexpression of BAK1 and/or its truncated forms correlated with the observed phenotype in *XVE-BAK1* seedlings (**Figure 3.1.6C**). Similar results were obtained with an independent homozygous *XVE-BAK1* line (**Figure S3.1.3**). These results indicate that BAK1 overexpression inhibits Arabidopsis growth already in the seedling stage and in the absence of elicitors. In addition BAK1 accumulation seems to trigger cell death which is different from the response triggered by flg22 treatment.


A
control
1 µM estradiol
1 µM flg22
1 µM estradiol
+ 1 µM flg22
WT
XVE-BAK1
line11-2

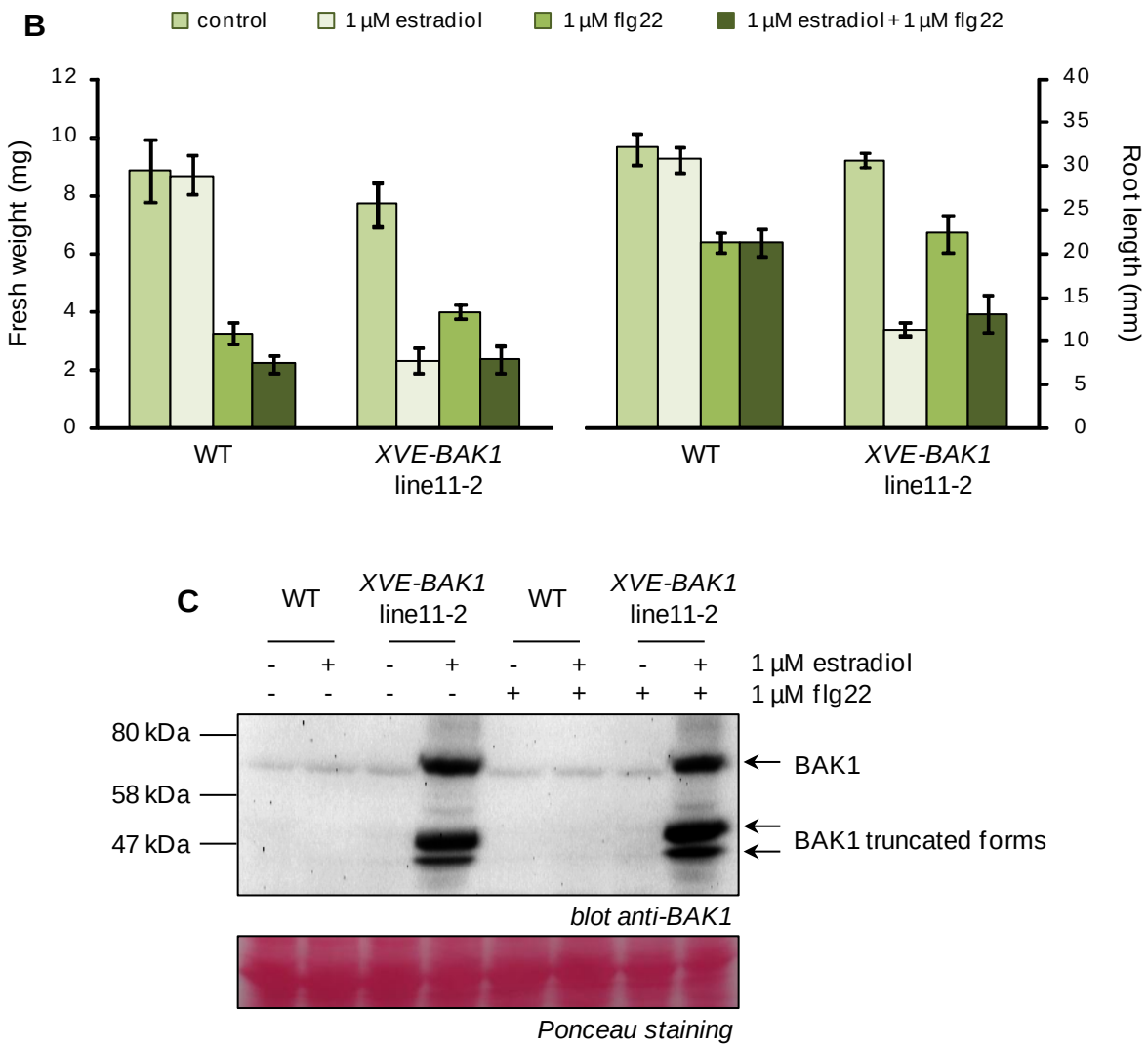
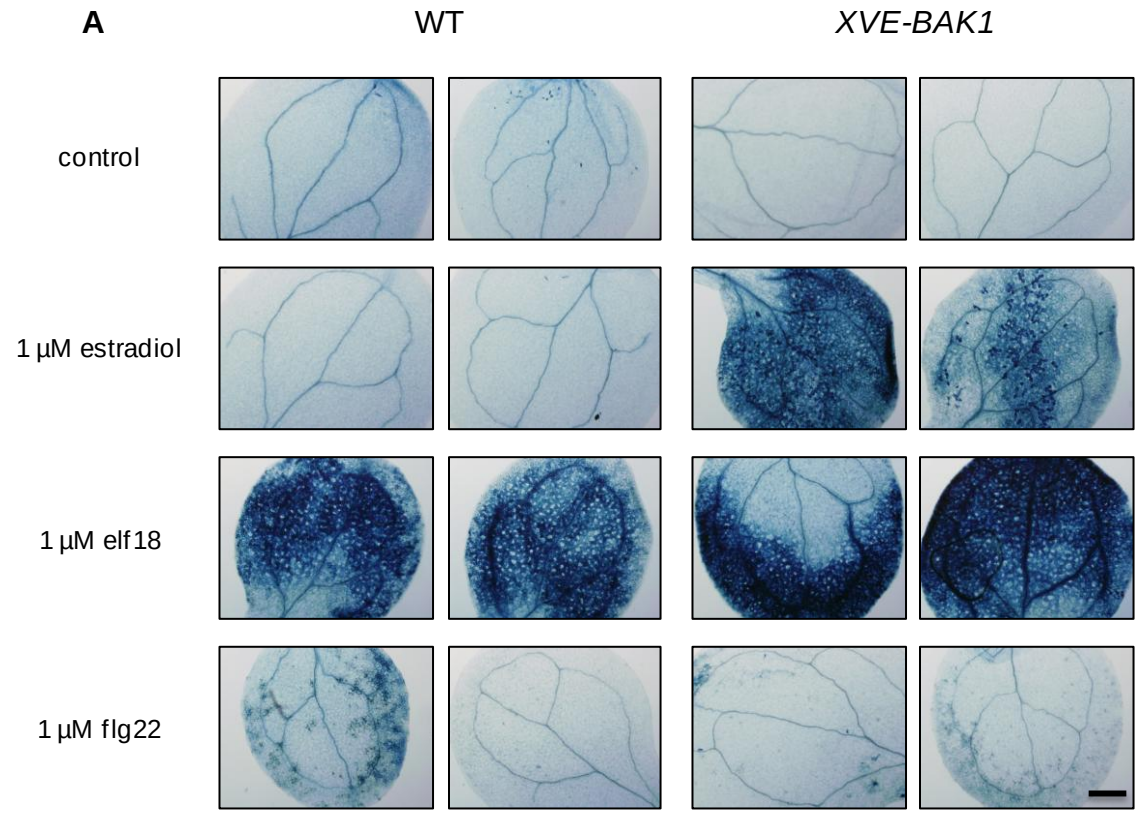


Figure 3.1.6: Seedling growth is inhibited by *BAK1* overexpression. Arabidopsis seedlings were grown on solid MS medium *in vitro* for six days and then transferred to liquid MS in presence of ethanol (control), 1 µM estradiol, 1 µM flg22 or double treatment for six days more. **A:** Two representative seedlings were photographed for each genotype and treatment. **B:** Fresh weight and root length were measured. Graphs correspond to the mean of six technical replicates and error bars represent standard error of the mean with n=6. Similar results were obtained in four independent experiments. **C:** Total proteins were extracted from seedlings and were analyzed by Western blot using anti-BAK1 antibodies (upper panel). Ponceau staining was made to detect the amount of protein present on the membrane (lower panel).

2x35S-BAK1 adult plants developed necrotic lesions on leaves (**Figure 3.1.1A**) therefore we decided to check whether it was due to cell death. For this purpose we used trypan blue staining assay, which is a tissue-staining approach to reveal dead mesophyll cells (Koch and Slusarenko, 1990). Six-day-old seedlings were grown for an additional three days in presence or absence of estradiol. *XVE-BAK1* seedlings treated with estradiol exhibited massive mesophyll cell death, which was absent from the estradiol treated wild type Col-0 seedlings and control treated seedlings (**Figure 3.1.7A**). Moreover we observed that overexpression of BAK1 and its truncated forms were linked to this cell death phenotype (**Figure 3.1.7B**). Treatments with MAMPs, elf18 and flg22, were performed as controls in comparison to estradiol treatment. It was shown previously that leaf infiltration of adult Arabidopsis plants with flg22 causes moderate cell death after 24h of treatment (Naito et al., 2008). We observed that elf18 treatment elicited cell death comparable to *XVE-BAK1* estradiol treated samples while flg22 treatment only provoked a very weak and not homogenous cell death phenotype (**Figure 3.1.7A**).



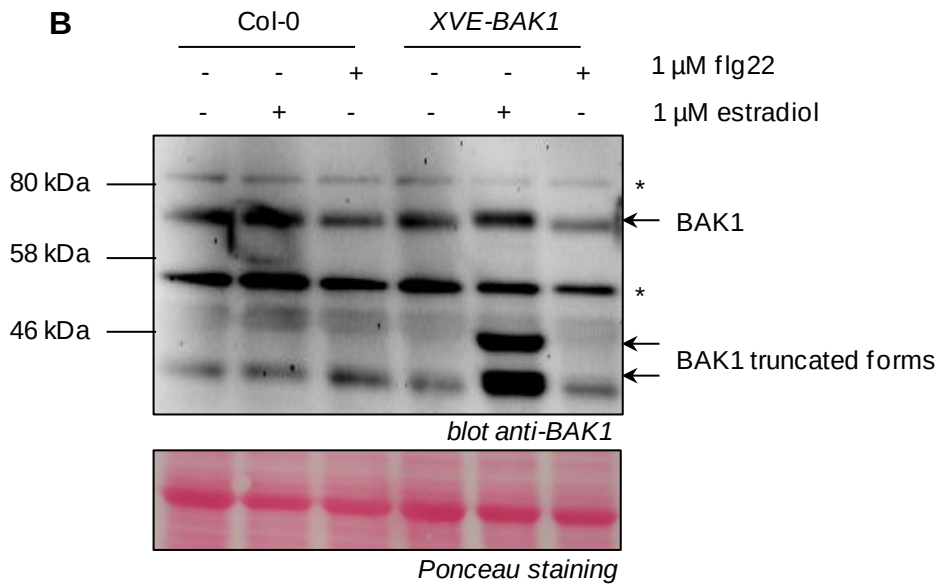


Figure 3.1.7: Overexpression of BAK1 induces mesophyll cell death in Arabidopsis seedlings. A: *XVE-BAK1* (line11-2) and wild type (Col-0) seedlings were grown on solid MS medium *in vitro* for six days. They were transferred to liquid MS medium and further grown for three more days in the presence of solvent, 1µM estradiol, 1µM elf18 or 1µM flg22. Seedlings were stained with a trypan blue solution and cotyledons analyzed by microscopy. For each treatment twelve seedlings were stained. Two representative pictures are shown for each treatment. The experiment was repeated four times with similar results. Scale bar corresponds to 0.5 mm. **B:** Total proteins were extracted from seedlings similarly treated as in A, but unstained and were analyzed by Western blot using antibodies raised against BAK1 (upper panel). * marks cross-reacting bands. Ponceau staining was made to detect the amount of protein present on the membrane (lower panel).

3.1.3.6 BAK1 overexpression enhances resistance to *Pseudomonas syringae* pv *tomato* (*Pto*) DC3000

In summary we observed activation of all defense responses tested including ethylene production, MAPK activation, up-regulation of defense genes expression, cell death as well as impairment in growth as a consequence of BAK1 overexpression. Thus, we were interested to test if BAK1 overexpression indeed increased resistance against pathogens in Arabidopsis. It was reported previously that the *bak1-5* mutant allele, which is only altered in immune signaling but not in BR-dependent development and cell death control, displayed increased susceptibility against virulent *Pto* DC3000, weakly virulent *Pto* DC3000 and non-adapted *P.*

syringae pv *tabaci* (*Pta*) 6605 strains (Roux et al., 2011; Schwessinger et al., 2011) showing that BAK1 is an important element in basal resistance.

The resistance to the virulent hemibiotrophic bacterium *Pto* DC3000 was examined in seedlings, which were pretreated with or without estradiol and/or flg22. Two days after inoculation bacterial proliferation was significantly reduced in estradiol treated *XVE-BAK1* samples, as well as flg22 treated samples, when compared to control treatments (**Figure 3.1.8A**; Ana Dominguez-Ferreras, unpublished results). As expected, estradiol induced the accumulation of BAK1 in the XVE-BAK1 line (**Figure 3.1.8B**). These results demonstrate that BAK1 overexpression results in an enhanced resistance to the hemibiotropic bacterial pathogen *Pto* DC3000.

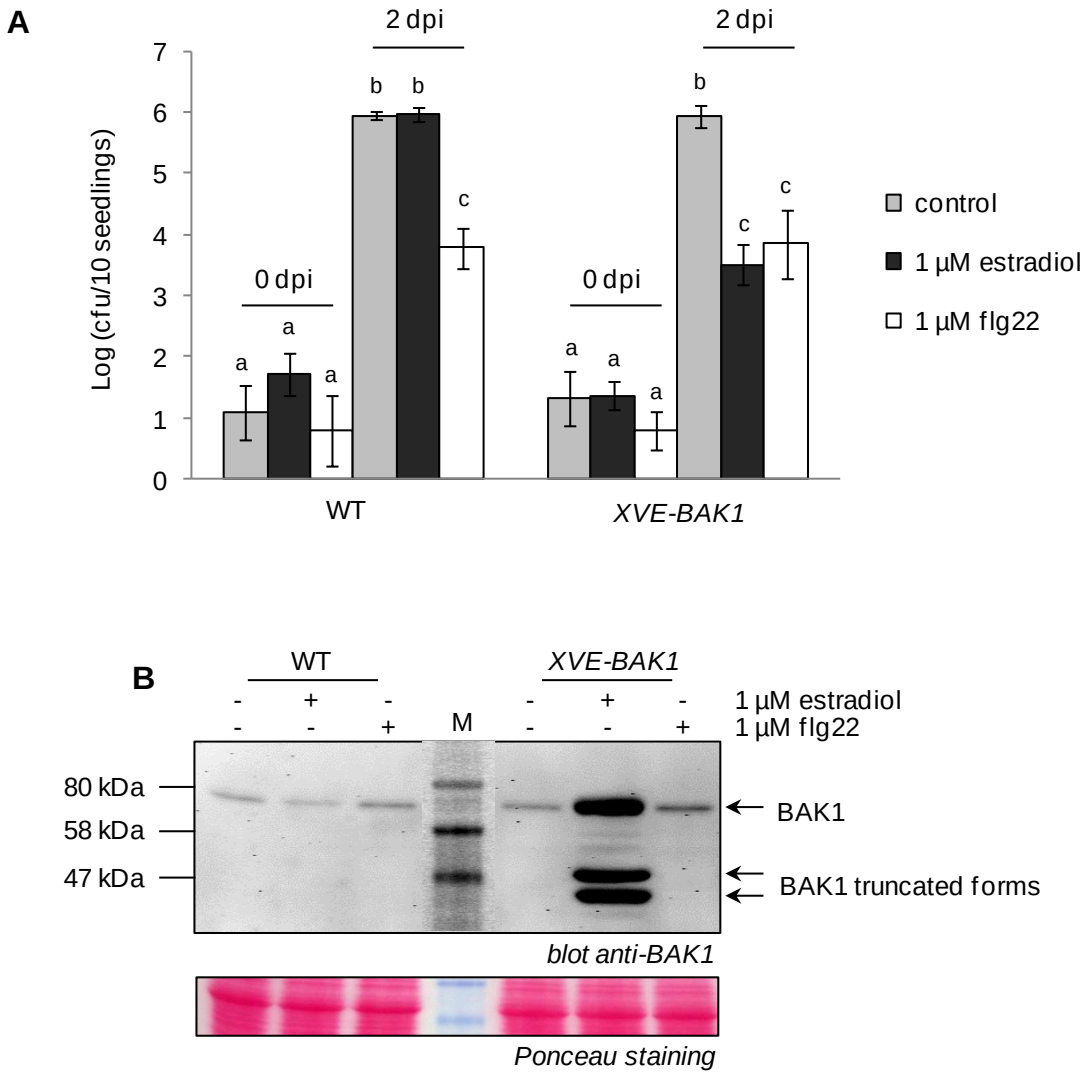


Figure 3.1.8: BAK1 overexpression restricts bacterial growth. A: Six-day-old seedlings were incubated for 24 hours in different solutions of estradiol and/or flg22 as indicated in the graph legend. They were inoculated by dipping into a solution of *Pseudomonas syringae* pv *tomato (Pto)* DC3000 for 1.5 hours (OD_{600}=0.01), washed three times and placed back into the solutions. Bacteria were counted after four hours for 0 dpi (day post inoculation) and after 48 hours for 2 dpi. Similar results were obtained in three independent experiments. a, b, c: difference is statistically significant (*p* value < 0.01, ANOVA). cfu: colony-forming unit. **B:** Total proteins were extracted from 2 dpi samples and analyzed by Western blot with antibodies raised against the C-terminal part of BAK1 (Schulze et al., 2010) (upper panel). Ponceau staining was done to check for equal loading of protein on the membrane (lower panel). M: protein marker.

3.1.3.7 Elevated temperature does not alleviate the growth defects caused by BAK1 overexpression

The phenotype induced by overexpression of BAK1 and/or its truncated forms includes defense responses, which are characteristic features of PTI but also of ETI. It was previously established that higher temperatures can suppress R protein-mediated cell death for example through inhibition of nuclear accumulation of R proteins such as SNC1 (Yang and Hua, 2004; Yang et al., 2006; Wang et al., 2009; Zhu et al., 2010). Indeed mutants of two interacting partners of BAK1, *bon1-1* and *bir1-1*, displayed cell death and constitutive defense responses at 22°C but this phenotype was rescued at 28°C (Gao et al., 2009; Wang et al., 2011). We also checked whether the phenotype caused by the overexpression of BAK1 depends on the temperature conditions. While the *bon1-1* growth phenotype was rescued by high temperature (28°C) in agreement with previous studies (Hua et al., 2001; Wang et al., 2011), the growth defect caused by BAK1 overexpression was not and became even more severe (**Figure S3.1.5**).

3.1.3.8 BAK1 overexpression induces transcriptional upregulation of BAK1 interacting partners

In response to BAK1 overexpression plant defense responses were activated and bacterial resistance was enhanced both independently of the presence of additional microbial elicitors. BAK1 is known to interact with several proteins to regulate different plant programs like defense, growth or cell death (Chinchilla et al., 2009). We then hypothesized that a change in BAK1 protein levels might trigger a change in the transcript and protein levels of interacting partners, which then may cause the observed phenotype. Thus we started with the investigation of potential transcriptional changes of several interacting partners of BAK1. Indeed, we could observe up-regulation of all tested genes, except *FLS2*, in *XVE-BAK1* seedlings when compared to wild type estradiol treated seedlings (**Figure 3.1.9**). Consistent with our results all these genes have been reported to be up-regulated by MAMP treatment (Navarro et al., 2004; Denoux et al., 2008; Boudsocq et al., 2010; Lu et al., 2010b). Thus except for *FLS2* results which were not conclusive because of the absence of significant differences between WT and XVE-BAK1, the regulation of expression of BAK1 partner genes after estradiol treatment

was comparable to the one observed during PTI signaling in XVE-BAK1 seedlings. These results indicate that BAK1 overexpression up-regulates the expression of its interacting partners, which then might contribute to the reported phenotypical changes.

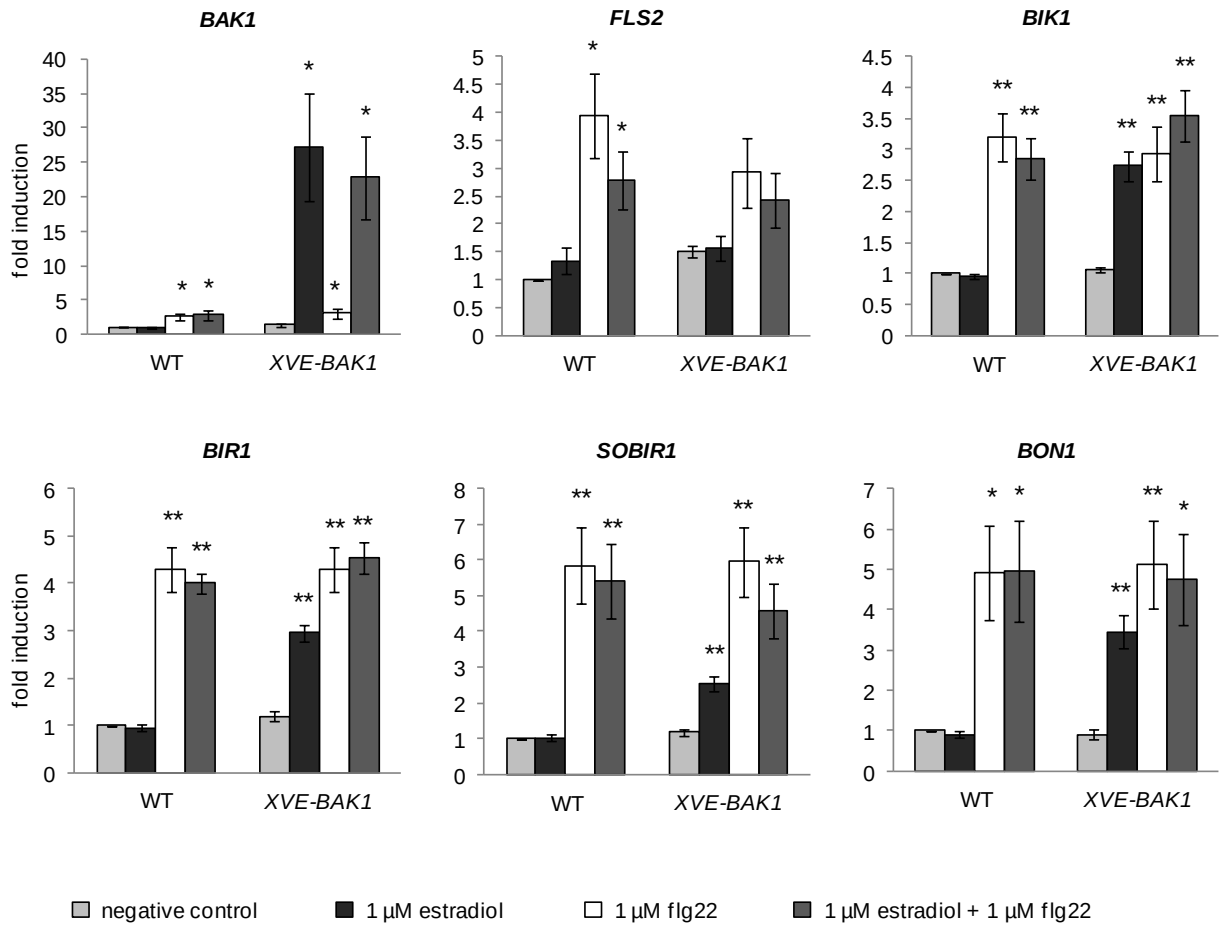


Figure 3.1.9: BAK1 overexpression as well as MAMP treatment induces changes in gene expression of BAK1-related proteins. Seven-day-old seedlings were treated with or without 1 µM estradiol for six hours and/or 1 µM flg22 for one hour. Transcript accumulation was measured by qRT-PCR analysis. For each treatment three technical replicates were performed. Transcript levels were normalized to the *EIF4a* gene and presented as relative to WT control. Graphs represent the mean of three biological replicates. Error bars correspond to standard error of the mean with n=3. "*" represents statistically significant difference compared to respective control treatment. ***:** 0.05>p value>0.01; ****:** 0.01>p value. ANOVA (Newman-Kleus post test) test was used to analyze the data.

3.1.3.9 Knock-out of SOBIR1 largely suppresses the BAK1 overexpression phenotype whereas lack of other selected defense-related genes does not.

Since gene expression analyses did not give us a clear candidate that might be responsible for the BAK1 overexpression-mediated phenotype we decided to study the BAK1 overexpression phenotype in a larger set of mutant backgrounds (**Table S3.1.1**). First, we investigated mutants affected in membrane receptors regulated by BAK1. A weak *bri1* mutant, *bri1-301*, deficient in BR signaling (Xu et al., 2008), and the *efr fls2* mutant (Nekrasov et al., 2009), affected in MAMP perception, were tested but neither could rescue the growth defect and necrosis induced by BAK1 overexpression (**Figure S3.1.4A and B**). Thus the phenotype is independent of the presence of BRI1, FLS2 and EFR receptors. We also tested elements acting downstream of receptors. The double mutant *bik1 pbl1* (Zhang et al., 2010), which lacks BIK1 and its closest homologue PBL1, important components of PTI signaling, showed a tendency to partially rescue the growth defect (Delphine Chinchilla, unpublished results). In adult plants, BAK1 expression caused in the *bik1 pbl1* background less lethality and leaf necrosis than in Col-0 wild type (WT) background while both contained similar levels of BAK1 protein. However these results were obtained once out of four experiments and cannot be considered as robust results.

To further elucidate whether the observed phenotype is the result of ETI activation we made a gene candidate approach by selecting a set of genes involved in ETI and transforming the respective mutants with the *2x35S-BAK1* construct (**Table S3.1.1**). T1 generations were screened for rescue of growth impairment and cell death symptoms caused by BAK1 over-accumulation. First, we tested the effect of the mutants *eds5-2* (*enhanced disease susceptibility 5-2*) (Volko et al., 1998), *sid2* (*isochorismate synthase*) (Wildermuth et al., 2001) and *npr1-5* (*nonexpresser of PR genes 1-5*) (Shah et al., 1997), which are defective in salicylic acid (SA) signaling, but none of these mutants were able to revert the growth impairment caused by BAK1 over-accumulation, indicating that SA accumulation may not be responsible for the observed phenotype (Delphine Chinchilla, unpublished data).

Next, we analyzed the involvement of *R* genes and regulators of *R* genes in the BAK1 overexpression phenotype (**Table S3.1.1**). We included the mutants *snc1-11* (*suppressor of*

npr1-1 constitutive 1-11) (Yang and Hua, 2004), affected in BON1-mediated temperature-dependent defense responses; *rps5-2* (*resistance to P. syringae protein 5-2*) (Warren et al., 1998) lacking RPS5, which recognizes modification of BIK1 and PBL kinases by the pathogenic *P. syringae* effector AvrPphB; *rpm1 rps2* (*resistance to P. syringae pv maculicola 1, resistance to P. syringae 2*) (Belkhadir et al., 2004), compromised in PTI responses negatively regulated by RIN4 (RPM1-INTERACTING PROTEIN 4) (Kim et al., 2005b). However T1 generation of the transformants still showed a stunted phenotype linked with BAK1 over-accumulation (Delphine Chinchilla, unpublished data). Similar results were obtained with mutants of regulators of *R* genes (Delphine Chinchilla, unpublished data). These include *eds1-2* (*enhanced disease susceptibility 1-2*) (Falk et al., 1999) and *pad4-1* (*phytoalexin deficient 4*) (Jirage et al., 1999) acting upstream of SA in ETI initiated by TIR-NB-LRR (Toll-interleukin-1-like nucleotide-binding LRR) type *R* genes such as for example *RPS4* and *RPS6* (Wiermer et al., 2005); *sag101-1* (*senescence-associated gene101-1*) mutated in *SAG101* an interacting partner of EDS1 involved together with PAD4 in TIR-NB-LRR triggered cell death (Feys et al., 2005); *ndr1-1* (*non-race-specific disease resistance 1-1*) (Century et al., 1995) affected in resistance conferred by CC-NB-LRRs (coiled-coil-NB-LRRs), the second major subset of R proteins, such as RPM1, RPS2, RPS5 (Aarts et al., 1998); *eta3* (*enhancer of tir1-1 auxin resistance*) (Gray et al., 2003) mutated in *SGT1b* (*SALICYLIC ACID GLUCOSYLTRANSFERASE 1*) gene, which regulates a sub-set of *R* genes of different structural classes recognizing several *Peronospora parasitica* isolates (Austin et al., 2002; Muskett and Parker, 2003). However, none of the tested *R* gene/regulator of *R* gene mutants were able to revert the growth defect induced by BAK1 overexpression so far.

The *sobir1-1* mutation strongly suppresses the cell death and constitutive-defense phenotype of the *bir1-1* null allele, lacking the BAK1 interacting partner BIR1 (Gao et al., 2009). Since our phenotype is reminiscent to the *bir1-1* phenotype we decided to examine whether *SOBIR1* could have a role in the BAK1 overexpression phenotype. Therefore we transformed the *sobir1-13* (SALK_009453) mutant with the *2x35S-BAK1,* construct and we found that mutation in *SOBIR1* reverted the stunting phenotype caused by BAK1 overexpression (**Figure 3.1.10A** and **Table S3.1.1**). None of the T1 plants overexpressing BAK1 and its truncated forms showed growth defects or cell death at six weeks after germination, and all plants could bolt and gave

seeds. Similar to WT lines, *sobir1-13* transgenic lines over-accumulated BAK1 (**Figure 3.1.10B**). This indicates that a mutation in *SOBIR1*, a positive regulator of cell death and defense responses, can rescue BAK1 overexpression-induced growth impairment and cell death.

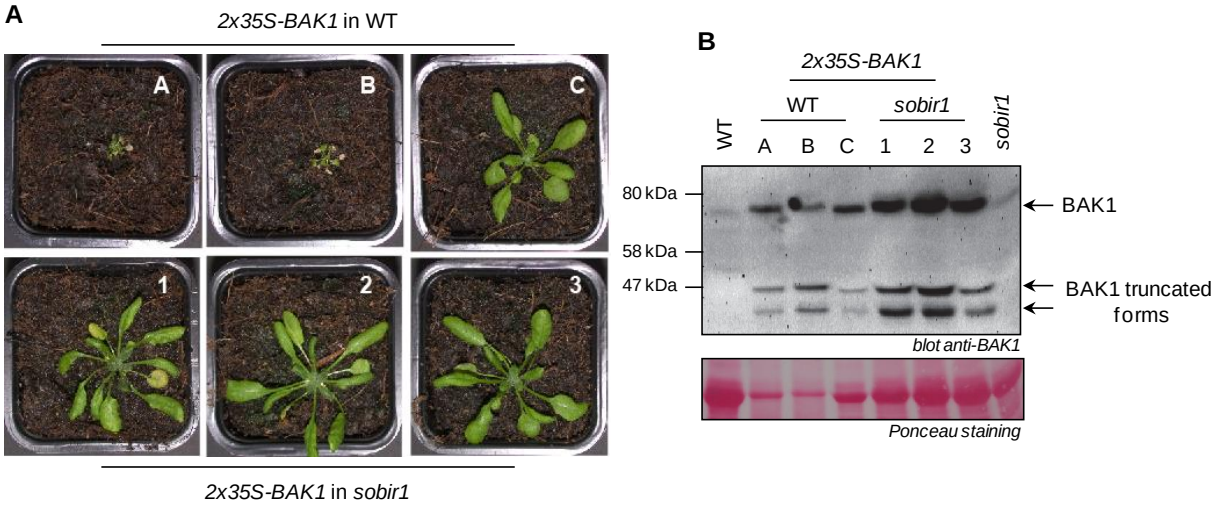


Figure 3.1.10: *sobir1-13* mutation rescues the BAK1 overexpression phenotype. *2x35S-BAK1* was stably transformed in Col-0 wild type (WT) and *sobir1-13* (SALK_009453) background. **A:** T1 generations were grown in short day conditions and pictures were taken after six weeks. **B:** Total proteins were extracted from leaf material and were analyzed by Western blot. Similar results were obtained in three independent experiments.

3.1.3.10 BAK1 homologs are able to induce the activated-defense phenotype similar to BAK1

As mentioned in the introduction, *BAK1* belongs to a five member multi-gene family called *SERK*s (*SOMATIC EMBRYOGENESIS RECEPTOR KINASE*) and shares up to 86 % similarity on the protein level with its homologs (Hecht et al., 2001; Boller and Felix, 2009). Null mutant of *BAK1* plants display only a reduced sensitivity to flg22, indicating a functional redundancy between BAK1 and its homologs in plant immunity (Chinchilla et al., 2007). Furthermore, by using the *bak1-5* mutant allele affected in PTI responses but not in BR-signaling and cell death control, the functional redundancy of BAK1 and SERK4 in PTI signaling and resistance of hemibiotrophic and biotrophic pathogens has been further supported (Roux et al., 2011).

Thus we investigated if other BAK1 homologs could activate the constitutive-defense phenotype in a similar manner as BAK1. Therefore we cloned the genomic sequence of *SERK4*

and *SERK1*, the closest and the most distantly related homologs of BAK1, respectively, under the control of the estradiol inducible *XVE* promoter. These constructs were transformed in *A. thaliana* wild type Col-0 plants, and ethylene accumulation measurement was used as a marker for the activated-defense phenotype in T1 generation. We observed that both *XVE-SERK1* and *XVE-SERK4* constructs were able to induce ethylene accumulation in a MAMP-independent manner upon estradiol treatment (**Figure 3.1.11A** and **C**). While over-accumulation of BAK1 and SERK4 was clearly detectable by Western blot with anti-BAK1 antibodies we never succeeded to detect SERK1 accumulation because anti-BAK1 antibodies cannot detect SERK1 (**Figure 3.1.11B** and **D;** Schulze et al., 2010)

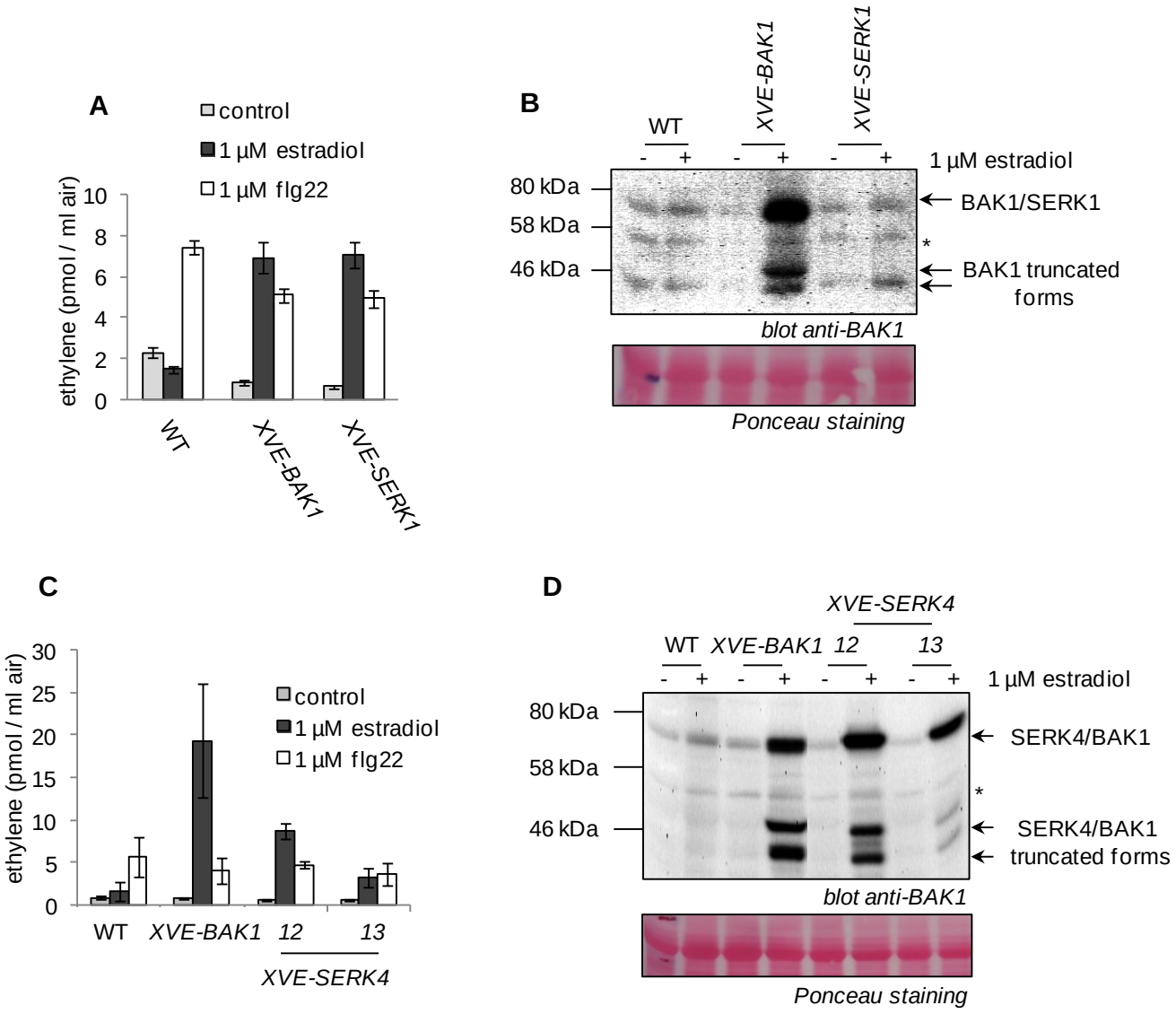


Figure 3.1.11: Overexpression of SERK1 and SERK4 induces ethylene biosynthesis in an MAMP-independent manner. *A. thaliana* wild type Col-0 plants were stably transformed with *BAK1*, *SERK1* and *SERK4 gDNA* sequences downstream of the estradiol-inducible *XVE* promoter. **A:** Leaf strips from three individual four-week-old T1 plants were pooled for each genotype to measure ethylene accumulation after overnight 1µM estradiol treatment or three hours 1µM flg22 treatment. Graph represents the mean of six technical replicates and error bars correspond to standard error of the mean with n=6. **B:** Material used for ethylene measurement was subsequently used for Western blot analysis with anti-BAK1 antibody to check for protein expression (upper panel). Equal amount of proteins present on the membrane was checked by Ponceau staining (lower panel). **C:** Ethylene assay was performed with wild type Col-0 and SERK4 (*XVE-SERK4*) T1 plants. BAK1 (*XVE-BAK1* line11-2) T3 plants were used as positive control for MAMP-independent ethylene accumulation. Leaf strips from four-week-old plants were treated overnight

with 1 µM estradiol or three hours with 1 µM flg22. Graph shows the mean of four technical replicates and error bars correspond to standard error of the mean with n=4. **D:** Expression of SERK4/BAK1 in presence or in absence of 1 µM estradiol was detected by anti-BAK1 antibodies (upper panel); Ponceau staining indicates the amount of proteins present on the membrane (lower panel). Asterisks (*) mark cross-reacting bands.

The constitutive ethylene phenotype was confirmed in T3 generation with homozygous BAK1 and SERK1 seedlings. In contrast to BAK1, six hours estradiol treatment did not enhance ethylene accumulation in T3 seedlings overexpressing SERK1, but after 24 hours the constitutive ethylene phenotype was induced by both proteins (**Figure S3.1.6A**). On the other hand seedling growth was equally reduced by SERK1 and BAK1 overexpression in presence of estradiol compared to wild type seedlings (**Figure S3.1.6B** and **C**). This reduction was even stronger than the one provoked by flg22. These results suggest that overexpression of SERK1 is able to activate early and late defense responses, but possibly the dynamic of the activation is different from this of BAK1. Collectively these results indicate that BAK1 homologues namely SERK1 and SERK4 may possess redundant roles in the constitutive-defense phenotype activated by BAK1 overexpression.

3.1.4 Discussion

One of the key actors in PTI signaling is the membrane-localized LRR-RLK BAK1. In the best characterized MAMP-induced signaling model, BAK1 interacts with the flagellin receptor FLS2 upon ligand binding, and both interacting partners get phosphorylated to induce downstream signaling cascades and plant defense (Chinchilla et al., 2007; Schulze et al., 2010). But BAK1 is also an important element of BR-dependent development in association with another LRR-RLK BRI1 (Li et al., 2002; Nam and Li, 2002). In addition to its two important functions in immunity and development, BAK1 is also a negative regulator of plant cell death control (He et al., 2007; Kemmerling et al., 2007).

Several former reports can be found in the literature on BAK1 overexpression. First, expression of *BAK1* cDNA under the control of the *CaMV 35S* promoter, showing 30 times greater mRNA levels than Col-0 wild type plants, was described to partially rescue the

phenotype of the weak *bri1-5* mutant (Li et al., 2002). In parallel, genomic *BAK1* driven by its own promoter expressed in Col-0 background phenocopied the overexpression of BRI1 with elongated leaves, petioles and enhanced BR sensitivity compared to wild type plants (Nam and Li, 2002). These results indicated that an increase in BAK1 expression could enhance BR-dependent plant growth. These previous reports are not concordant with our findings, where we observed growth defects, cell death and constitutive activation of defense responses by overexpression of BAK1, although we cannot exclude that this difference may be due to different experimental conditions used in different laboratories. More recently it was described that overexpression of a functional Arabidopsis *BAK1* ortholog, *Oryza sativa BAK1*, in rice reduced the plant height average about 40% compared to wild type rice and increased considerably the host resistance against blast fungus *Magnaporthe grisea* infection (Hu et al., 2005; Li et al., 2009). Additionally, during the course of our study, it was published that *Arabidopsis* BAK1 expression under its endogenous promoter resulted in a growth defect with inappropriate cell death responses and constitutive accumulation of PATHOGENESIS-RELATED 1 (PR1) protein in Col-0 wild type background but not in *bak1-3* null mutant background (Belkhadir et al., 2011). The authors demonstrated that increased levels of BRI1 were able to suppress the BAK1 overexpression phenotype, and they suggested that BR signaling might has a role in this phenotype. Nevertheless the characterization of this phenotype remains very limited in this study.

3.1.4.1 BAK1 overexpression in Arabidopsis activates PTI

Surprisingly, our study revealed that overexpression of *BAK1* caused a drastic phenotype in Arabidopsis: developmental defect, leaf cell death, plant lethality (**Figure 3.1.1, 3.1.6-7, S3.1.1, S3.1.3-4**), elicitor-independent activation of defense responses (**Figure 3.1.2-7**) and increased resistance against hemibiotrophic bacterial pathogen *Pto* DC3000 (**Figure 3.1.8**). Two of the observed activated defense responses induced by BAK1 overexpression are the phosphorylation of MAPKs and the accumulation of the stress hormone ethylene. Both were observed after six hours of estradiol treatment in a correlation with the accumulation of BAK1 protein (**Figure 3.1.2-3**). Consistently with MAPK activation, we could observe induction of transcriptional changes upon estradiol treatment of *XVE-BAK1* seedlings. We tested the

expression of three defense marker genes. While *FRK1* is specific for the MAPK cascade and *PHI-1* for the CDPK cascade, *NHL10* is activated synergistically by MAPKs and CDPKs (Boudsocq et al., 2010). *FRK1* and *NHL10* showed up-regulation after BAK1 overexpression independently of MAMP treatment (**Figure 3.1.4**) confirming that MPK6/MPK3 signaling cascade was activated in these plants. In case of *PHI-1*, which was shown to be rapidly induced after flg22 perception (Boudsocq et al., 2010), a significant increase in transcript levels was neither detected after estradiol treatment nor after treatment with flg22. These variable results could be due to the difference in the approach. Boudsocq and collaborators used protoplast and analyzed the transcript levels after 30 minutes flg22 treatment whereas here seedlings were used and transcript levels were determined at six hours estradiol and one hour flg22 treatment. Thus it cannot be concluded if BAK1 accumulation also triggers the CDPK cascade, which might be an intriguing difference to PTI elicited by flg22. However, these results demonstrate that BAK1 overexpression triggers expression of defense genes, which at least partially depend on the MAPKs MPK3 and MPK6.

The biological role of MAPK activation, the up-regulation of defense gene expression and the accumulation of ethylene is to improve the plant's resistance to pathogens. Hence, to test this idea we performed a pathogen assay on Arabidopsis plants overexpressing BAK1. Our results confirm the role of BAK1 in pathogen resistance previously demonstrated with *bak1* mutants in Arabidopsis and in *N. benthamiana* plants (Heese et al., 2007; Roux et al., 2011). Notably, it was described before that *bak1-3* and *bak1-4* mutants developed necrotic lesions upon *Pto* DC3000 infection but their resistance, as measured by quantitative analysis, was not altered compared to wild type plants (Kemmerling et al., 2007). In contrast, the same mutants were more susceptible to necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola* and showed increased resistance to biotrophic oomycete *Hyaloperonospora arabidopsidis* (Kemmerling et al., 2007). However, it is difficult to interpret these results regarding the role of BAK1 in disease resistance since these *bak1* mutants exhibit deregulation not only in immune responses but also in cell death and hormone signaling (Li et al., 2002; Chinchilla et al., 2007; He et al., 2007; Heese et al., 2007; Kemmerling et al., 2007). To overcome this issue, the *bak1-5* mutant was used, which is only altered in immune signaling (Schwessinger et al., 2011). Plants

expressing the BAK1-5 protein displayed increased susceptibility compared to wild type plants against adapted and non-adapted hemibiotrophic *P. syringae* strains and also against weakly virulent isolates of biotrophic oomycete *Hyaloperonospora arabidopsidis* (Roux et al., 2011; Schwessinger et al., 2011). In accordance with a positive role of BAK1 in immunity, we showed that over-accumulation of the wild type BAK1 in seedlings increased the resistance against pathogenic *Pto* DC3000 (**Figure 3.1.8**). Since the constitutive overexpression of BAK1 led to premature plant death and the inducible BAK1 expression was hardly reproducible in entire adult plants it was difficult to perform other pathogen assays, for example with biotrophic oomycetes or necrotrophic bacteria.

3.1.4.2 Possible role of truncated forms of BAK1

It is not clear if the form of BAK1 causing our phenotype is the full-length protein or a truncated BAK1 form since we could observe accumulation of a full length and two truncated forms, containing the C-terminal part of BAK1, in both constitutive and inducible overexpressor lines (**Figures 3.1.1-2, 3.1.6-8, 3.1.10, S3.1.1-3**). The presence of BAK1 forms of lower masses could be due to degradation of the full-length protein. It is also possible that these BAK1 forms are the products of different transcriptional or post-transcriptional changes like alternative splicing, and that they are naturally present in WT plants but below the detection limit of our experimental procedure. A previous study compared cDNA sequences submitted in "The Arabidopsis Information Resource" (TAIR) database with full-length cDNA clones of the entire Arabidopsis *LRR-RLK* subfamily genes and revealed that several members of this subfamily undergo alternative splicing (Gou et al., 2010). These alternatively spliced transcripts could be present in the same tissue or in different tissues or could be produced under different environmental conditions and therefore increase the functional and regulatory diversity of the given gene. Interestingly it was recently reported that *Medicago truncatula SERK3* (*MtSERK3*), possibly also involved in defense, possesses seven splice variants (Nolan et al., 2011). However, up to date, no existence of splice variants was reported for Arabidopsis *BAK1*. Further investigation of different BAK1-derived constructs could help to establish its structure-function relation (3.2 Chapter 2: Structure-function analysis of BAK1).

In contrast to the hypothesis, that BAK1 truncated forms might be the product that elicits the constitutive defense response, our results also support an inhibitory role of the truncated forms. In estradiol treated samples, ethylene production decreased to the basic level at 27 hours treatment while in doublly treated samples (estradiol and flg22), the production stayed as high as in flg22 treated samples (**Figure 3.1.3**). This indicates that the decrease of ethylene production in estradiol treated XVE-BAK1 seedlings was not due to the exhaustion of the biosynthetic pathway of ethylene. Moreover, at 27 hours over-accumulation of BAK1, BAK1 itself and its truncated forms were still present at an even higher level than at six hours (**Figure 3.1.2**). Since truncated forms of BAK1 accumulate over time, a possible explanation for the disappearance of the phenotype could be a negative feedback control by these truncated forms on downstream element(s) shutting down BAK1-triggered responses.

3.1.4.3 The BAK1 overexpression phenotype is independent of the known membrane-localized interaction partners of BAK1

To elucidate the genetic determinant(s) of the BAK1 overexpression phenotype, the *2x35S-BAK1* construct was introduced into different mutant backgrounds. First we hypothesized that the over-accumulation of BAK1 might lead to aberrant, ligand-independent interaction with PRRs like FLS2 and EFR, which then could trigger a constitutive defense response. But our results demonstrate that the growth phenotype and leaf necrosis shown by BAK1 overexpressors seem to be independent of the presence of PRRs such as FLS2 and EFR (**Figure S3.1.4B**). However BAK1 likely regulates several PRRs which remain unknown and using single mutants of those may not allow us to test this hypothesis.

So we searched for potential interactors of BAK1 which may act as PRRs using a proteomic approach: The PRRs for endogenous elicitor perception, PEPR1 (PEP RECEPTOR 1) and PEPR2 (PEP RECEPTOR 2), were found to co-immunoprecipitate with BAK1 in *XVE-BAK1* estradiol-treated seedlings (Delphine Chinchilla, unpublished results). Thus, an over-accumulation of BAK1 might trigger an aberrant activation of the *At*Pep-PEPR signaling pathway which then induces PTI and causes the observed phenotypes. This might work either by direct phosphorylation and activation of the PEPRs by BAK1 (Schulze et al., 2010) or the excess of BAK1 could mimick the presence of MAMPs. Detection of MAMPs is known to trigger an

upregulation of *PEPR* and *PROPEP* transcription and is supposed to promote a release of *At*Peps to enhance the immune response initiated by the detection of MAMPs (Yamaguchi et al., 2010). However the *pepr1 pepr2* double mutant was unable to rescue the growth phenotype caused by BAK1 overexpression (Delphine Chinchilla, unpublished results). Moreover, the results of the co-IP experiments appear to be unspecific since repetitions of the co-IP experiment including WT plants as control showed an interaction of PEPRs with BAK1 already in wild type plants. For the moment there is no evidence which supports that the *At*Pep-PEPR-system takes part in the formation of the constitutive defense phenotype of the BAK1 overexpression plants.

In addition we investigated the involvement of another ligand-binding receptor, BRI1, which is also an interacting partner of BAK1. Its expression showed reduced levels in *XVE-BAK1* seedlings upon estradiol treatment, but this decrease was also observed in response to MAMP treatment (flg22), so it is difficult to conclude if this effect is direct or indirect (**Figure 3.1.5**). Moreover, the *bri1-301* mutation was not able to revert the growth defect and cell death observed when constitutively overexpressing BAK1, indicating that the observed phenotype is independent of the presence of a fully functional BR receptor (**Figure S3.1.4A**). This genetic approach did not allow to conclude if BAK1 acts via ligand-binding interacting partners to induce growth impairment, cell death and lethality possibly by modifying downstream signaling element(s).

Next we addressed the role of known signaling elements that act downstream of the ligand binding receptors. After flg22 perception and FLS2-BAK1 receptor complex formation and phosphorylation, BIK1 and its close homolog PBL1 are phosphorylated and BIK1 is released from the receptor complex (Lu et al., 2010b; Zhang et al., 2010). *BIK1* expression levels were induced about three fold by BAK1 overexpression as well as by flg22 treatment, indicating the activation of defense signaling (**Figure 3.1.9**). Interestingly we found a tendency that the combined *bik1 pbl1* knock-out mutations could rescue the BAK1 overexpression induced growth defect suggesting that functional BIK1 and/or PBL1 are involved in the BAK1 overexpression phenotype (data not shown). However these results were difficult to reproduce. Indeed, the Arabidopsis genome possesses 29 homologs of BIK1 and PBL1 sharing functional redundancy (Zhang et al., 2010), which might mask the effect of *bik1 pbl1* mutation on BAK1

overexpression in our growth conditions. To further test the involvement of BIK1 in the BAK1 overexpression phenotype we could introduce the *XVE-BAK1* construct in BIK1-HA expressing plants and monitor BIK1 phosphorylation status, detected as band shift with anti-HA antibodies (Lu et al., 2010b). This could give an indication that the PTI pathway is involved in our phenotype since BIK1 phosphorylation seems to be required for MAMP and DAMP signaling (Zhang et al., 2010; Liu et al., 2013).

3.1.4.4 Is the BAK1 overexpression phenotype a result of ETI activation?

Cell death, such as observed upon BAK1 overexpression, is a characteristic reaction of constitutive R protein activation. However none of the tested R protein mutants or mutants of regulators of R proteins such as PAD4, EDS1 and NDR1 were able to rescue the activated-defense phenotype induced by BAK1 overexpression (Delphine Chinchilla, unpublished data and **Table S3.1.1**). Additionally elevated temperature (28 °C) did not revert the phenotype and made it even more sever (**Figure S3.1.5**). A recent study investigated the effect of temperature on plant immune response (Cheng et al., 2013). The authors demonstrated that relatively elevated temperatures (23 – 32 °C) inhibit ETI but promote PTI responses (Cheng et al., 2013). According to these findings, our results would point into the direction of PTI activation.

Importantly, we found that the knock out *sobir1-13* mutation reverted almost entirely the growth impairment and cell death phenotype of BAK1 overexpressors (**Figure 3.1.10 and Table S3.1.1**). Additionally, *SOBIR1* expression was up-regulated by BAK1 overexpression (**Figure 3.1.9**). *SOBIR1* was identified in a suppressor screen to revert the constitutive-defense phenotype of *bir1-1* (Gao et al., 2009). The *SOBIR1* gene encodes an LRR-RLK which does not seem to interact with BIR1 and BAK1 (Gao et al., 2009; Liebrand et al., 2013). Its overexpression leads to cell death activation and induction of defense responses therefore SOBIR1 was proposed to be a positive regulator of cell death and defense responses in *R* gene mediated resistance (Gao et al., 2009). Regarding these results one hypothesis could be that the integrity of BAK1 or the BAK1 complex is under the control of an *R* gene(s). Modification of BAK1 leads to activation of this *R* gene(s), which then activates the *SOBIR1*-dependent resistance pathway. Interestingly, it was shown that SOBIR1 interacts with two RLPs in tomato, Ve1 and Cf-4 mediating resistance to the fungal pathogens *Verticillium dahlia* and *Cladosporium fulvum*,

respectively (Liebrand et al., 2013). In addition, SOBIR1 together with BAK1 were shown to control defense against necrotrophic fungal pathogens mediated by RLP30 (RECEPTOR-LIKE PROTEIN30) / SCFE1 (SCLEROTINIA CULTURE FILTRATE ELICITOR1), a newly identified PRR / MAMP pair (Zhang et al., 2013). Similarly, SOBIR1 is also required for the functionality of a recently identified Arabidopsis RLP, ReMAX (RECEPTOR OF eMax), which recognizes eMax (enigmatic MAMP of *Xanthomonas*), a bacterial MAMP from Xanthomonas (Jehle et al., 2013a; Jehle et al., 2013b). These recent findings point into the direction that SOBIR1 is most likely involved in PTI. Therefore more data are needed to support the idea of BAK1 being under the control of an R protein, which causes the observed aberrant phenotype of BAK1 accumulation.

SOBIR1 is also called EVERSHED and has been connected to the process of abscission possibly by modulating molecule secretion through the Golgi apparatus (Leslie et al., 2010; Lewis et al., 2010). Additionally, in *Nicotiana benthamiana* SOBIR1 was shown to be required for the accumulation and the stability of RLPs Ve1 and Cf-4 (Liebrand et al., 2013). Thus another possible hypothesis is that SOBIR1 could affect the amount/stability of BAK1 in the plasma membrane and therewith suppress its phenotype based on over-accumulation. One way to confirm this would be to test if BAK1-GFP is localized at the membrane in the *sobir1-13* mutant compared to the wild type.

Regarding our results there is no evidence for ETI activation would be required for the BAK1 overexpression phenotype. Nevertheless the activation of ETI is not so unlikely since BAK1 is a target of effectors due to its broad importance in immunity (Shan et al., 2008; Chinchilla et al., 2009; Cheng et al., 2011).

3.1.4.5 Overexpression of SERK proteins induce the activated defense phenotype

We also investigated the role of two BAK1 homologs, namely SERK1 and SERK4, in the constitutive-defense phenotype. Overlapping functions of the SERK family members in distinct biological processes were already demonstrated several times (He et al., 2007; Albrecht et al., 2008; Roux et al., 2011; Gou et al., 2012). SERK1 involvement in defense was already confirmed in two plant species. Rice SERK1 was proposed to positively regulate resistance against *Magnaporthe grisea* blast fungus (Hu et al., 2005). Additionally tomato SERK1 is required for the functioning of an NB-LRR resistence protein Mi-1 responsible for resistance against

herbivores (nematodes and insects) and also for Ve1-mediated fungal *Verticillium* resistance (Fradin et al., 2011; Mantelin et al., 2011). Here we found that SERK1 is involved in defense also in Arabidopsis. The *XVE-SERK1* seedlings showed constitutive ethylene accumulation and seedling growth inhibition upon estradiol treatment (**Figure 3.1.11** and **S3.1.6**). Interestingly after six hours estradiol treatment SERK1 samples in contrast to BAK1 did not show enhanced ethylene accumulation while at 24 hours both proteins induced ethylene accumulation in an elicitor-independent manner. This difference could be due to the different accumulation dynamic of these two proteins. Unfortunately this could not be tested since we don't have suitable antibodies to detect SERK1. Another possibility is a preferential association of BAK1 and SERK1 with an unknown interacting partner, as it was shown for SERK family members with FLS2 and with EFR (Roux et al., 2011), to induce the activated-defense phenotype. Moreover SERK4 overexpression also induced constitutive ethylene accumulation (**Figure 3.1.11C** and **D**). These results demonstrated that SERK proteins are able to induce the activated-defense phenotype however further analysis are needed to fully explore this redundancy among the members of the SERK family.

3.1.4.6 Concluding remarks

Our current results indicate that BAK1 overexpression may override mechanisms of negative regulation of PRRs. Recently SOBIR1 emerged as a potential regulator for several PRRs from the RLP class (Jehle et al., 2013a; Jehle et al., 2013b; Zhang et al., 2013). RLPs lack the cytoplasmic kinase domain and may require additional proteins such as for example SOBIR1 to transmit the extracellular stimuli into the cytoplasm (**Figure 3.1.12A**). We found that the the *sobir1-13* mutation rescued the growth defect and cell death induced by BAK1 overexpression (**Figure 3.1.10** and **Table S3.1.1**). In a hypothetical model one could imagine that in absence of microbes, PRRs or molecules associated with PRRs, in the case of RLPs, SOBIR1, might be inhibited by an unknown inhibitor (**Figure 3.1.12A**). Upon elicitor recognition BAK1 is recruited to the RLP-SOBIR1 complex and this may lead to the release of the unknown inhibitor (**Figure 3.1.12A**). In case of BAK1 overexpression, extra molecules of BAK1 may saturate the repressing system (**Figure 3.1.12B**). For example there is too little amount of inhibitors to repress the BAK1 activity. This allows the kinase domain of BAK1 to reach/modify more easily the catalytic

domain of SOBIR1 and thus causes constitutive defense responses despite the absence of elicitor molecules. BAK1 overexpressors showed massive cell death, a hallmark of ETI, but none of the mutations affecting *R*-gene or regulators of *R*-genes tested in this study had an effect on this phenotype (**Table S3.1.1**). These results may indicate that more than one R gene is involved in the BAK1 overexpression phenotype. Thus we cannot exclude that BAK1 overexpression and/or formation of truncated forms, somehow, induces *R* gene(s)-dependent resistance pathway(s) to activate ETI. Finally, since BAK1 is involved in multiple biological processes it should be mentioned that all the programs including development, defense (PTI/ETI) and cell death regulated by BAK1 are potentially modified by its overexpression and thereby may contribute to the observed phenotype in parallel of the activation of immunity pathway. These open hypotheses need to be further explored in the future to fully understand the complex role of BAK1 in plant defense.

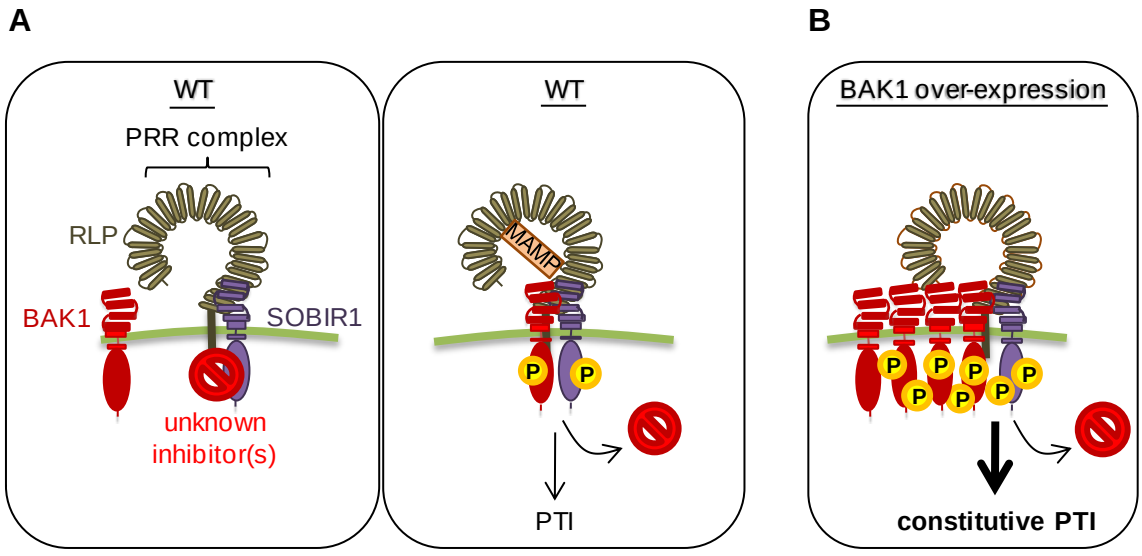


Figure 3.1.12: BAK1 overexpression overcomes the normal repression of PRRs. **A**: In a hypothetical model the PRR complex containing SOBIR1 could be repressed by an unknown inhibitor in absence of elicitors. In presence of a MAMP, BAK1 is recruited in the receptor complex, to induce the phosphorylation of the interacting partners and the release of the unknown inhibitor leading to PTI activation. **B**: Overexpression of BAK1 may saturate the normal repressing system. **WT**: wild type; **PRR**:

pattern recognition receptor; **RLP**: receptor-like protein; **BAK1**: BRI1-ASSOCIATED KINASE 1; **SOBIR1**: SUPPRESSOR OF BIR1-1; **MAMP**: microbe-associated molecular pattern; **P**: phosphorylation; **PTI**: pattern-tiggered immunity.

3.1.5 Supporting information for Chapter 1: BAK1 overexpression leads to constitutive defense responses in *Arabidopsis thaliana*

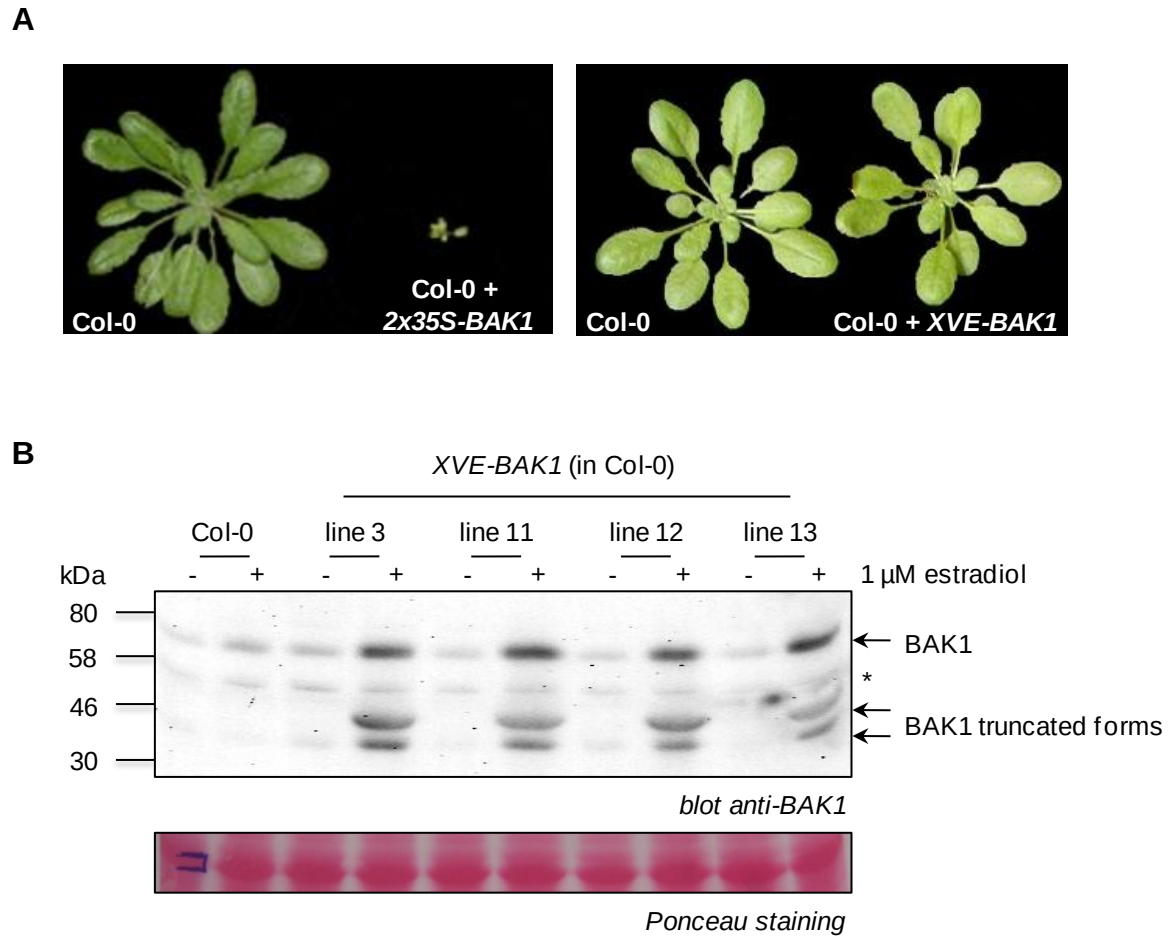


Figure S3.1.1: Development of first generation plants of *2x35S-BAK1* and *XVE-BAK1* stably transformed in Col-0 background. A: Col-0 and *2x35S-BAK1* plants were grown in short day conditions for 6 weeks. The *2x35S-BAK1* plant shows a developmental defect compared to the wild type plant (left picture). Col-0 and *XVE-BAK1* plants were grown in short day conditions for five weeks. In absence of estradiol, *XVE-BAK1* plants and wild type plants develop the same way (right picture). **B:** Total proteins were extracted from T2 seedlings treated or not with estradiol (1 µM) overnight and were analyzed by Western blot using antiBAK1 antibodies (upper panel). *marks cross-reacting bands. Ponceau staining was made to detect the amount of protein on the membrane (lower panel).

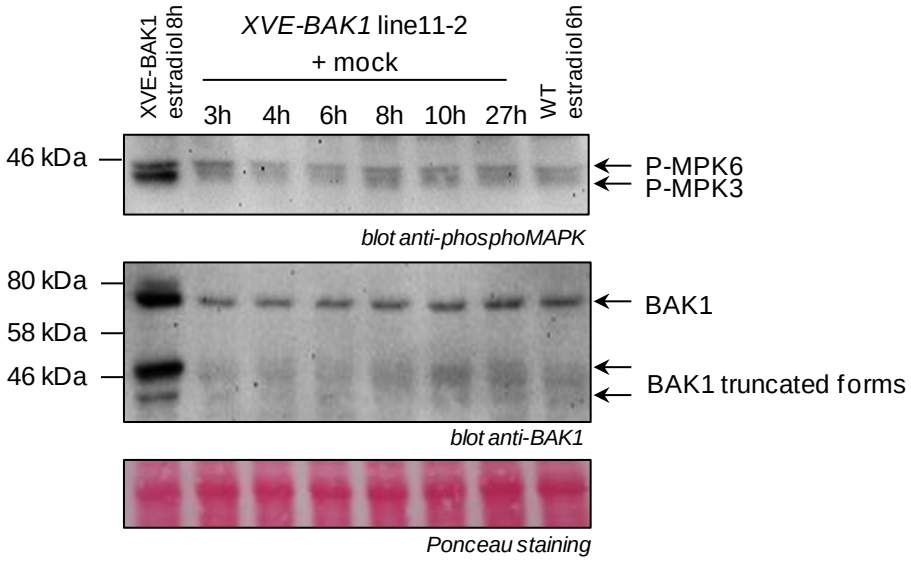
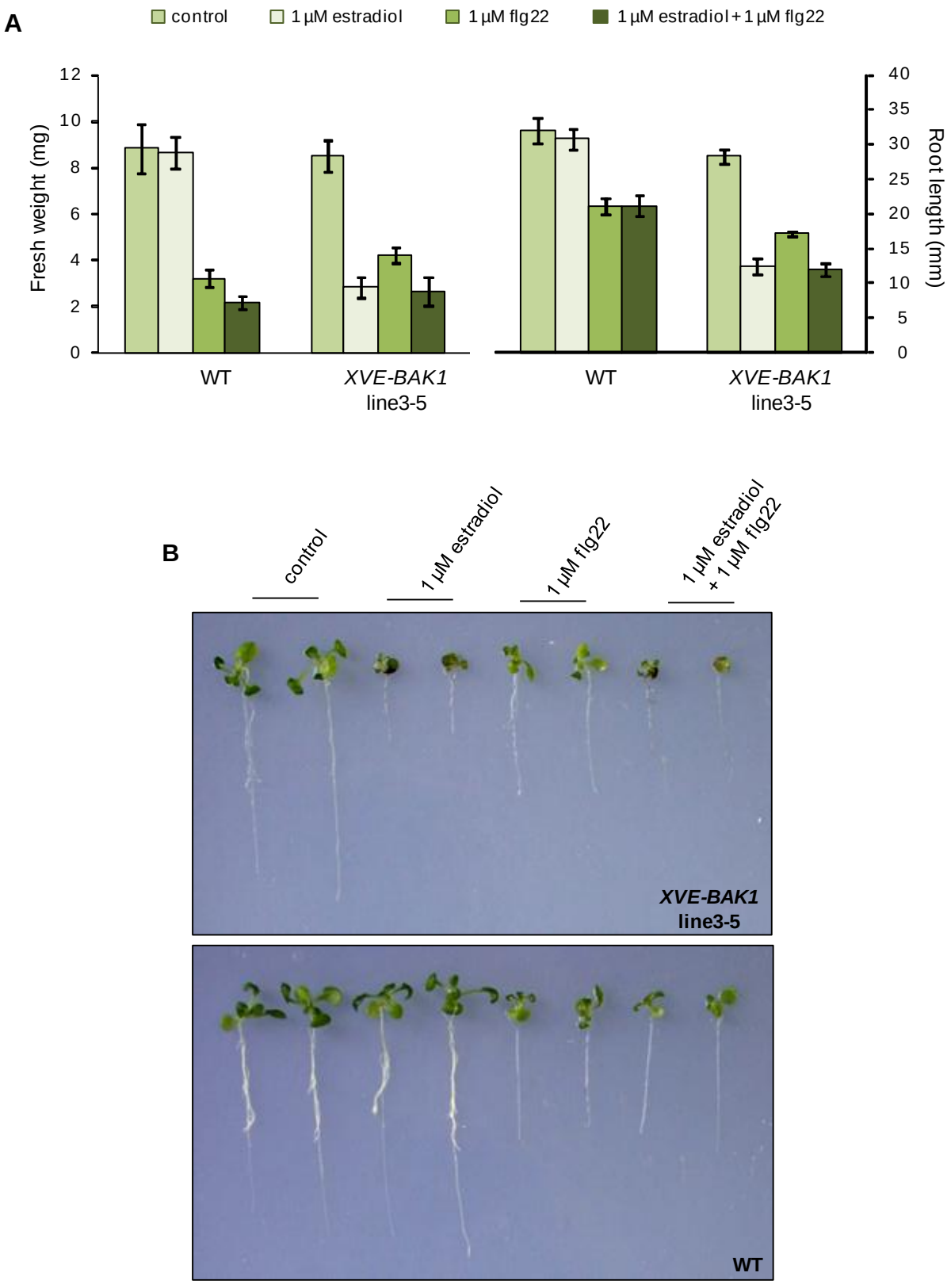


Figure S3.1.2: BAK1 overexpression is required for MAPK activation in *XVE-BAK1* seedlings. Western blot analysis were done with total protein extracts from six-day-old *XVE-BAK1* and wild type (WT) seedlings treated with 1 µM estradiol for eight hours (left- and right most lanes) or XVE-BAK1 seedlings treated with mock solution (ethanol at the same concentration as used in the estradiol treatments). MAPK activation was detected with antibodies recognizing the phosphorylated forms of MAPKs (upper panel) in *XVE-BAK1* seedlings treated for three, four, six, eight, ten and 27 hours with 1 µM estradiol. Accumulation of BAK1 and its truncated forms were detected by BAK1 antibody able to recognize the C-terminal part of BAK1 (middle panel). Ponceau staining was done on the membrane to verify the amount of protein extract in each well (lower panel). This experiment was performed four times and showed similar results.


A
control
1 µM estradiol
1 µM flg22
1 µM estradiol + 1 µM flg22
Fresh weight (mg)
Root length (mm)
0
2
4
6
8
10
12
0
5
10
15
20
25
30
35
40
WT
XVE-BAK1 line3-5
WT
XVE-BAK1 line3-5
B
control
1 µM estradiol
1 µM flg22
1 µM estradiol + 1 µM flg22
XVE-BAK1 line3-5
WT

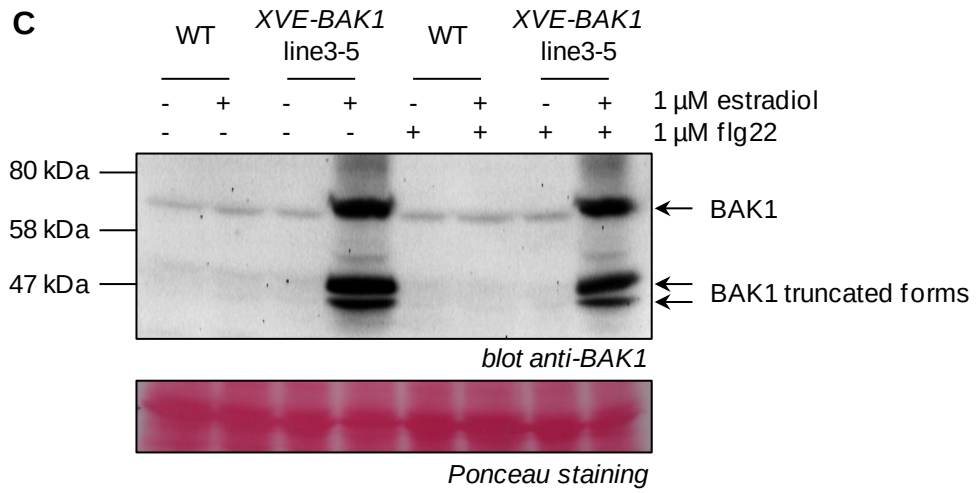


Figure S3.1.3: BAK1 overexpression induces seedling growth inhibition in absence of elicitor. Seedling growth inhibition assay was repeated with an independent *XVE-BAK1* line (line3-5). **A:** Fresh weight and root length were measured. Graphs represent the mean of six technical replicates, error bars correspond to standard error of mean with n=6. In four independent experiments similar results were obtained. **B:** Two representative seedlings per treatment were analyzed. **C:** Total proteins were extracted from seedlings and analyzed by Western blot.

Figure S3.1.4: BAK1 overexpression phenotype is independent of BRI1, FLS2 and EFR. A: *2x35S-BAK1* was transformed in wild type (WT) and *bri1-301* mutant. T1 generation was grown in short day conditions for six weeks. **B:** *2x35S-BAK1* was transformed in wild type (WT) and *efr fls2* background. T1 generation was grown in short day conditions for six weeks.

Table S3.1.1: A *sobir1* mutation reverts the BAK1 overexpression phenotype but mutation of the other selected defense-related genes does not. *2x35S-BAK1* construct was stably transformed in different Arabidopsis mutant backgrounds. T1 generations were screened for BAK1 overexpression phenotype (Delphine Chinchilla, unpublished data). LRR-RLK: leucine-rich repeat - receptor-like kinase; BR: brassinosteroid; MAMP: microbe-associated molecular pattern; DAMP: damage-associated molecular patterns, PTI: pattern-triggered immunity; SA: salicylic acid; R: resistance; CC: coiled-coil, TIR: toll interleukin 1 receptor, NB-LRR: nucleotide binding - leucine-rich repeat.

mutant	reference	caracteristic of the mutation	BAK1 overxpression phenotype
bri1-301	Xu et al., 2008	LRR-RLK, BR signaling	yes
efr fls2	Nekrasov et al., 2009	LRR-RLK, MAMP perception	yes
pepr1 pepr2	Liu et al., 2013	LRR-RLK, DAMP perception	yes
bik1 pbl1	Zhang et al., 2010	PTI signaling	partial rescue ?
eds5-2	Volko et al., 1998	SA signaling	yes
sid2	Wildermuth et al., 2001	SA signaling	yes
npr1-5	Shah et al., 1997	SA signaling	yes
snc1-11	Yang and Hua, 2004	R gene	yes
rps5-2	Warren et al., 1998	R gene	yes
rpm1 rps2	Belkhadir et al., 2004	R gene	yes
eds1-2	Falk et al., 1999	regulator of CC-NB-LRRs	yes
pad4-1	Jirage et al., 1999	regulator of CC-NB-LRRs	yes
sag101-1	Feys et al., 2005	regulator of CC-NB-LRRs	yes
ndr1-1	Century et al., 1995	regulator of TIR-NB-LRRs	yes
eta3	Gray et al., 2003	regulator of CC-NB-LRRs	yes
sobir1-13	Gao et al., 2009	LRR-RLK, suppressor of *bir1-1* phenotype	rescue

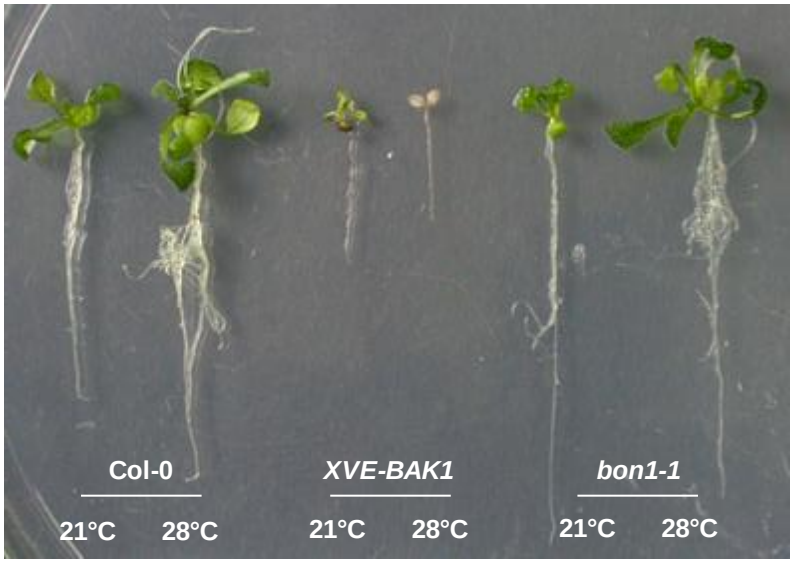


Figure S3.1.5: High temperature does not rescue BAK1 overexpression phenotype. Six-day-old Col-0, *XVE-BAK1* line11-2 and *bon1-1* (Hua et al., 2001) seedlings grown on solid MS plates were transferred in liquid MS medium supplemented or not with 1 µM estradiol. Seedlings were placed in 21°C and 28°C for six days. Picture shows estradiol treated samples at 21°C / 28°C. Two independent experiments show similar results. The *bon1-1* mutant was used as a positive control for the high temperature rescue of the growth phenotype induced by defense activation (Wang et al., 2011).

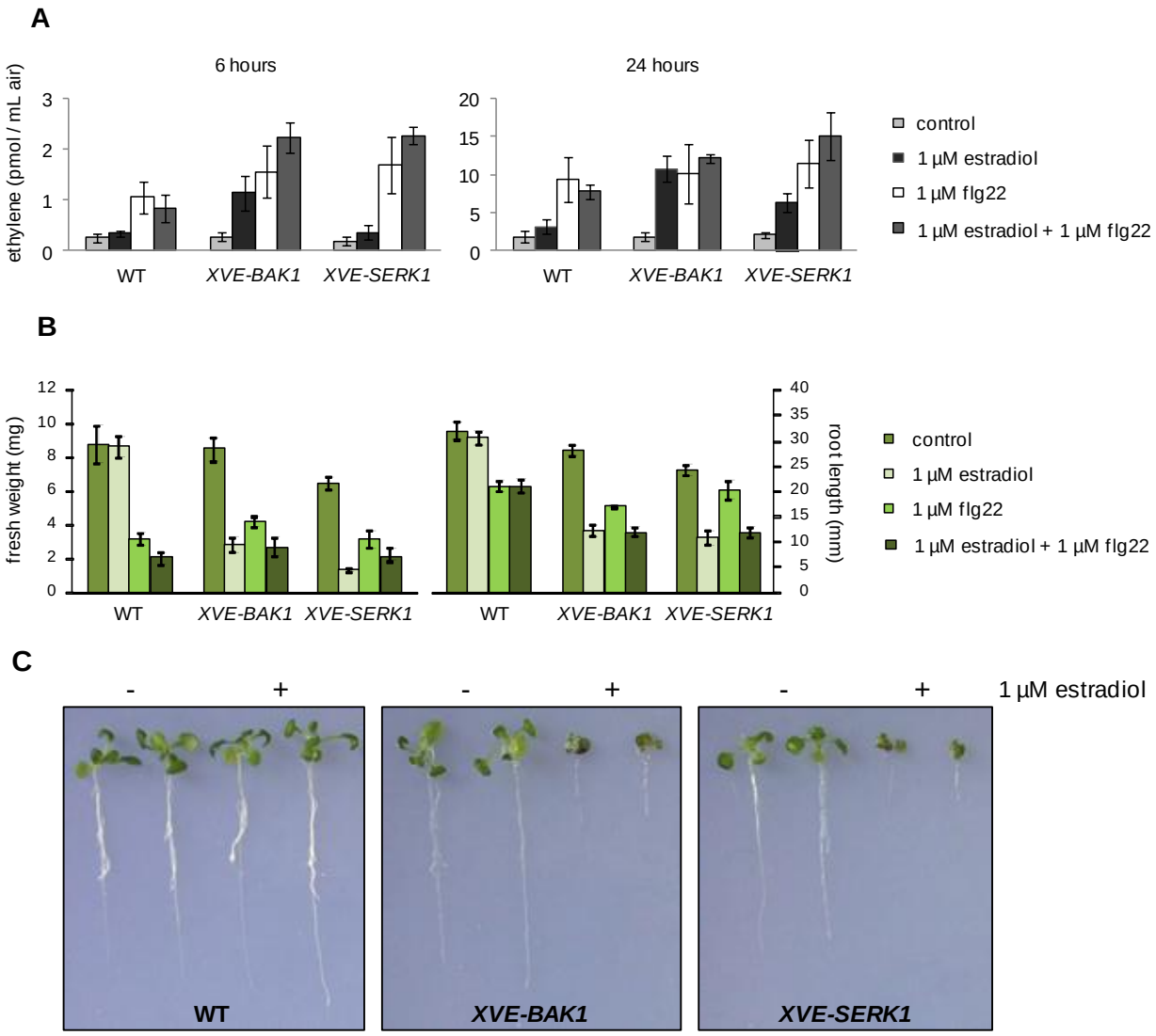


Figure S3.1.6: SERK1 activates defense responses in a MAMP-independent manner. A: wild type (Col-0), BAK1 (*XVE-BAK1* line 3-5) and SERK1 (*XVE-SERK1* line 6-2) homozygous T3 seedlings were used for ethylene assay in presence of control or 6 hours / 24 hours 1µM estradiol treatment or 1µM flg22 treatment or double treatment. The graphs represent the mean of four technical replicates and error bars correspond to standard deviation with n=4. B: The seedling growth inhibition assay was performed with the same lines. Fresh weight and root length were measured after 6 days of incubation with treatments used in A. C: Pictures were taken after 6 days of incubation in presence or in absence of 1 µM estradiol.

3.2 Chapter 2: Structure-function analysis of BAK1

3.2.1 Abstract

Plant immunity is triggered by pattern-recognition receptors (PRRs) upon the detection of microbe-associated molecular patterns (MAMPs). These PRRs belong mainly to the large class of receptor-like kinases (RLKs) which consist of an extracellular binding domain, a transmembrane domain and an intracellular domain. Although most RLKs seem to bind MAMPs or other ligands directly, some RLKs act as co-receptors to regulate the response. The Arabidopsis RLK BAK1 (BRI1-ASSOCIATED KINASE 1) is a known interaction partner of the MAMP-sensing receptors FLAGELLIN SENSING 2 (FLS2) and EF-TU RECEPTOR (EFR). Intriguingly, BAK1 overexpression leads to constitutive activation of defense responses along with growth impairment, leaf necrosis and premature death (Chapter 1). Together with the elevated protein levels of BAK1, truncated versions have been detected suggesting that breakdown products might be responsible for the impact of BAK1 overexpression on plant defense and development.

Here we investigated the involvement of different domains of BAK1 in the constitutive defense phenotype by expression of BAK1-derived constructs in Arabidopsis. BAK1 versions, which lack the C-terminal tail or display a reduced kinase activity still induced constitutive ethylene accumulation upon overexpression. Likewise, the overexpression of BAK1 extracellular domain anchored to the plasma membrane (BAK1anchor-ex) was also able to induce the same ethylene phenotype. In contrast, overexpression of BAK1 kinase deficient form suppressed the developmental defects caused by BAK1 overexpression whereas loss of the whole intracellular domain caused developmental defects distinct from the ones caused by full-length BAK1. Finally, the accumulation of soluble BAK1 extracellular or intracellular domain alone did neither induce the constitutive defense phenotype nor developmental defects.

In conclusion, overexpression of the BAK1 extracellular domain located at the plasma membrane is already sufficient to trigger the constitutive defense response, supporting the

hypothesis that the BAK1 extracellular domain interacts with other, yet unidentified RLKs for defense response induction.

3.2.2 Introduction

The first line of plant immunity involves pattern recognition receptors (PRRs) able to recognize conserved molecular signatures called microbe-associated molecular patterns (MAMPs) characteristic for microorganisms (Mackey and McFall, 2006; Zipfel, 2008; Boller and Felix, 2009). PRRs located at the plasma membrane transduce the extracellular stimuli into the cytoplasm to initiate downstream signaling cascades and induce defense responses such as ethylene accumulation, production of reactive oxygen species (ROS), activation of mitogen-activated protein (MAP) kinases or inhibition of seedling growth (Boller and Felix, 2009). All these reactions finally result in pattern-triggered immunity (PTI), which confers successful resistance against a broad range of pathogens (Zipfel et al., 2004; Jones and Dangl, 2006; Boller and Felix, 2009). Several PRRs belong to the receptor-like kinase (RLK) family, members of which have a prominent role in the initiation of cellular signaling to regulate plant processes as diverse as disease resistance, self/non-self recognition, regulation of development and hormone perception (Torii, 2004; Johnson and Ingram, 2005). RLK functions have been intensively studied in the last 20 years. However our knowledge on how perception of extracellular stimuli by RLKs is transmitted to the cell, and how appropriate plant responses are induced is still limited.

The leucine-rich repeat (LRR)-RLK subfamily is the largest subfamily of RLKs with more than 200 members in the *Arabidopsis thaliana* genome (Dievart and Clark, 2003). Two well characterized PRRs in Arabidopsis, FLS2 (FLAGELLIN SENSING 2) and EFR (ELONGATION FACTOR TU RECEPTOR) responsible for the recognition of bacterial flagellin and elongation factor Tu, respectively, belong to the LRR-RLK subfamily (Gomez-Gomez and Boller, 2000; Torii, 2004; Zipfel et al., 2006; Boller and Felix, 2009). Most of the LRR-RLKs are involved in ligand-protein and/or protein-protein interactions. The extracellular part of the LRR-RLKs is composed of a hydrophobic N-terminus including a signal peptide and an LRR domain with variable number of LRR copies, in certain cases assumed to be responsible for ligand binding (Shiu and Bleecker, 2001b; Kinoshita et al., 2005; Chinchilla et al., 2006; Albert and Felix, 2010). This extracellular

LRR domain is flanked by two characteristic double-cysteine motifs (Dievart and Clark, 2003). A single trans-membrane domain separates the ecto-domain from the intracellular Serine/Threonine (Ser/Thr) kinase domain and a C-terminal tail. Alignment of distinct kinase domain sequences supported by crystal structure determinations revealed that, despite their similar structures, protein kinases can be divided in two groups dependent on the presence or absence of a conserved RD (arginine-aspartate) motif in the catalytic loop of their kinase domain (Johnson et al., 1996). Interestingly most of the identified PRRs belong to the non-RD kinase family, as it is the case for FLS2 and EFR (Dardick and Ronald, 2006). Non-RD kinases are often less active and they are not able to auto-phosphorylate the activation loop and to maintain the correct conformation for catalysis (Johnson et al., 1996). This suggests that non-RD kinases are regulated through alternative mechanisms than RD kinases.

After respective ligand perception FLS2 and EFR both associate with the plasma membrane localized RLK, BAK1 (BRI1-ASSOCIATED KINASE 1) (Chinchilla et al., 2007; Heese et al., 2007). The interacting partners may phosphorylate each other to activate the downstream signaling cascade leading to PTI (Boller and Felix, 2009; Schulze et al., 2010). BAK1 was originally identified as the interacting partner of another LRR-RLK, the BRI1 (BRASSINOSTEROID INSENSITIVE 1) receptor to enhance brassinosteroid (BR)-dependent plant development (Li et al., 2002; Nam and Li, 2002). Additionally, BAK1 was also reported to be a negative regulator of cell death control (He et al., 2007; Kemmerling et al., 2007). Similar to FLS2, EFR and BRI1, BAK1 also presents a characteristic LRR-RLK structure (**Figure 3.2.1**). Its extracellular part contains a short LRR domain followed by a membrane spanning region, an intracellular serine/threonine kinase domain and a highly conserved C-terminus. But in contrast to FLS2 and EFR, BAK1 belongs to the RD kinase family, and possesses a strong kinase activity (Dardick and Ronald, 2006).

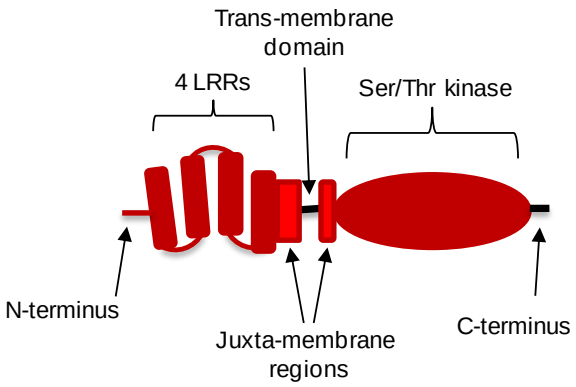


Figure 3.2.1: Schematic structure of BAK1. LRR: leucine rich repeat, **Ser:** serine, **Thr:** threonine.

Previously we showed in Arabidopsis that overexpression of BAK1 resulted in growth defect, leaf necrosis, premature death together with a constitutive activation of defense responses (Chapter 1). This phenotype occurred in a MAMP-independent manner and independently of the presence of FLS2, EFR and BRI1 (Chapter 1: **Figure S3.1.4**). Protein expression analysis of BAK1 overexpression lines showed the accumulation of putative truncated forms of BAK1 in addition to the full-length protein (Chapter 1: **Figure 3.1.1**). So we hypothesized that these or one of these truncated forms might be responsible for the activated-defense phenotype.

In this study, we wanted to find out, which structural part(s) of BAK1 are important to induce the observed constitutive-defense phenotype. Therefore we overexpressed BAK1-derived constructs under both the strong constitutive *2xCaMV 35S* and the estradiol-inducible *XVE* promoters. Analysis of these constructs stably transformed in Arabidopsis revealed that the C-terminal tail of BAK1 was not required and the extracellular or the intracellular domains of BAK1 alone were not sufficient to activate a constitutive defense response. More diverse results were obtained for the kinase-deficient version of BAK1: overexpression of this construct did not induce the growth phenotype characteristic to BAK1 overexpression but still induced constitutive ethylene accumulation. Furthermore, the extracellular domain anchored to the plasma membrane, but not the BAK1 intracellular part fused to the trans-membrane domain, was able to activate immune responses upon overexpression. However the growth phenotype induced by the overexpression of BAK1 ecto-domain anchored to the plasma membrane was different from the one induced by full-length BAK1 overexpression. These results suggest that the BAK1 ecto-domain localized in the plasma membrane is already sufficient to initiate a constitutive defense response. How this is accomplished in detail needs further investigation.

3.2.3 Results

3.2.3.1 BAK1 C-terminus is not determinant for the activated-defense phenotype

BAK1 belongs to the five members SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) group (SERK1 to SERK5), therefore it is also called SERK3 (Hecht et al., 2001). Protein sequence alignments of the SERK family members revealed that in the C-terminus (C-ter) the last four

amino acids (SGPR) are highly conserved (Seraine Beeler master work; Delphine Chinchilla personal communication). Indeed functional complementation studies of *bak1-4* null mutant with C-terminally tagged BAK1 showed defects in the induction of defense responses triggered by MAMPs but did not perturb ligand-dependent heterodimerisation between FLS2 and BAK1 (Delphine Chinchilla unpublished data; Ntoukakis et al., 2011). These data suggest that the C-terminus might have an important function in PTI signaling. In our study the construct lacking the SGPR motif was called "BAK1Δ". Upstream of the SGPR motif, the protein sequence of BAK1 is divergent from those of SERK1/2 and SERK4/5 (Boller and Felix, 2009). In addition to BAK1Δ, we constructed another C-ter-truncated version "BAK1Δ580" where we inserted a stop codon by site directed mutagenesis in position 580, because from this position the protein sequence of the SERK homologs is more divergente (**Figure 3.2.2**).

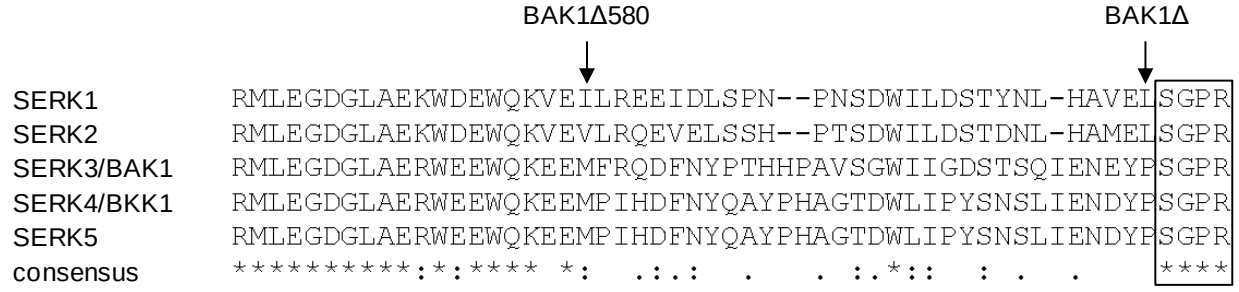


Figure 3.2.2: Amino acid sequence alignment of the C-terminus of SERK1 to SERK5. Protein sequences of SERK family members were aligned using the T-Coffee multiple sequence alignment tool: http://www.tcoffee.org (Di Tommaso et al., 2011). Black frame mark the highly conserved SGPR motif. Black arrows indicate the position of stop codons introduced in BAK1Δ and BAK1Δ580 respectively. In the consensus sequence an asterisk (*****) represents conserved amino acids in all sequences, a colon (**:**) indicates a position composed of amino acids with similar physicochemical properties, a dot (**.**) represents a position where semi-conserved substitutions are observed.

To analyze the role of the C-terminal region of BAK1 in plant defense regulation, we investigated *A. thaliana* Col-0 ecotype plants expressing the BAK1Δ constructs and BAK1 under the control of the estradiol-inducible *XVE* promoter. We selected ethylene accumulation as standard bioassay to determine which constructs were able to activate defense responses in a MAMP-independent manner. Samples for each genotype were pooled from three individual T1 plants and leaf strips were treated or not with estradiol overnight. A three hours 1 µM flg22

treatment was performed as positive control for MAMP-induced defense response. Plants containing estradiol-inducible BAK1Δ and BAK1Δ580 constructs produced similar levels of ethylene in presence of estradiol as BAK1 (**Figure 3.2.3A**). The accumulation level of the proteins encoded by the constructs was assessed by Western blot analysis (**Figure 3.2.3B**). BAK1Δ and BAK1Δ580 displayed the same protein pattern with the putative truncated forms as the BAK1 construct. Our results show that the C-terminal tail of BAK1 is not required for the induction of constitutive ethylene production suggesting that it is not involved in the activation of the constitutive defense response.

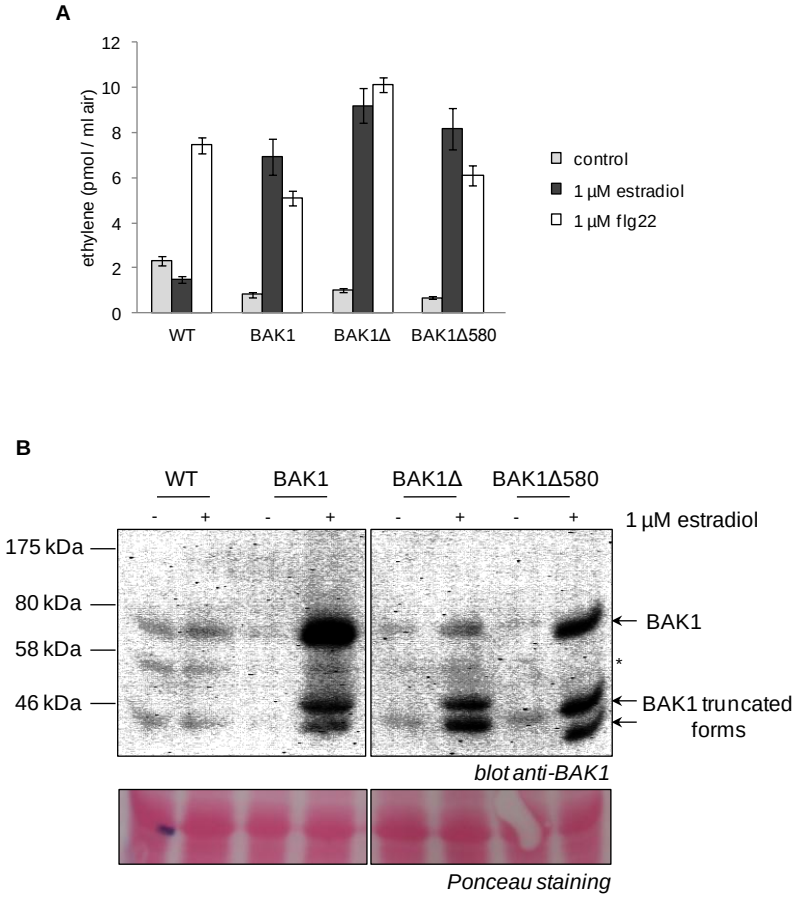
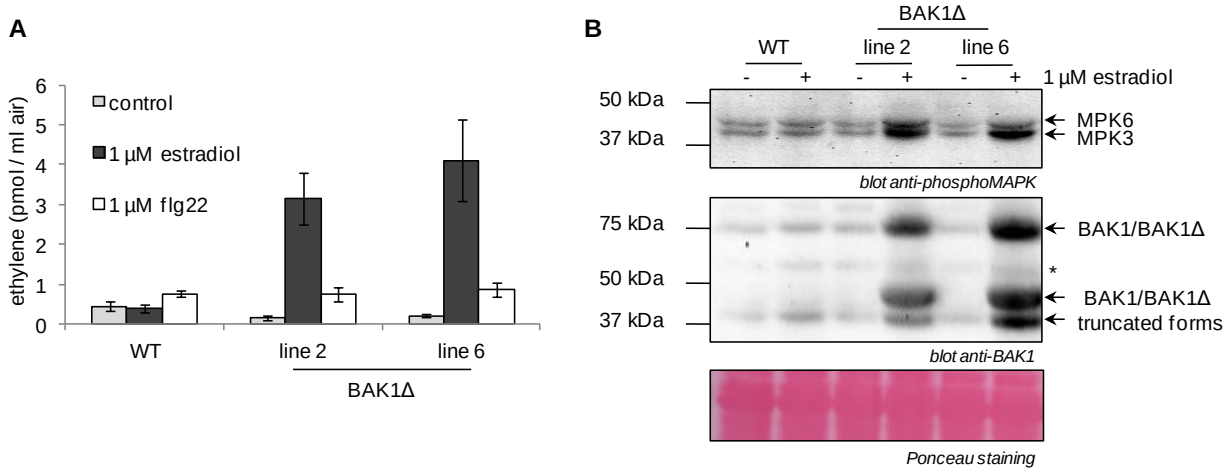


Figure 3.2.3: In *A. thaliana* C-terminal truncated BAK1 constructs are able to induce ethylene accumulation in a MAMP-independent manner. *A. thaliana* ecotype Col-0 (WT) transformed with BAK1, BAK1 C-terminally truncated versions were used for ethylene biosynthesis measurement. All the constructs were expressed under the estradiol inducible *XVE* promoter. **WT:** Col-0; **BAK1:** *XVE-BAK1*; **BAK1Δ:** *XVE-BAK1Δ*; **BAK1Δ580:** *XVE-BAK1Δ580.* **A:** Samples were pooled from three different T1 plants for each genotype and were treated with solvents (control), with 1 µM estradiol overnight or with 1

µM flg22 for three hours. Six technical replicates were prepared for each treatment. Error bars represent standard error of mean (n=6). **B:** Samples from ethylene bioassay were used for Western blot analysis with anti-BAK1 antibody (upper panels). Ponceau staining was performed to detect the amount of protein present on the membrane (lower panels). Asterisk (*) marks cross-reacting bands.

We further investigated the function of the conserved SGPR motif. In T2 generation the constitutive ethylene phenotype was confirmed with two independent lines, BAK1Δ-2 and BAK1Δ-6 (**Figure 3.2.4A**). Subsequently we used these two lines to test MAPK activation, another early defense response induced by pathogens (Nuhse et al., 2000; Asai et al., 2002). In estradiol treated samples accumulating the BAK1Δ protein, MPK6 and MPK3 were hyperphosphorylated in comparison to the control treatment, while in wild type Col-0 (WT) samples there was no difference between control and estradiol treated samples (**Figure 3.2.4B**). Inhibition of seedling growth was also assayed. In presence of estradiol in BAK1Δ lines fresh weight of seedlings was reduced compared to WT seedlings (**Figure 3.2.4C**). Pictures taken of BAK1Δ-2 seedlings showed that both shoots and roots were affected by overexpression of BAK1Δ. We did not observe significant differences in the activated-defense phenotype triggered by BAK1 and by BAK1Δ overexpression.



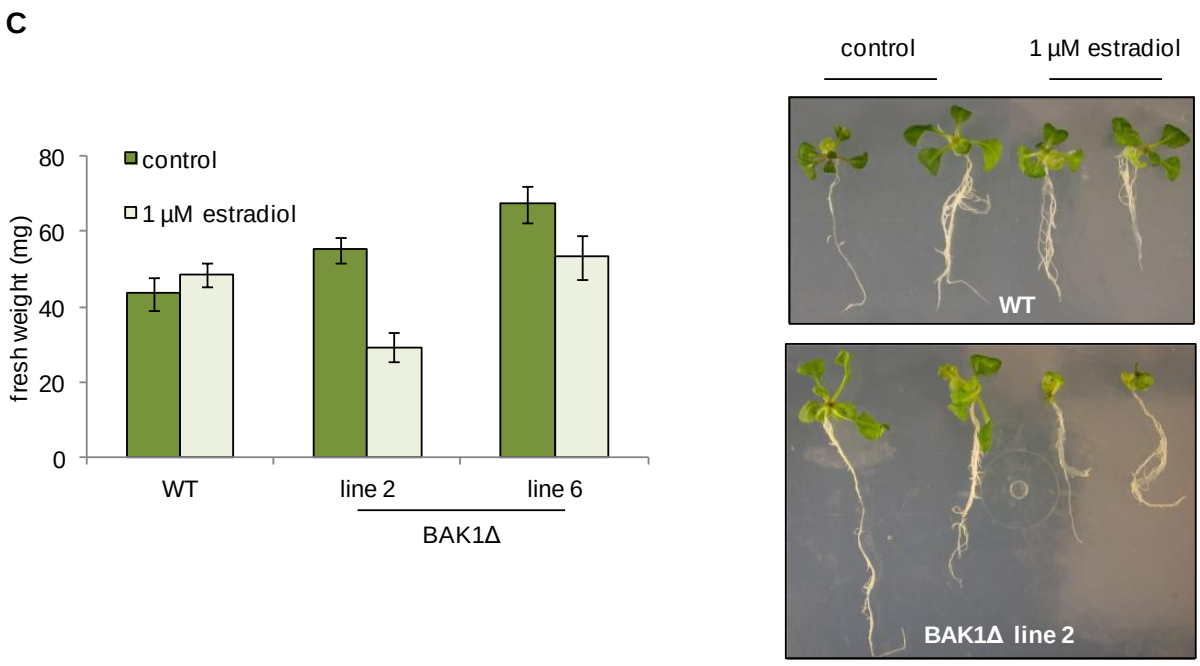


Figure 3.2.4: BAK1Δ induces constitutive defense responses in a MAMP-independent manner. Constructs were driven by the estradiol-inducible *XVE* chimeric promoter. **A:** Ethylene bioassay was performed with a pool of 7 T2 plants for each genotype. Leaf strips were treated with control or with 1 µM estradiol overnight or with 1 µM flg22 for three hours. Error bars correspond to standard deviation with n=4. **B:** Total proteins were extracted from two-week-old seedlings treated or not with 1 µM estradiol overnight. MAPK activation was detected by antibodies recognizing phosphorylated forms of MAPKs (upper panel). Accumulation of BAK1 and its truncated forms was detected with anti-BAK1 antibody (middle panel). Asterisk (*) marks cross-reacting bands. Ponceau staining was performed to detect the amount of protein present on the membrane (lower panel). **C:** Seedling growth assay was performed with T2 seedlings. Fresh weight of individual seedlings was measured after 10 days of germination in presence or in absence of 1 µM estradiol. Graph represents the mean of 10 technical replicates and error bars correspond to standard error of mean (n=10).

3.2.3.2 BAK1 kinase activity is not required to induce the constitutive ethylene phenotype

We observed that in the intracellular part of BAK1 the C-ter domain does not seem to be determinant for the induction of ethylene accumulation in an elicitor-independent manner (**Figure 3.2.3** and **3.2.4**). In the next step we tested whether BAK1 kinase activity was necessary or not to induce a constitutive ethylene accumulation. Therefore we used a BAK1 kinase deficient (BAK1-KD) mutant carrying two amino acid substitutions in the kinase domain: one in the ATP-binding site position 317 (K→E) and another one in the kinase sub-domain XI position

537 (Q→R) (Schulze et al., 2010). Both BAK1 and BAK1-KD could form a stable complex with FLS2 upon flg22 elicitation. However BAK1-KD was not able to restore the flg22 responsiveness of a *bak1-4* mutant while the wild type form of BAK1 did, indicating that BAK1 kinase activity is important for PTI signaling (Schulze et al., 2010). *A*. *thaliana* Col-0 wild type plants were transformed with the *BAK1-KD* construct under the control of the *2xCaMV 35S* promoter (hereafter referred to as *2x35S-BAK1-KD*) to check whether BAK1 kinase activity has a role in the growth phenotype previously observed with *2x35S-BAK1*. We observed that plants accumulating BAK1-KD did not show necrosis after 6 weeks of germination and after nine weeks they displayed a *bri1*-like phenotype with rounder leaves and shorter petioles (Delphine Chinchilla unpublished results). Notably, in contrast to wild type BAK1 overexpressing lines, BAK1-KD overexpression lines were able to produce seeds. These results suggest that kinase activity is required for the stunting phenotype induced by BAK1 overexpression. We also transformed plants with *BAK1-KD* construct under the control of the estradiol inducible *XVE* promoter (hereafter referred to as *XVE-BAK1-KD*) and ethylene accumulation was measured. From 13 independent transgenic lines analyzed, 11 lines showed the constitutive ethylene phenotype (**Figure 3.2.5A**) and this was in correlation with the overexpression of BAK1-KD and truncated forms (**Figure 3.2.5B**). These results indicate that BAK1 kinase activity is not required for constitutive ethylene accumulation induced by BAK1 overexpression.

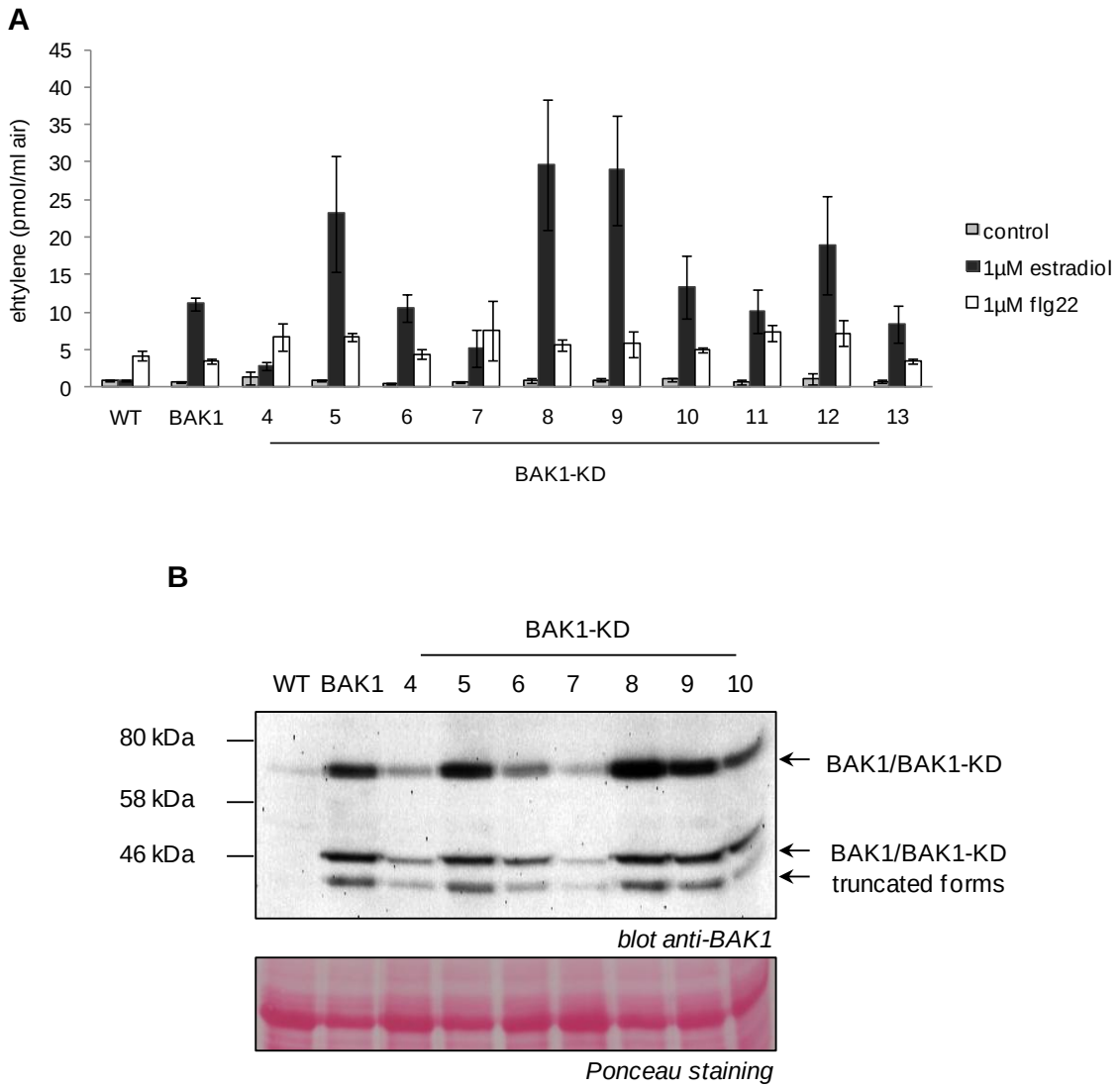


Figure 3.2.5: BAK1 kinase activity is not required to induce the activated-defense phenotype observed in plants overexpressing BAK1. A: Leaf strips from four-week-old Col-0 wild type (WT), BAK1 (*XVE-BAK1* line 11-2, T3 plants) and BAK1-KD (*XVE-BAK1-KD*, T1 plants) plants were used to measure ethylene biosynthesis in presence of solvents (control), overnight 1 µM estradiol treatment or three hours 1 µM flg22 treatment. Graph represents the mean of four technical replicates and error bars correspond to standard deviation with n=4. **B:** BAK1/BAK1-KD accumulation was detected by antibodies anti-BAK1 in estradiol treated leaf material after overnight incubation (upper panel). Ponceau staining was performed to detect the amount of protein present on the membrane (lower panel).

3.2.3.3 Overexpression of the plasma membrane anchored BAK1 ecto-domain causes phenotypes similar but not identical to BAK1 overexpression

To further investigate structural features of BAK1 involved in the activated defense phenotype we prepared additional BAK1-derived constructs. “BAK1ex” corresponds to ecto-domain of BAK1 and “BAK1anchor-ex” is composed of the ecto-domain and the predicted trans-membrane domain of BAK1. We also cloned the cytoplasmic domain of BAK1 called “BAK1in” as well as the signal peptide fused to the trans-membrane domain plus the intracellular domain called “BAK1anchor-in” (**Figure 3.2.6**).

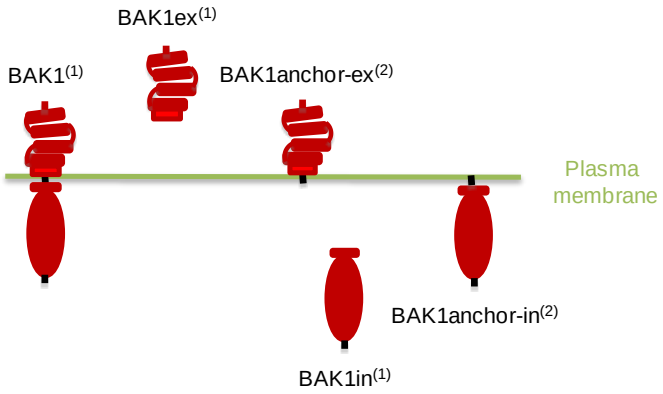


Figure 3.2.6: Schematic representation of BAK1-derived constructs. BAK1ex: BAK1 ecto-domain; **BAK1anchor-ex:** BAK1 ecto-domain with the predicted trans-membrane domain; **BAK1in:** juxta-membrane and kinase domain with the C-terminus; **BAK1anchor-in:** cytoplasmic domain of BAK1 with the predicted trans-membrane domain; (**1**): BAK1, BAK1ex and BAK1in were cloned and provided by Seraina Beeler; (**2**): BAK1anchor-ex and BAK1anchor-in were cloned from *BAK1 cDNA* and provided by Ana Domínguez-Ferreras.

To further study which part(s) of BAK1 are determinant for the constitutive-defense phenotype, BAK1anchor-ex (GFP fusion protein), BAK1anchor-in, BAK1ex and BAK1in were expressed under the control of the estradiol inducible *XVE* promoter in *A. thaliana* plants and T1 plants were used to measure ethylene accumulation upon estradiol treatment. The expression of BAK1ex and BAK1in constructs was detectable but in contrast to BAK1, they were not able to induce ethylene production in a MAMP-independent manner, indicating that extracellular or intracellular parts of BAK1 alone or the kinase domain attached to the membrane are not sufficient to induce constitutive defense responses (**Figure 3.2.7A** and data

not shown). The BAK1anchor-ex and BAK1anchor-in constructs were able to accumulate but only BAK1anchor-ex was able to provoke constitutive ethylene accumulation upon estradiol treatment (**Figure 3.2.7B**, Ana Domínguez-Ferreras unpublished data). These results indicate that the trans-membrane domain and the extracellular domain of BAK1 are both required to induce constitutive ethylene accumulation independently of elicitors.

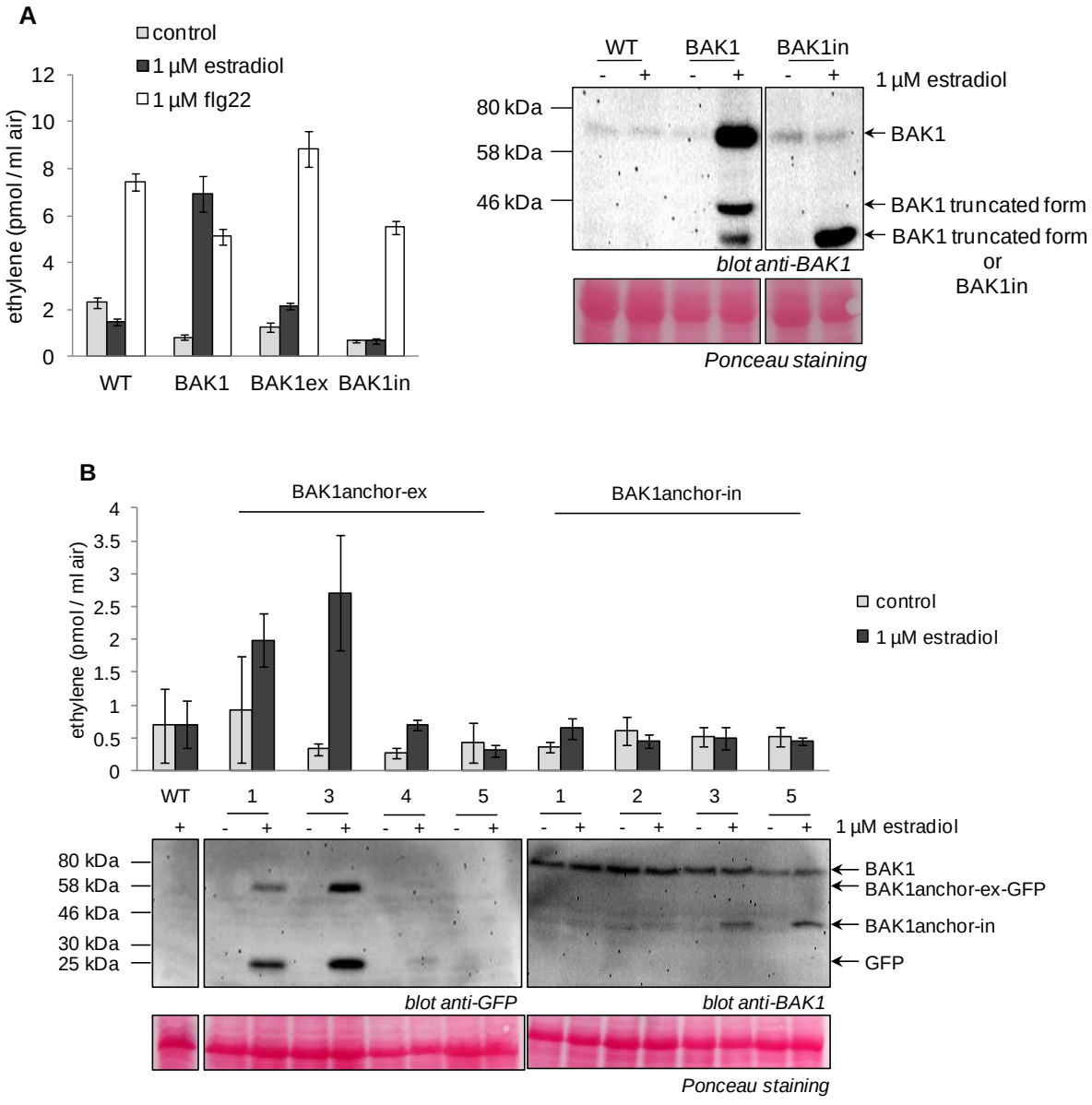


Figure 3.2.7: BAK1 extracellular domain anchored in the plasma membrane is required to trigger constitutive ethylene production. WT: Col-0; **BAK1:** *XVE-BAK1*; **BAK1ex:** *XVE-BAK1ex*; **BAK1in:**

XVE-BAK1in; **BAK1anchor-ex:** *XVE-BAK1anchor-ex-GFP*; **BAK1anchor-in:** *XVE-BAK1anchor-in*. **A:** Leaf strips from four-week-old WT, BAK1, BAK1ex and BAK1in plants were treated or not with 1 µM estradiol overnight and ethylene accumulation was measured. Three hours flg22 treatment was performed as elicitor induced ethylene accumulation control. For each genotype leaf strips from three T1 plants were pooled together. Graphs represent the mean of six technical replicates and error bars correspond to the standard error of the mean with n=6. Leaf material was collected and analyzed by Western blot with antibodies anti-BAK1. **B:** Four-week-old individual T1 plants were use to measure ethylene accumulation in presence or in absence of 1 µM estradiol, and to analyze gene expression by Western blot with antibodies recognizing the C-terminal domain of BAK1 and anti-GFP antibodies. Experience presented in figure B was performed by Ana Domínguez-Ferreras. Graphs represent the mean of four technical replicates and error bars correspond to standard deviation with n=4.

In Arabidopsis, the overexpression of BAK1anchor-ex under the control of the *2xCaMV 35S* promoter induced a growth defect phenotype, as described below, while BAK1anchor-in expressing plants remained as WT plants (**Figure 3.2.8A**). Plants accumulating BAK1anchor-ex (**Figure 3.2.8B**) displayed stunted stature and developed narrower leaves than wild type plants. However growth defects provoked by overexpression of BAK1anchor-ex were distinct from those induced by full-length BAK1 overexpression. BAK1anchor-ex plants, in contrast to BAK1 overexpressors, did not present leaf necrosis and lethality, and they were able to bolt. However they never set seeds. These results suggest that BAK1 extracellular domain anchored in the plasma membrane is only partially responsible for the phenotype induced by BAK1 overexpression.

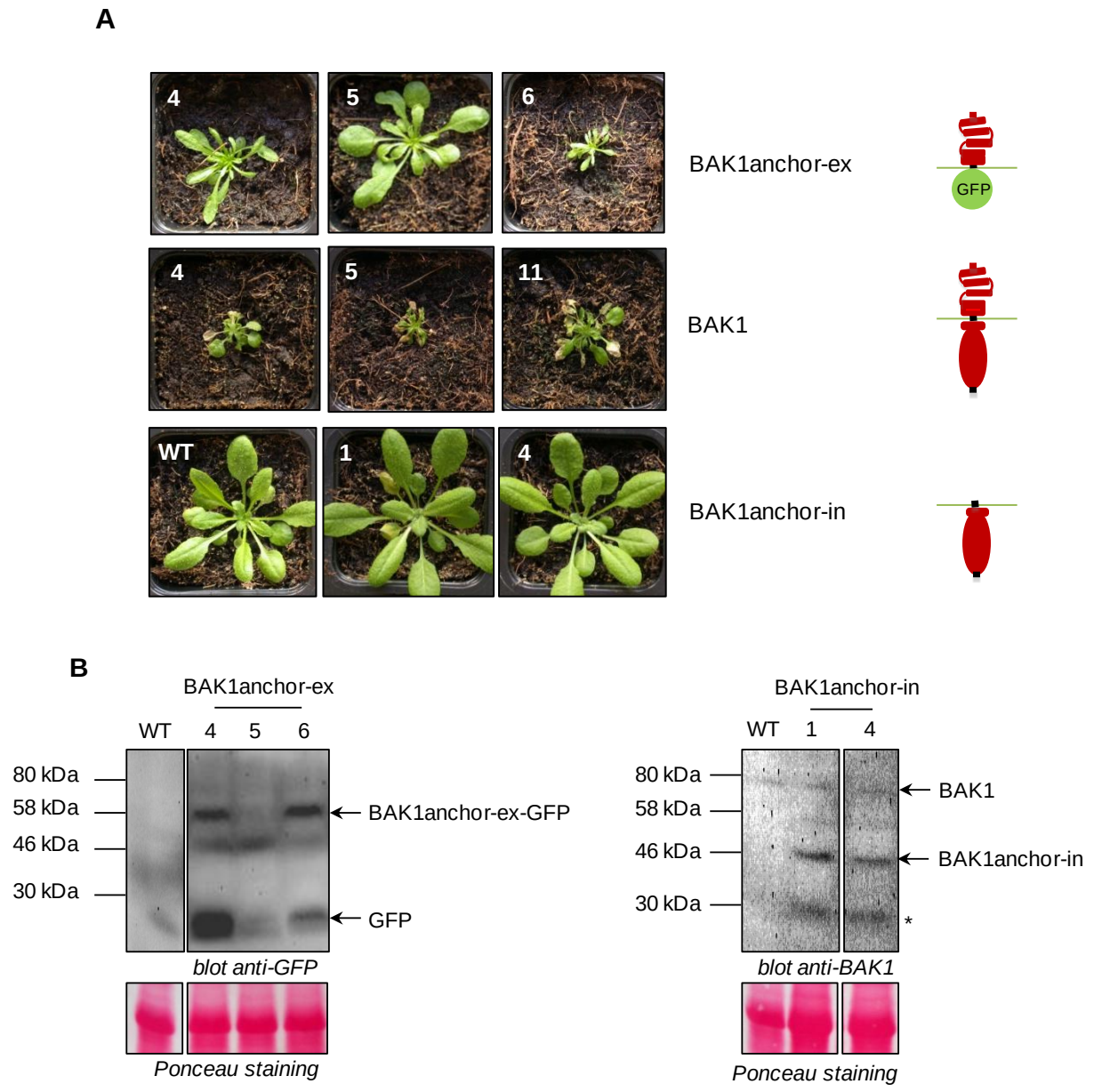


Figure 3.2.8: Constitutive overexpression of BAK1 extracellular domain anchored in the plasma membrane provokes developmental defects. (Experiment performed by Ana Domínguez-Ferreras) **A** Col-0 wild type**,** BAK1 (*2x35S-BAK1 gDNA*), BAK1anchor-ex (*2x35S-BAK1anchor-ex-GFP cDNA*) and BAK1anchor-in (*2x35S-BAK1anchor-in cDNA*) T1 plants were photographed after seven weeks of germination. **B:** Total proteins were extracted from individual T1 plants and Western blot analyses were performed with anti-GFP antibodies to detect protein expression (upper panels). Equal amount of proteins on the membrane was revealed by Ponceau staining (lower panels). Asterisk (*) marks BAK1 truncated forms.

3.2.4 Discussion

In *A. thaliana* plants overexpression of BAK1 caused developmental defects, leaf necrosis, premature death and constitutive, MAMP-independent activation of defense responses (Results: Chapter 1). This phenotype could be correlated with the accumulation of full-length BAK1 protein as well as with the appearance of BAK1-derived proteins of lower size. Thus, one of our hypotheses to explain the BAK1 overexpression-mediated phenotype focused on the accumulation of these truncated forms of BAK1. We assumed that the plant triggers defense responses upon detection of the accumulation of these BAK1 breakdown products. But first, we attempted to rule out the possibility that these are splicing variants of BAK1 with distinct functions instead of truncated BAK1 proteins. Recently it was reported that *Medicago truncatula* BAK1 (*Mt*SERK3) possesses 7 splice variants (Nolan et al., 2011). However, we observed that overexpression of gDNA and cDNA sequences of BAK1 presented the same protein pattern in Western blot analysis giving evidence that the observed protein bands are most likely not splice variants of BAK1 (Ana Domínguez-Ferreras personal communication).

Next, we assumed that these truncated forms might correlate with certain domains of BAK1. Thus we constructed different truncated and mutated forms of BAK1 to analyze which of these forms still have the ability to elicit phenotypes similar to the ones caused by BAK1 overexpression. In other words, which domains contribute to the constitutive defense phenotype and/or the developmental changes.

In previous studies it was shown that BAK1 kinase activity is not required for heteromeric complex formation between BAK1 and its ligand-binding interacting partners BRI1 and FLS2 but it is crucial for the activation of receptor complexes and for induction of appropriate downstream signaling cascades (Wang et al., 2008; Schulze et al., 2010; Schwessinger et al., 2011). Moreover BAK1 kinase activity is also involved in cell death control since a kinase dead version of BAK1 did not rescue *bak1-4 bkk1* lethality (Wang et al., 2008). These results indicate that the kinase activity of BAK1 is indispensable for these regulatory functions. In accordance to this we found in our study that BAK1 kinase activity was required to induce the growth phenotype and lethality induced by BAK1 overexpression (Delphine Chinchilla unpublished results). But we also found that BAK1 kinase activity was not required for constitutive ethylene

accumulation (**Figure 3.2.5**). However we should take into consideration that the BAK1 mutant that we used in this study may be not kinase-dead (Schulze et al., 2010) and the possible remaining kinase activity may be sufficient to activate constitutive ethylene biosynthesis. Analysis of other defense responses, such as seedling growth inhibition and cell death would be necessary with a kinase-dead version of BAK1, to further dissect the role of BAK1 kinase-activity in the activated-defense phenotype.

Overexpression of the C-terminally truncated variants, BAK1Δ or BAK1Δ580, led to an enhanced accumulation of the stress hormone ethylene, induced MAPK activation and inhibited seedling growth similar to full length BAK1, indicating that the C-terminal tail is dispensable for the activation of defense responses (**Figure 3.2.3** and **3.2.4**). Even removal of the whole intracellular domain (BAK1anchor-ex) did not impair the ability of defense response activation, such as ethylene production (**Figure 3.2.7B**). Notably, it has been shown for the RLK FLS2, that the N-terminal cysteine pair is essential for processing, stability, binding activity and for FLS2-FLS2 association underlining the importance of the LRR-RLK's extracellular domain for its functions (Dunning et al., 2007; Sun et al., 2012).

However, the growth phenotype induced by full-length BAK1 and by BAK1anchor-ex is very different (**Figure 3.2.8**). At this stage of the analysis no leaf cell death and no plant lethality were observed in BAK1anchor-ex plants, which indicate that the presence of the intracellular domain of BAK1 could be important in the cell death phenotype. This should be further investigated by trypan blue staining for example in seedlings overexpressing BAK1anchor-ex and BAK1-KD constructs. Collectively these results indicate that the overexpression of BAK1 extracellular domain attached to the plasma membrane is sufficient to activate early defense features such as ethylene accumulation but the induction of cell death, which is a later and a more radical defense response probably depends on BAK1 kinase activity. Moreover, it has been shown previously that BAK1 trans-membrane and kinase domains are essential for its interaction with AvrPto, a bacterial effector probably targeting BAK1 in the aim of suppressing PTI (Shan et al., 2008). Since we showed before that knock-out of *SOBIR1* largely suppressed the BAK1 overexpression phenotype indicating that BAK1 might also be linked to ETI, which is associated with cell death, it is possible that BAK1 domains are involved in different defense

pathways. The extracellular domain attached to the plasma membrane plays a role in PTI whereas the intracellular domain might be important for ETI. In contrast, overexpression of BAK1 intracellular domain attached to the plasma membrane was not able to induce constitutive ethylene accumulation (**Figure 3.2.7**) and did not induce growth impairments nor cell death (**Figure 3.2.8**). Thus if the BAK1 intracellular domains plays indeed a role in ETI, it seems to be still dependent on the presence of the extracellular domain for proper function. However we cannot exclude the possibility that in BAK1anchor-in plants, the absence of the phenotype is due to the cleavage of the signal peptide and therefore the truncated protein is not properly targeted to the plasma membrane. To check this we could prepare plasma membrane cell fractions followed by Western blot analysis, or BAK1anchor-in could be expressed as GFP fusion protein in *Nicotiana benthamiana* leaves and analyzed by confocal microscopy.

Finally, the localization of both, extra- and intracellular domains, to the plasma membrane seems to be crucial since overexpression of BAK1 soluble extracellular or intracellular domains alone did neither trigger a constitutive defense response or cell death nor changes in plant development (**Figure 3.2.7A** and data not shown). Indeed, localization of GFP-tagged BAK1anchor-ex was checked in transgenic Arabidopsis plants and found to be present in the plasma membrane (Delphine Chinchilla, unpublished data).

In summary, all domains of BAK1 contribute to the BAK1 overexpression phenotype. The extracellular domain attached to the plasma membrane seems to be sufficient to trigger a constitutive PTI response, whereas the intracellular domain is involved in cell death regulation and plant development but needs the presence of the extracellular domain for full activity. It will be intriguing to uncover the molecular mechanisms behind this distinct involvement of BAK1. It is possible that the simultaneous involvement of BAK1 in several signaling pathways prevents the uncovering of a clear domain-to-function connection, neither in development nor in innate immunity. In the future these BAK1-derived constructs will be used and tested to determine their interaction with known PRRs such as FLS2 and other regulators such as BIK1.

4 FINAL DISCUSSION

In our study, we tried to overexpress BAK1 in *Arabidopsis thaliana* plants to elucidate its role in innate immunity. Our initial strategy was to overexpress full-length BAK1 under a constitutive promoter to investigate if it is a limiting factor for BR/MAMP signaling if both stimuli are present. This might have explained why treatments with MAMPs like flg22 lead to growth inhibition, a phenotype reminiscent of impaired BR signaling (Gomez-Gomez et al., 1999; Albrecht et al., 2011; Lin et al., 2013). Surprisingly we found, in contrast to previous studies (Li et al., 2002; Nam and Li, 2002), that constitutive overexpression of wild-type BAK1 induced a severe phenotype in Arabidopsis. This includes developmental arrest, necrosis, sterility and constitutive activation of defense responses (3 Results: 3.1 Chapter 1).

Prolonged activation of defense responses can impose negative effects on the plant fitness (Lorrain et al., 2003; Tian et al., 2003; Korves and Bergelson, 2004; Liew et al., 2005) and many mutants with constitutive activation of resistance exhibit growth defects and spontaneous lesions (Shirano et al., 2002; Yang and Hua, 2004; Gao et al., 2009). Thus we hypothesized that constitutive overexpression of *BAK1* somehow triggers constitutive defense response despite a lack of elicitors leading to the observed phenotype.

By using an inducible *BAK1* construct (*XVE-BAK1*) we found that already a transient accumulation of BAK1 protein triggers defense responses independent of the presence of MAMPs (**Figure 3.1.2-7**). Additionally, resistance to the hemibiotrophic bacterial pathogen *Pto* DC3000 was increased (**Figure 3.1.8**).

Taken together, this is a very surprising finding. BAK1 has been described as an important co-receptor used by many PRRs and BRI1 but has not been connected to the possibility that it could trigger PTI or ETI on its own. In this chapter, different models will be proposed that try to bring the observed phenotype, the collected molecular data and the published knowledge about BAK1 function together, and to come up with new ideas about the role of BAK1 in plant immunity and development.

4.1 BAK1 overexpression may override mechanisms of negative regulation

BAK1 transcription is five times induced by flg22 perception compared to control samples (**Figure 3.1.4**) indicating that higher levels of *BAK1* transcription and BAK1 protein, respectively, might contribute to plant immunity. Following this idea, plants will also need means to reduce BAK1 levels after pathogens have been defeated to prevent a constitutive activation of the immune system. Two potential mechanisms might exist:

First, BAK1 endocytosis and subsequent degradation could be responsible for the regulation of the amount of BAK1. Indeed it was proposed that BAK1 is involved in BRI1 endocytosis (Russinova et al., 2004). To date, there is no evidence that MAMP treatment induces BAK1 endocytosis as for FLS2 (Robatzek et al., 2006; Chinchilla et al., 2007). Assuming that BAK1 is internalized together with FLS2 or BRI1 after perception of flg22 or BR, respectively, the removed amount of BAK1 from the plasma membrane seems to be little compared to the whole pool of BAK1. Additionally BAK1 was shown not to be a rate-limiting factor in PRR and BRI1 signaling (Albrecht et al., 2011) thus, BAK1 endocytosis might not play a dominant role in the negative control of BAK1-mediated PTI.

Second, it was shown in Arabidopsis that two typical E3 ubiquitin-protein ligases PUB12 (PLANT U-BOX 12) and PUB13 (PLANT U-BOX 13), constitutively co-immunoprecipitating with BAK1, are recruited in the FLS2 receptor complex upon flg22 treatment and target FLS2 for degradation (Lu et al., 2011) (**Figure 4.1A**). Although they do not target BAK1 for degradation, other yet unknown E3 ubiquitin-protein ligases might be involved here (**Figure 4.1B**). For example the three Arabidopsis E3 ligases, PUB22/23/24 negatively regulate flagellin signaling which could work via BAK1 degradation (Trujillo et al., 2008).

However, activation of BAK1-dependent signaling pathways might not be necessary for enhanced BAK1 degradation since the lower molecular weight bands we detected using the anti-BAK1 antibody also appeared in the absence of MAMPs when BAK1 expression was induced (**Figures 3.1.1-2, 3.1.6-8**). Thus BAK1 might not only be degraded after MAMP or BR perception but there could be a constant turnover dependent on BAK1 abundance.

In summary, the constitutive defense response triggered by BAK1 overexpression might be caused by an over-accumulation of BAK1 due to insufficient degradation routes (**Figure 4.1B**). How the simple over-accumulation of BAK1 could activate PTI or ETI will be discussed in the next chapter.

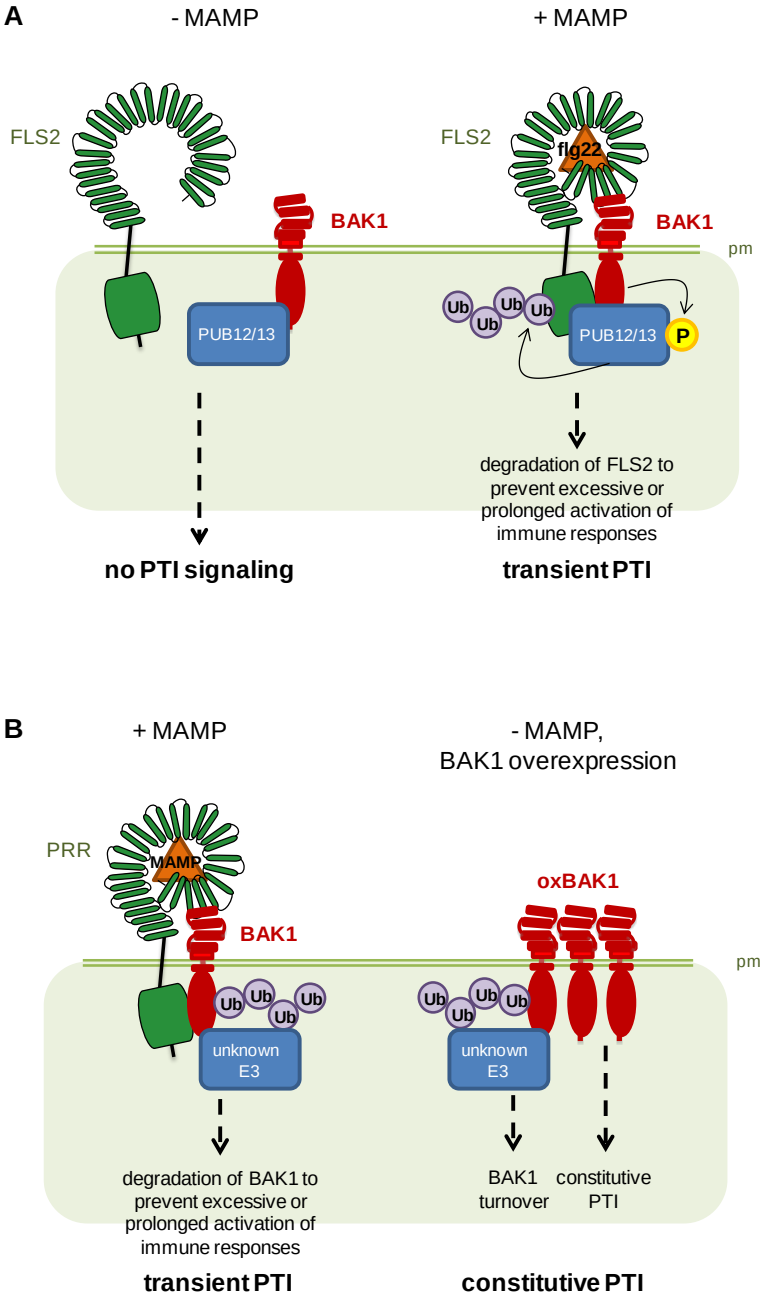


Figure 4.1: Model to explain how BAK1 overexpression could interfere with negative regulatory mechanisms of PTI. A: In case of receptor complex activation E3 ubiquitine ligases PUB12/13 target FLS2 for degradation to prevent constitutive activation of PTI (Lu et al., 2011). **B:** The effect of E3

ubiquitin ligase possibly targeting BAK1 for degradation is not sufficient to offset the BAK1 overexpression phenotype. **oxBAK1:** BAK1 overexpression**; PRR:** pattern recognition receptor; **MAMP:** microbe-associated molecular patterns; **P:** phosphate; **PTI:** pattern-triggered immunity; **pm:** plasma membrane; **E3:** ubiquitin-protein ligase; **Ub:** ubiquitin.

4.2 Constitutive PTI might be based on aberrant activation of BAK1-interacting receptors

BAK1 forms ligand-dependent heteromeric complexes with several receptors of conserved microbial or danger signatures, such as FLS2, EFR, PEPR1 and 2 (Chinchilla et al., 2007; Heese et al., 2007; Postel et al., 2010; Schulze et al., 2010; Roux et al., 2011). Therefore a logical scenario is that BAK1 over-accumulation induces an aberrant association between BAK1 and BAK1-interacting receptors and promotes their phosphorylation, which then could activate the downstream signaling cascade leading to PTI. To test this hypothesis, BAK1 was overexpressed in the *efr fls2* mutant lacking two BAK1-interacting MAMP receptors, and in *pepr1 pepr2* mutant lacking the two DAMP receptors. These mutants were not able to rescue the BAK1 overexpression phenotype (**Figure S3.1.4B** and Delphine Chinchilla, unpublished results). Thus, these known BAK1-interacting PRRs either do not take part in the generation of the observed phenotype, or they act redundantly, or single or double knock-outs are insufficient to suppress their effect. However BAK1 was described to be involved in signaling induced by additional microbial patterns, such as bacterial cold shock protein csp22 or *Phytophtora infestans* elicitor INF1 (Heese et al., 2007; Chaparro-Garcia et al., 2011). Hence we cannot exclude that further unknown receptors from Arabidopsis interact with BAK1 and, in case of BAK1 overexpression, lead to PTI activation (**Figure 4.2A**). Interestingly we found that overexpression of the BAK1anchor-ex construct, lacking the whole kinase domain, also displayed the constitutive ethylene phenotype and a growth defect but no necrosis (**Figure 3.2.7B and 3.2.8**). Complex formation between BAK1 and FLS2/EFR does not need the kinase activity of BAK1 (Schulze et al., 2010; Schwessinger et al., 2011). Thus, the BAK1 extracellular and/or the transmembrane domain may be sufficient to recruit the unknown receptor(s). The receptor activation needs to be independent of the BAK1 kinase domain. Since the current

models suggest that BAK1 kinase activity is required for receptor activation (Schulze et al., 2010; Gou et al., 2012), one could imagine that other members of the SERK family, are recruited in this complex and activate the unknown receptor (**Figure 4.2B**). However, the growth phenotype induced by BAK1 overexpression is different from the one induced by overexpression of BAK1anchor-ex (**Figure 3.2.8**). Hence, BAK1 kinase-domain is required for the full activation of the BAK1 overexpression phenotype.

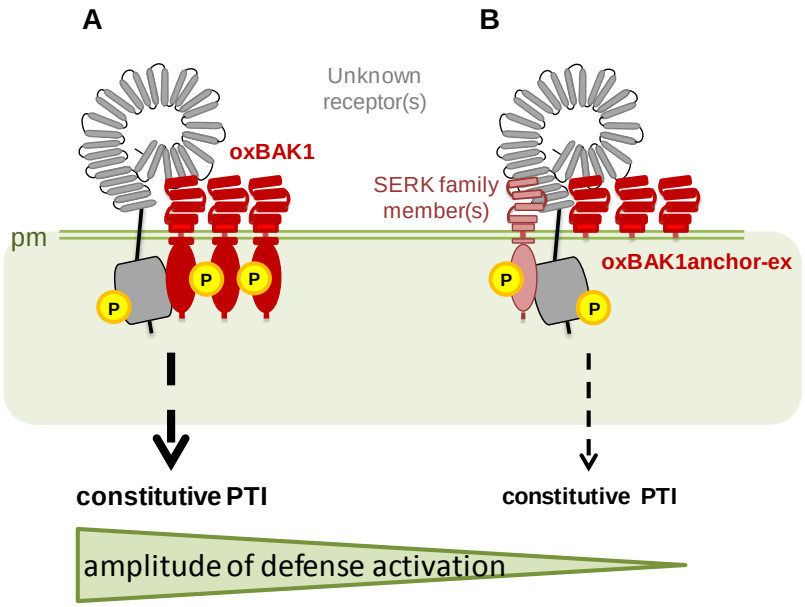


Figure 4.2: BAK1 overexpression might activate unknown receptor(s) leading to PTI. A: In case of BAK1 overexpression (oxBAK1), BAK1 interacts and phosphorylates unknown receptor(s) leading to PTI activation. **B:** Overexpression of BAK1 extracellular domain anchored to the plasma membrane (oxBAK1anchor-ex) is sufficient to interact with unknown receptor(s) and possibly recruits another member of the SERK family. Consequently this leads to PTI activation. **P:** phosphorylation; **PTI:** pattern-triggered immunity.

4.3 Strong kinase activity of BAK1 induces the downstream signaling cascade leading to constitutive PTI

The so called RD kinases possess a negatively charged aspartate (D) and a positively charged arginine (R) residues in their kinase domain. Phosphorylation of the kinase activation loop neutralizes the positively charged R residue resulting in kinase activation (Johnson et al.,

1996). These kinases have a strong kinase activity and are able to autophosphorylate their activation loop. BAK1 belongs to the RD kinase family (Dardick and Ronald, 2006). Therefore one can imagine that without further stimulus a certain percentage of BAK1 is already present in the phosphorylated stage but could be constitutively dephosphorylated by a phosphatase to keep it "inactive" and therewith prevent an aberrant activation of PTI (**Figure 4.3A left**). In the case of flg22 perception, BAK1 associates with FLS2 leading to cross-phosphorylation which increases the amount of phosphorylated BAK1. This could be further increased by a simultaneous deactivation of the phosphatase facilitated by FLS2-BAK1 activated downstream components which would allow full activation of PTI. After FLS2 endocytosis the signaling pathway might get deactivated. Then, the phosphatase might recover to keep BAK1 in its dephosphorylated state to completely block PTI (**Figure 4.3A**).

In case of BAK1 over-accumulation the number of phosphorylated BAK1 proteins might increase above a certain threshold, which might be sufficient to phosphorylate considerable numbers of downstream signaling elements such as BIK1 and PBL1. Following the above described flow of events these downstream signaling elements could deactivate the phosphatase in a negative feedback loop and therewith further increase the amount of phosphorylated BAK1 molecules. Since BAK1 protein levels cannot be reduced efficiently by the plant's degradation routes (as hypothesized in chapter 4.1), this will eventually lead to a constitutive activation of PTI (**Figure 4.3B**). However, this model is difficult to be reconciled with my findings, since BAK1anchor-ex lacks the kinase domain but still partially induces the BAK1 overexpression phenotype.

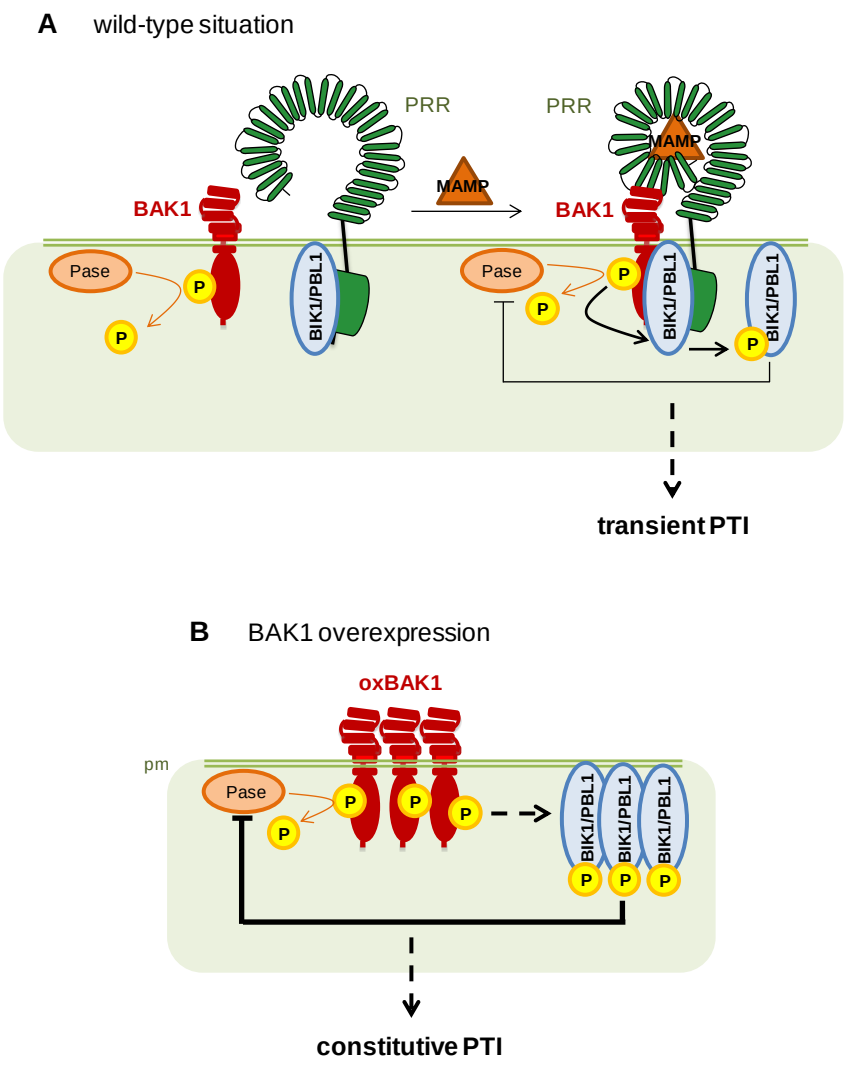


Figure 4.3: BAK1 overexpression could activate the downstream signaling cascade and induce constitutive PTI. A: Possible phosphorylation events in wild type context. **B:** Hypothetical phosphorylation events in case of BAK1 overexpression. **PRR:** pattern recognition receptor; **MAMP:** microbe-associated molecular pattern; **Pase:** phosphatase; **P:** phosphorylation; **oxBAK1:** BAK1 overexpression; **PTI**: pattern-triggered immunity; **pm:** plasma membrane. Black lines represent direct events and dashed lines several events.

4.4 BAK1 overexpression phenotype could be due to ETI activation

BAK1 and other proteins involved in MAMP receptor complexes like FLS2, EFR or BIK1 are known to be targeted by effectors (Shan et al., 2008; Zhang et al., 2010; Cheng et al., 2011;

Feng et al., 2012) (**Figure 4.4A**). Co-IP studies performed in protoplasts indicate that AvrPto and AvrPtoB target BAK1 and interfere with the formation of FLS2-BAK1 and BRI1-BAK1 complexes (Gohre et al., 2008; Shan et al., 2008). In contrast, it was recently shown by protoplast- and plant-based co-IP and BiFC (bimolecular fluorescence complementation) assays that FLS2 but not BAK1 is targeted by AvrPto (Xiang et al., 2011). However, BAK1 is a central protein for PTI and well conserved within the plant kingdom. Therefore it is very likely that BAK1 is targeted by effectors, and most likely also guarded by one or even several R-proteins.

Further support for an involvement of BAK1 in ETI comes from the report that BAK1 interacts with BIR1 and BON1 (Gao et al., 2009; Wang et al., 2011). Both *bir1-1* and *bon1-1* mutants display constitutive defense responses and cell death which are partially reversible by high temperature (28 °C) and mutations in genes (*PAD4*, *EDS1*, *SNC1*, *LCD*s) typically associated with ETI signaling. In addition BAK1 overexpression induces massive cell death and plant lethality (**Figure 3.1.1 and 3.1.7**) which could be similar to the hypersensitive response, a hallmark of ETI. But neither mutations in different *R* genes or regulators of *R* genes nor elevation of the growth temperature to 28 °C was able to rescue the phenotype (**Figure S3.1.5 and Table S3.1.1**). However, these results do not exclude that BAK1 itself or BAK1-containing multimeric complexes are guarded by an *R* gene. Since BAK1 appears as a key component of multiple defense as well as developmental pathways, it is possible that several *R* genes monitor BAK1 integrity. Thus knocking out a single R gene might not be sufficient to suppress the BAK1 overexpression phenotype.

Interestingly, knock-out of *SOBIR1* suppressed the BAK1 overexpression phenotype (**Figure 3.1.10**). *SOBIR1* was proposed to be involved in ETI, but also in PTI as well as in organ abscission by regulation of molecule secretion through the Golgi apparatus (Gao et al., 2009; Leslie et al., 2010; Lewis et al., 2010; Liebrand et al., 2013; Zhang et al., 2013). Therefore the link between BAK1 and SOBIR1 revealed in our study does neither support nor contradict a connection of BAK1 to ETI.

Taken together, despite clear evidence for a link between BAK1 and ETI it is tempting to hypothesize that the phenotype observed for BAK1 overexpression is at least partially due to

constitutive ETI activation. Notably, if BAK1 over-accumulation triggers (in addition) ETI it would do that without the presence or activity of an effector. How could this work?

Two things are changed in BAK1 overexpression plants. First, the BAK1 protein level is elevated and second, putative degradation products are present or more abundant than in wild type plants. Effectors were shown to induce the degradation of PTI components (Nomura et al., 2006; Gohre et al., 2008; Gimenez-Ibanez et al., 2009b), and might target BAK1 as wel. Thus, the guarding in case of BAK1 might work by monitoring the presence or abundance of BAK1 degradation products (**Figure 4.4A**). For example a putative R-protein might need to bind to a specific BAK1 break-down product or intermediate product to activate ETI. In wild type plants these break-down products or intermediates are absent or their presence is very low and thus insufficient to activate ETI via the *R*-gene product. In case of the BAK1 overexpressing plants these break-down products are present or more abundant; the R protein binds to these products in sufficient amounts and activates ETI (**Figure 4.4B**).

Most of the R proteins are intracellular. Thus the putative R-protein would most likely guard the intracellular part of BAK1 which consists mainly of the kinase domain. Analysis of plants overexpressing BAK1anchor-ex showed that the absence of BAK1 kinase-domain did not trigger cell death although it produced an altered growth phenotype (**Figure 3.2.8**). Thus if BAK1 over-accumulation would indeed trigger ETI via the detection of elevated amounts of BAK1-kinase-domain-derived degradation products it seems to only partially contribute to the observed phenotype.

Taken together, the BAK1 overexpression phenotype is most likely based on multiple alterations in distinct signaling pathways including PTI, ETI and maybe also BR signaling.

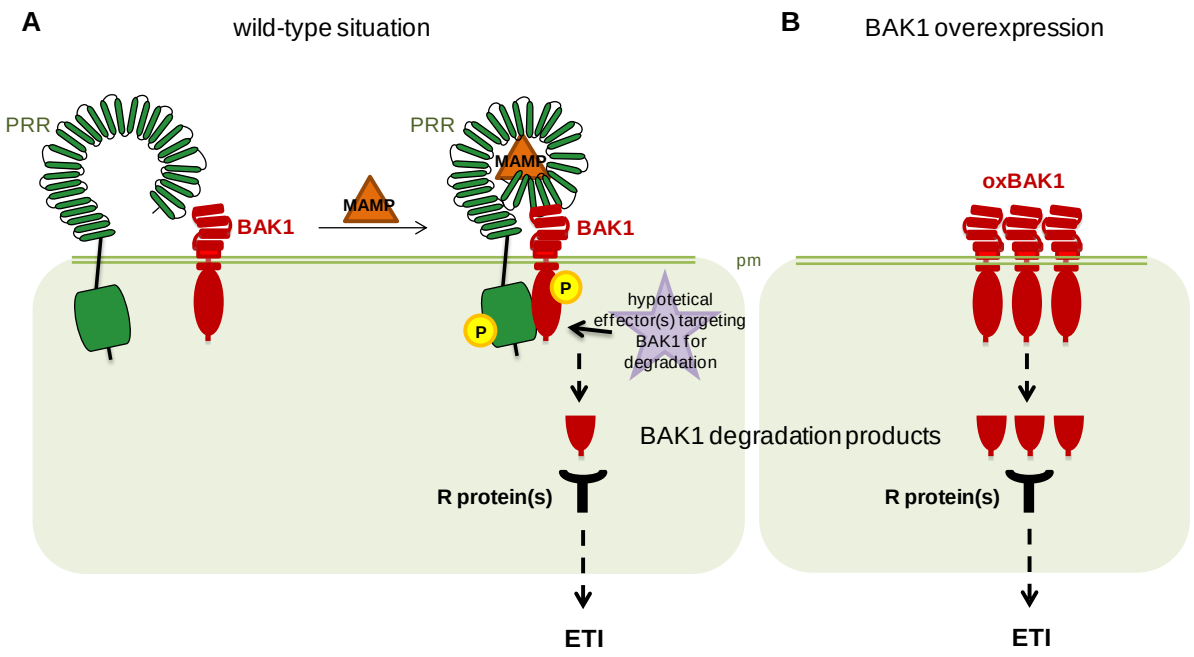


Figure 4.4: BAK1 overexpression phenotype might involve one or several resistance (R) proteins to activate ETI signaling. A: BAK1 seems to be a logical target of effectors and therefore its integrity could be monitored by one or several R proteins through detection of BAK1 degradation products. **B:** BAK1 overexpression could induce accumulation of BAK1-derived degradation products which can be detected by R protein(s) and lead to ETI. **PRR:** pattern recognition receptor; **MAMP:** microbe-associated molecular pattern; **oxBAK1:** BAK1 overexpression; **P:** phosphorylation; **pm:** plasma membrane **ETI**: effector triggered immunity. Black lines represent direct events and dashed lines several events.

Although PTI and ETI share many signaling components, ETI triggers quicker and stronger defense responses than PTI does (Jones and Dangl, 2006). For example, ETI but not PTI is associated with HR. However, it has been demonstrated that flg22 may also induce cell death in Arabidopsis under certain conditions (Naito et al., 2008). We showed that treatment with elf18 did the same (**Figure 3.1.7**). These results may argue for the existence of a continuum between PTI and ETI (Boller and Felix, 2009; Thomma et al., 2011). This potential lack of a sharp separation of PTI and ETI makes it further difficult to assign the BAK1 overexpression phenotype to either PTI or ETI.

4.5 Are there other BAK1-dependent pathways additionally involved in the formation of the BAK1 overexpression phenotype?

Since BAK1 overexpression induced developmental defects and cell death we cannot exclude that other pathways regulated by BAK1 are influenced by its overexpression and contribute to the observed phenotype. For example, does BAK1 accumulation affect the BR pathway? Our results demonstrated that *BRI1* expression is decreased by BAK1 overexpression (**Figure 3.1.5**), which may indicate an inhibition of the BR pathway. In contrast, *CPD* expression was also inhibited (**Figure 3.1.5**), which is a sign for BR pathway activation (Mathur et al., 1998). On the one hand it has been described that BR pathway activation results in the methylation of a phosphatase that in turn dephosphorylates the internalized pool of ligand-activated BRI1 and marks it for degradation (Wu et al., 2011) (**Figure 4.5A**). Therefore one could imagine that BAK1 overexpression amplifies this negative feedback loop and inhibits BR signaling (**Figure 4.5A**). On the other hand a recent study revealed that additionally to PRRs, BIK1 constitutively interacts with BRI1 (Lin et al., 2013). BIK1 is phosphorylated and released from BRI1 in a ligand-dependent and a BAK1-independent manner; the latter presents a difference compared to mechanism of PRR-BIK1 dissociation (Lin et al., 2013) (**Figure 4.5B**). *Bik1* mutants display compromised MAMP responses hence BIK1 is considered as positive regulator of innate immunity (Lu et al., 2010b; Zhang et al., 2010). In contrast, *bik1* mutants show various BR hypersensitive phenotypes, therefore BIK1 seems to be a negative regulator in BR signaling (Lin et al., 2013). Regarding these it is a possibility that BAK1 overexpression induces accumulation of BIK1 (**Figure 3.1.9**), which consequently inhibits BR signaling (**Figure 4.5B**). In both cases inhibition of BR signaling would lead to developmental defects. Since PTI and BR signaling pathways are connected at the signaling level (feedback) (Albrecht et al., 2011; Belkhadir et al., 2011; Lin et al., 2013), manipulation of one likely affects the other, but maybe to a lesser extent than the direct manipulation.

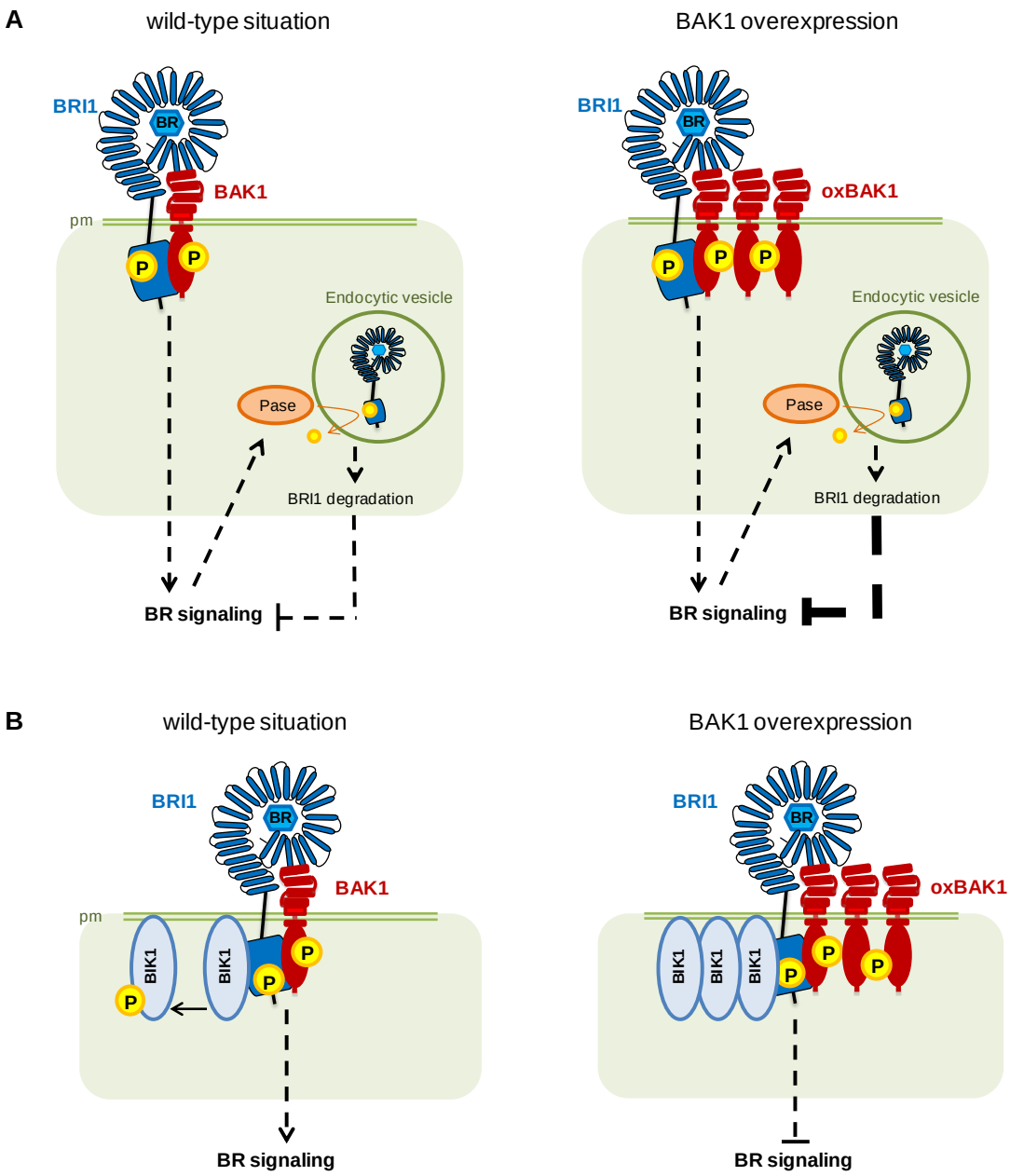


Figure 4.5: BAK1 overexpression might interfere with BR signaling and inhibit plant development. **A:** In wild type, BR pathway activation induces a negative feedback loop via BRI1 degradation (left schema). BAK1 overexpression might amplify the negative feedback loop and inhibit BR-signaling (right schema). **B:** BIK1, a negative regulator of BRI1 is released from BRI1 and phosphorylated in a ligand-dependent manner (left schema) (Lin et al., 2013). Due to BAK1 overexpression BIK1 might accumulate as well and inhibit BR-signaling (right schema). **BR:** brassinosteroids; **Pase:** phosphatase; **P:** phosphorylation; **oxBAK1:** BAK1 overexpression; **pm:** plasma membrane. Black lines represent direct events and dashes several events.

4.6 Conclusion

This study further underlines the role of BAK1 as a central integrator of immune and developmental signaling pathways. Due to its multiple functions and potential redundancy with other SERKs in one or the other aspect the precise dissection of events following the over-accumulation of BAK1 turned out to be much more difficult than expected. However, it is obvious that BAK1 accumulation causes at least a constitutive defense phenotype which might be coupled via BRI1 to disturbed BR signaling. Regarding the former, a constitutive activation of PTI seems to be the likely scenario but data from this study as well as published data support the idea that in addition ETI might get constitutively activated by BAK1 accumulation.

5 OUTLOOK

5.1 Looking forward to BAK1 molecular signaling

BAK1 emerged in the last years as one of the most intensively studied proteins in the plant field. However, as becomes apparent from my thesis, BAK1 is so centrally involved in many different signaling pathways that much more work is needed to grasp the full picture of the BAK1-related signaling networks. Here, I will focus on further experiments related to uncovering the underlying mechanisms of the BAK1 overexpression phenotype, but which will surely also contribute to the understanding of BAK1 functions.

First, it would be useful to understand the correlation between BAK1 quantity and the activated defense phenotype as well as the deleterious effect on plant development. Is there a certain threshold or do the alterations manifest gradually? Plants expressing BAK1 under the control of its endogenous promoter in wild-type background did not present a stunted phenotype and developmental defects (Delphine Chinchilla, unpublished results) but they displayed small necrotic lesions and accumulation of PR1 protein (Belkhadir et al., 2011). Thus using either an inducible promoter or the integration-site-dependent variability of the strength of the *CaM35S* promoter might lead to a set of transgenic plants showing diverse phenotypes.

Analysis of BAK1 protein levels in these plants could already give an indication about the “BAK1 amount” – “induced phenotype” correlation.

BIK1 phosphorylation seems to be a required step in PTI activation (Lu et al., 2010b; Zhang et al., 2010; Liu et al., 2013). Thus, overexpression of BAK1 in BIK1-HA plants and checking the BIK1 phosphorylation status revealed by band shift in Western blot analysis (Lu et al., 2010b) will give an indication if PTI activation is indeed involved in the BAK1 overexpression phenotype.

Additionally a suppressor screen could be performed to investigate important components of the BAK1 overexpression phenotype, which could shed light on the involved pathways. Here, a pool of homozygous XVE-BAK1 seeds should be mutagenized. Since BAK1 accumulation (in this case after addition of estradiol) strongly impairs proper plant growth, mutants suppressing the phenotype should develop more biomass and could thus be easily identified among the treated seedlings. However, this method is extremely time consuming and labor intensive and even more difficult when multiple loci are involved in the phenotype. Additionally, we also noticed that the XVE system has problems for the transgene induction over several generations possibly due to the silencing of the XVE system.

Finally, to determine the involvement of BR signaling in the BAK1 overexpression phenotype, signaling elements downstream of the BRI1 receptor could be investigated. For example the phosphorylation status of BES1, a BR-specific transcription factor (Yin et al., 2002; Mora-Garcia et al., 2004), should be determined in BAK1 overexpression plants. In case of activation of BR signaling pathway, a strong increase of unphosphorylated BES1 can be detected by immunoblot analysis with anti-BES1 antibody. Moreover it was briefly described by Belkhadir and collaborators that an increased level of BRI1 was able to rescue their comparably mild BAK1 overexpression phenotype (Belkhadir et al., 2011). It would be interesting to see whether our overexpression phenotype could be rescued by BRI1 overexpression as well.

5.2 Could pattern-triggered immunity be used to improve crop resistance?

The impact of plant pathogens on crop yield is a major constraint in agriculture (Peterson and Higley, 2001; Dangl et al., 2013). Diseases can be controlled by pesticides, but there is an increasing need to reduce chemical input in the field to reduce their potential impact on the environment and human health. Sustainable agricultural methods are therefore focused on the genetic potential of plants to control pathogens. To this aim *R* genes have been widely used in plant breeding, and genetic engineering approaches to increase disease resistance of crop plants (Dennis et al., 2008). However, in general, this method ensures resistance against one given pathogen race or strain, which possesses the corresponding effector protein (Stuiver and Custers, 2001; Dennis et al., 2008). Additionally, it confers only a short-term efficiency because of the rapid evolution of pathogens resulting in the brake-down of *R* genes in the field (Pretorius et al., 2000; Hovmoller et al., 2008). Hence, resistance strategies acting on long-term and against a broad range of pathogens are required to resolve this issue. Because PTI is activated upon recognition of conserved and essential pathogen molecules, which cannot be easily mutated or lost, actors of PTI signaling could be good candidates to improve durable disease control. Therefore, advances in our understanding of the molecular basis of PTI together with microbial strategies to overcome PTI, may lead to new methods for engineering durable disease resistance in crop plants. For example, it was demonstrated that tomato plants transformed with EFR (EF-TU RECEPTOR) (Zipfel et al., 2006), a PRR specific to the Brassicaceae, acquired resistance against bacterial pathogens including *Agrobacterium*, *Xanthomonas*, and *Ralstonia* species (Lacombe et al., 2010). Furthermore, it was reported that overexpression of *Oryza sativa* BAK1 enhanced resistance to blast fungus *Magnaporthe grisea* infection in transgenic rice (Hu et al., 2005; Li et al., 2009). Similarly we found that overexpression of BAK1 increased the hemibiotrophic bacterial pathogen *Pto* DC3000 resistance in Arabidopsis (**Figure 3.1.10**). These results suggest that enhancement of PTI could provide an opportunity to improve plant immunity. However, additionally to increased resistance to pathogens, BAK1 overexpression provoked growth defects and in our case it was also linked with massive cell death, which are not considered as advantageous traits in agriculture (Li et al., 2009; **Figure**

3.1.1). Another important issue is that the sustainability of this type of engineered immunity can be compromised as some microbial effectors are known to suppress elements of PTI (Shan et al., 2008; Xiang et al., 2008). In the future, to take advantage from PTI in crop protection, we need to understand in detail biological processes and molecular mechanisms involving PTI signaling components, such as BAK1.

ANNEX

Table 2.1: Oligonucleotides used for cloning, colony PCR, site directed mutagenesis and sequencing.

name	sequence 5' – 3'	gene or vector	orientation
Att adapter fwd	GGGGACAAGTTTGTACAAAAAAGCAGGCT	pDONR^{TM}207	forward
Att adapter rev	GGGGACCACTTTGTAGAAGAAAGCTGGGT	pDONR^{TM}207	reverse
pDONR207for	TCGCGTCGCTAGCATGGATCTC	pDONR^{TM}207	forward
pDONR207rev	GTAACATCAGAGATTTTGAGACAC	pDONR^{TM}207	reverse
BAK1 atg B1	AAAAAGCAGGCTCCATGGAACGAAGATTCC	BAK1 + attB1	forward
BAK1-Cdel rev	AGAAAGCTGGGTGGGGGTATTCGTTTTCGATC	BAK1 + attB2	reverse
BAK1AttB2	AGAAAGCTGGGTGTCTTGGACCCGAGGGG	BAK1 + attB2	reverse
BAKrevA	GGATAAGGTAGTTGAGAGCC	BAK1	reverse
N534523primer1	GGTGCTTCAAAGTTGGGATGC	BAK1	forward
BAK1-1	ATCTGACGGAATTGGTGAGC	BAK1	forward
BAK1-2	TCAAGGCTTAAGTCCACTG	BAK1	reverse
BAK1-3	TTGGTGGTTAGGCGTC	BAK1	forward
BAKrevB	CCGGCATGAACCGTAATTCAGTC	BAK1	reverse

BAK4Trans5’	CCTCTCACCGGAGATATTCCTGT	BAK1	forward
BAK3Trans3’	CGGCGTCCTGGTGAAGAAACTAC	BAK1	reverse
BAK1-4	TGCAGCTGAAGAGGACCC	BAK1	forward
BAK4Trans3’	GCCAATCGACTACCATGAAATCACCGGC	BAK1	reverse
BAKrevC	GGGATGTACCGATTACCTTC	BAK1	reverse
BAK1-5	TTCCGTCCAATGGTTGCC	BAK1	forward
BAK1-6	GTCTTTGGGTATGGAGTC	BAK1	forward
BAK1-7	CTGAGAGATGGGAAGAG	BAK1	forward
AttB1SERK1	AAAAAGCAGGCTAAATGGAGTCGAGTTATGTGGTGTTTATCTTACTTTC	SERK1 + attB1	forward
AttB2SERK1stop	AGAAAGCTGGGTGTTACCTTGGACCAGATAACTCAACGGCGTGCAAATTG	SERK1 + attB2	reverse
AttR1SERK4	AAAAAGCAGGCTAAATGACAAGTTCAAAAATGGAAC	SERK4 + attB1	forward
AttR2SERK4	AGAAAGCTGGGTGTTATCTTGGACCCGAGGGG	SERK4 + attB2	reverse
BAK1MutSTP4010 for	AAGGAGGAAATGTAGAGACAAGATTTC	BAK1	forward
BAK1MutSTP4010 rev	GAAATCTTGTCTCTACATTTCCTCCTT	BAK1	reverse
BAK1MutSTP4071-5	CTGGATCATTGGCTAGTCCACTTCCCAG	BAK1	forward
BAK1MutSTP4071-3	CTGGGAAGTGGACTAGCCAATGATCCAG	BAK1	reverse
FLS2addSTPfor	GATCGAGAAGTTTAGAAGGGTGGGCGC	FLS2	forward

FLS2addSTPrev	GCGCCCACCCTTCTAAACTTCTCGATC	FLS2	reverse
Chim1Seq2	ACCGGCTGAAATAGGAAAGT	FLS2	forward
FLS2rev600	CAAATGTTTGTAGCAGCTGG	FLS2	reverse
Chim1Seq3	GCTGAGATCGGAAACTGC	FLS2	forward
FLS2-1080	GGAACTTGACAGTCCTAACGGTG	FLS2	forward
Chim1Seq4	CTTAAACTCCTGGACCTGTC	FLS2	forward
FLS2for2	ATGATATCTTCAACTGTTCAAACTTGG	FLS2	forward
Chim1Seq5	CAGGTCAAATTCCTGCCTTG	FLS2	forward
Chim1Seq6	CCCTCAGAGCTTCGGGAAC	FLS2	forward
Chim1Seq7	ACCGGATTTGGATTCAGC	FLS2	forward
FLS2-951	GGGGTTATCAGAAAACCATTTGG	FLS2	forward
FLS2-for2704	CTAAAGGAATTCTCTGCAGAATC	FLS2	forward
FLS2-3020	GTGACCGCGTTGCTCACGTAAGCG	FLS2	forward
FLS2-END	GAGGCTATTGAAGACTTTCTG	FLS2	forward
SERK1-1	GAGGCCTGTTTGAATAGTTCTC	SERK1	forward
SERK1-2	GTTCCAGAGCTTGGTGTGCTCAAG	SERK1	forward
SERK1-2 rev	CTTGAGCACACCAAGCTCTGGAAC	SERK1	reverse

SERK1-3	GGGTCAATTCCTATGTCACTG	SERK1	forward
SERK1-4	GACCTATGTGGACCTGTTACAAG	SERK1	forward
SERK1-5	GGAGCAATAGCTGGTGGAGTTGCTG	SERK1	forward
SERK1-5 rev	GCAACTCCACCAGCTATTGCTCCAG	SERK1	reverse
SERK1-6	GGTTTGGGAAAGTCTACAAGGG	SERK1	forward
SERK1-7	GCTCGAGGTTTGTCTTACCTAC	SERK1	forward
SERK1-8 rev	GGTTTTTGTTGGGGTCAAATG	SERK1	reverse
SERK4-1	GTGATGCTTTGACTCAGCTG	SERK4	forward
SERK4-1rev	CAGCTGAGTCAAAGCATCAC	SERK4	reverse
SERK4-2	GCAGTACTTGTAAGCCATC	SERK4	forward
SERK4-3	GGTAATCACCAATCAGTGCC	SERK4	forward
SERK4-4	GTGTTGCGGCTGCAAAATG	SERK4	forward
SERK4-5	GGGGAGATACCTGAGGAGC	SERK4	forward
SERK4-5rev	GCTCCTCAGGTATCTCCCC	SERK4	reverse
SERK4-6	GGGAGATGGTTGTTATAGGC	SERK4	forward
SERK4-7	GAAGTAGTTAGAGACTCAG	SERK4	forward
SERK4-8	GGGGAAATTCCAATGACTTTG	SERK4	forward

SERK4-8rev	CAAAGTCATTGGAATTTCCCC	SERK4	reverse
SERK4-9	GGATCTTCCCGAACCTCCG	SERK4	forward
SERK4-10	GTGGAAAGAAATTTAGTGG	SERK4	forward
SERK4-11	GTGGGACACAAAGATCTATG	SERK4	forward
SERK4-12	GTTTAGCCGATGGCAATCTAG	SERK4	forward
SERK4-12rev	CTAGATTGCCATCGGCTAAAC	SERK4	reverse
SERK4-13	CACCGGGATGTTAAAGCTGC	SERK4	forward
SERK4-14	CTGGGTGTGATTAGGTGAAAG	SERK4	forward
RPL4for	GGTGGTGCGATAGTGGTGGAGG	RPL4	forward
RPL4rev	CTCATTCTTAAGCTTCCTCTGACC	RPL4	reverse

Table 2.2: Primers used for qRT-PCR analysis. Beacon Designer 2.0 was used to designed primers for qRT-PCR analysis with target T_m= 60 °C +/- 1 °C; primer length range from 18 to 25 pb; amlpcon length from 100 to 250 pb. FLS2 primers were designed by Dr. Dagmar R. Hann and FRK1, PHI1, NHL10 and EIF4a are described in (Boudsocq et al., 2010).

name	sequence 5' – 3'	gene or vector	orientation
EIF4a qF	TCATAGATCTGGTCCTTGAAACC	EIF4a	forward
EIF4a qR	GGCAGTCTCTTCGTGCTGAC	EIF4a	reverse
BAK1qF	TGTCCTGACGCTACAAGTTCTGG	BAK1	forward
BAK1qR	AGCAACTCCTCCCGCAATCG	BAK1	reverse
FRK1qF	CGGTCAGATTTCAACAGTTGTC	FRK1	forward
FRK1qR	AATAGCAGGTTGGCCTGTAATC	FRK1	reverse
PHI1qF	TTGGTTTAGACGGGATGGTG	PHI1	forward
PHI1qR	ACTCCAGTACAAGCCGATCC	PHI1	reverse
NHL10qF	TTCCTGTCCGTAACCCAAAC	NHL10	forward
NHL10qR	CCCTCGTAGTAGGCATGAGC	NHL10	reverse
AtFLS2qPCRfw	TGCTCACGTAAGCGATTTTG	FLS2	forward
AtFLS2qPCRrev	CTGTTTCGTCATCAGCTCCA	FLS2	reverse
qBIK1for	GCGAGCGTTGGATCATAACAGAC	BIK1	forward

qBIK1rev	ATTCTCACGGCTTCTTCAGGTAGG	BIK1	reverse
BON1qF	ATTGGCTTCACGAGACATTGTTCAG	BON1	forward
BON1qR	ACGAGACGAGATCATTCATGGAGG	BON1	reverse
qBIR1for	TGTGACTTGTTGGCATGATGATGAG	BIR1	forward
qBIR1rev	GGTGCTAATGTTGGCTGGTAAAGG	BIR1	reverse
qSOBIR1for	ACAAACCCAACAACAGCACTACATC	SOBIR1	forward
qSOBIR1rev	ACACCGAGAAGACAAACCCAGAG	SOBIR1	reverse
BRI1qF	TCTTGTGCCTCTTCTTGGTTATTGC	BRI1	forward
BRI1qR	TCTCTGTGGATGATATGCGGACTG	BRI1	reverse
CPDqF	CTAACGGTGAAGCAGTTGATGAGC	CPD	forward
CPDqR	AAGCAACGCCGCAAGCATATC	CPD	reverse

LITERATURE

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.

Abramovitch, R.B., Janjusevic, R., Stebbins, C.E., and Martin, G.B. (2006). Type III effector AvrPtoB requires intrinsic E3 ubiquitin ligase activity to suppress plant cell death and immunity. Proc Natl Acad Sci U S A **103,** 2851-2856.

Adams, J.A. (2003). Activation loop phosphorylation and catalysis in protein kinases: is there functional evidence for the autoinhibitor model? Biochemistry **42,** 601-607.

Albert, M., and Felix, G. (2010). Chimeric receptors of the Arabidopsis thaliana pattern recognition receptors EFR and FLS2. Plant Signal Behav **5,** 1430-1432.

Albert, M., Jehle, A.K., Mueller, K., Eisele, C., Lipschis, M., and Felix, G. (2010). Arabidopsis thaliana pattern recognition receptors for bacterial elongation factor Tu and flagellin can be combined to form functional chimeric receptors. J Biol Chem **285,** 19035-19042.

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. (2011). 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.

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.

Alfano, J.R., and Collmer, A. (2004). Type III secretion system effector proteins: double agents in bacterial disease and plant defense. Annu Rev Phytopathol **42,** 385-414.

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.

Austin, M.J., Muskett, P., Kahn, K., Feys, B.J., Jones, J.D., and Parker, J.E. (2002). Regulatory role of SGT1 in early R gene-mediated plant defenses. Science **295,** 2077-2080.

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.

Bar, M., Sharfman, M., and Avni, A. (2011). LeEix1 functions as a decoy receptor to attenuate LeEix2 signaling. Plant Signal Behav **6,** 455-457.

Bar, M., Sharfman, M., Ron, M., and Avni, A. (2010). BAK1 is required for the attenuation of ethylene-inducing xylanase (Eix)-induced defense responses by the decoy receptor LeEix1. Plant J **63,** 791-800.

Bartels, S., Lori, M., Mbengue, M., van Verk, M., Klauser, D., Hander, T., Boni, R., Robatzek, S., and Boller, T. (2013). The family of Peps and their precursors in Arabidopsis: differential expression and localization but similar induction of pattern-triggered immune responses. J Exp Bot **64,** 5309-5321.

Belkhadir, Y., Nimchuk, Z., Hubert, D.A., Mackey, D., and Dangl, J.L. (2004). Arabidopsis RIN4 negatively regulates disease resistance mediated by RPS2 and RPM1 downstream or independent of the

NDR1 signal modulator and is not required for the virulence functions of bacterial type III effectors AvrRpt2 or AvrRpm1. Plant Cell **16,** 2822-2835.

Belkhadir, Y., Jaillais, Y., Epple, P., Balsemao-Pires, E., Dangl, J.L., and Chory, J. (2011). Brassinosteroids modulate the efficiency of plant immune responses to microbe-associated molecular patterns. Proc Natl Acad Sci U S A **109,** 297-302.

Bogdanove, A.J. (2002). Protein-protein interactions in pathogen recognition by plants. Plant Mol Biol **50,** 981-989.

Boller, T. (1995). Chemoperception of microbial signals in plant cells. Annu. Rev. Plant Physiol. Plant Mol Biol. **46,** 189-214.

Boller, T., and He, S.Y. (2009). Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. Science **324,** 742-744.

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.

Bolton, M.D., van Esse, H.P., Vossen, J.H., de Jonge, R., Stergiopoulos, I., Stulemeijer, I.J., van den Berg, G.C., Borras-Hidalgo, O., Dekker, H.L., de Koster, C.G., de Wit, P.J., Joosten, M.H., and Thomma, B.P. (2008). The novel Cladosporium fulvum lysin motif effector Ecp6 is a virulence factor with orthologues in other fungal species. Mol Microbiol **69,** 119-136.

Boudsocq, M., Willmann, M.R., McCormack, M., Lee, H., Shan, L., He, P., Bush, J., Cheng, S.H., and Sheen, J. (2010). Differential innate immune signalling via Ca(2+) sensor protein kinases. Nature **464,** 418-422.

Boutrot, F., Segonzac, C., Chang, K.N., Qiao, H., Ecker, J.R., Zipfel, C., and Rathjen, J.P. (2010). Direct transcriptional control of the Arabidopsis immune receptor FLS2 by the ethylene-dependent transcription factors EIN3 and EIL1. Proc Natl Acad Sci U S A **107,** 14502-14507.

Bowling, S.A., Guo, A., Cao, H., Gordon, A.S., Klessig, D.F., and Dong, X. (1994). A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. Plant Cell **6,** 1845-1857.

Broekaert, W.F., Terras, F.R., Cammue, B.P., and Osborn, R.W. (1995). Plant defensins: novel antimicrobial peptides as components of the host defense system. Plant Physiol **108,** 1353-1358.

Brooks, D.M., Bender, C.L., and Kunkel, B.N. (2005). The Pseudomonas syringae phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in Arabidopsis thaliana. Mol Plant Pathol **6,** 629-639.

Brutus, A., Sicilia, F., Macone, A., Cervone, F., and De Lorenzo, G. (2010). A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. Proc Natl Acad Sci U S A **107,** 9452-9457.

Bush, D.S. (1995). Calcium regulation in plant cells and its role in signalling. Annu Rev Plant Physiol Plant Mol Biol **27,** 953-967.

Cai, R., Lewis, J., Yan, S., Liu, H., Clarke, C.R., Campanile, F., Almeida, N.F., Studholme, D.J., Lindeberg, M., Schneider, D., Zaccardelli, M., Setubal, J.C., Morales-Lizcano, N.P., Bernal, A., Coaker, G., Baker, C., Bender, C.L., Leman, S., and Vinatzer, B.A. (2011). The plant pathogen Pseudomonas syringae pv. tomato is genetically monomorphic and under strong selection to evade tomato immunity. PLoS Pathog **7,** e1002130.

Cano-Delgado, A., Yin, Y., Yu, C., Vafeados, D., Mora-Garcia, S., Cheng, J.C., Nam, K.H., Li, J., and Chory, J. (2004). BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in Arabidopsis. Development **131,** 5341-5351.

Caplan, J., Padmanabhan, M., and Dinesh-Kumar, S.P. (2008). Plant NB-LRR immune receptors: from recognition to transcriptional reprogramming. Cell Host Microbe **3,** 126-135.

Century, K.S., Holub, E.B., and Staskawicz, B.J. (1995). NDR1, a locus of Arabidopsis thaliana that is required for disease resistance to both a bacterial and a fungal pathogen. Proc Natl Acad Sci U S A **92,** 6597-6601.

Chaparro-Garcia, A., Wilkinson, R.C., Gimenez-Ibanez, S., Findlay, K., Coffey, M.D., Zipfel, C., Rathjen, J.P., Kamoun, S., and Schornack, S. (2011). The receptor-like kinase SERK3/BAK1 is required for basal resistance against the late blight pathogen phytophthora infestans in Nicotiana benthamiana. PLoS One **6,** e16608.

Chen, X., Chern, M., Canlas, P.E., Jiang, C., Ruan, D., Cao, P., and Ronald, P.C. (2010). A conserved threonine residue in the juxtamembrane domain of the XA21 pattern recognition receptor is critical for kinase autophosphorylation and XA21-mediated immunity. J Biol Chem **285,** 10454-10463.

Cheng, C., Gao, X., Feng, B., Sheen, J., Shan, L., and He, P. (2013). Plant immune response to pathogens differs with changing temperatures. Nat Commun **4,** 2530.

Cheng, W., Munkvold, K.R., Gao, H., Mathieu, J., Schwizer, S., Wang, S., Yan, Y.B., Wang, J., Martin, G.B., and Chai, J. (2011). Structural analysis of Pseudomonas syringae AvrPtoB bound to host BAK1 reveals two similar kinase-interacting domains in a type III Effector. Cell Host Microbe **10,** 616-626.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T., and Felix, G. (2006). The Arabidopsis receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. Plant Cell **18,** 465-476.

Chinchilla, D., Shan, L., He, P., de Vries, S., and Kemmerling, B. (2009). One for all: the receptor-associated kinase BAK1. Trends Plant Sci **14,** 535-541.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, 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.

Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. Cell **124,** 803-814.

Clarke, C.R., Chinchilla, D., Hind, S.R., Taguchi, F., Miki, R., Ichinose, Y., Martin, G.B., Leman, S., Felix, G., and Vinatzer, B.A. (2013). Allelic variation in two distinct Pseudomonas syringae flagellin epitopes modulates the strength of plant immune responses but not bacterial motility. New Phytol **200,** 847-860.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J **16,** 735-743.

Clouse, S.D. (1996). Molecular genetic studies confirm the role of brassinosteroids in plant growth and development. Plant J **10,** 1-8.

Clouse, S.D. (2011). Brassinosteroid signal transduction: from receptor kinase activation to transcriptional networks regulating plant development. Plant Cell **23,** 1219-1230.

Clouse, S.D., and Sasse, J.M. (1998). BRASSINOSTEROIDS: Essential Regulators of Plant Growth and Development. Annu Rev Plant Physiol Plant Mol Biol **49,** 427-451.

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.

Collmer, A., Lindeberg, M., Petnicki-Ocwieja, T., Schneider, D.J., and Alfano, J.R. (2002). Genomic mining type III secretion system effectors in Pseudomonas syringae yields new picks for all TTSS prospectors. Trends Microbiol **10,** 462-469.

Conrath, U. (2006). Systemic acquired resistance. Plant Signal Behav **1,** 179-184.

Cui, H., Wang, Y., Xue, L., Chu, J., Yan, C., Fu, J., Chen, M., Innes, R.W., and Zhou, J.M. (2010). Pseudomonas syringae effector protein AvrB perturbs Arabidopsis hormone signaling by activating MAP kinase 4. Cell Host Microbe **7,** 164-175.

Cunnac, S., Lindeberg, M., and Collmer, A. (2009). Pseudomonas syringae type III secretion system effectors: repertoires in search of functions. Curr Opin Microbiol **12,** 53-60.

Curtis, M.D., and Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol **133,** 462-469.

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

Danna, C.H., Zhang, X.C., Khatri, A., Bent, A.F., Ronald, P.C., and Ausubel, F.M. (2012). FLS2-Mediated Responses to Ax21-Derived Peptides: Response to the Mueller et al. Commentary. Plant Cell **24,** 3174-3176.

Danna, C.H., Millet, Y.A., Koller, T., Han, S.W., Bent, A.F., Ronald, P.C., and Ausubel, F.M. (2011). The Arabidopsis flagellin receptor FLS2 mediates the perception of Xanthomonas Ax21 secreted peptides. Proc Natl Acad Sci U S A **108,** 9286-9291.

Dardick, C., and Ronald, P. (2006). Plant and animal pathogen recognition receptors signal through non-RD kinases. PLoS Pathog **2,** e2.

Dardick, C., Schwessinger, B., and Ronald, P. (2012). Non-arginine-aspartate (non-RD) kinases are associated with innate immune receptors that recognize conserved microbial signatures. Curr Opin Plant Biol **15,** 358-366.

de Jonge, R., and Thomma, B.P. (2009). Fungal LysM effectors: extinguishers of host immunity? Trends Microbiol **17,** 151-157.

de Torres, M., Mansfield, J.W., Grabov, N., Brown, I.R., Ammouneh, H., Tsiamis, G., Forsyth, A., Robatzek, S., Grant, M., and Boch, J. (2006). Pseudomonas syringae effector AvrPtoB suppresses basal defence in Arabidopsis. Plant J **47,** 368-382.

De Wit, P.J., Mehrabi, R., Van den Burg, H.A., and Stergiopoulos, I. (2009). Fungal effector proteins: past, present and future. Mol Plant Pathol **10,** 735-747.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., and Ryals, J. (1994). A central role of salicylic Acid in plant disease resistance. Science **266,** 1247-1250.

Dennis, E.S., Ellis, J., Green, A., Llewellyn, D., Morell, M., Tabe, L., and Peacock, W.J. (2008). Genetic contributions to agricultural sustainability. Philos Trans R Soc Lond B Biol Sci **363,** 591-609.

Denoux, C., Galletti, R., Mammarella, N., Gopalan, S., Werck, D., De Lorenzo, G., Ferrari, S., Ausubel, F.M., and Dewdney, J. (2008). Activation of defense response pathways by OGs and Flg22 elicitors in Arabidopsis seedlings. Mol Plant **1,** 423-445.

Di Tommaso, P., Moretti, S., Xenarios, I., Orobitg, M., Montanyola, A., Chang, J.M., Taly, J.F., and Notredame, C. (2011). T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. Nucleic Acids Res **39,** W13-17.

Dievart, A., and Clark, S.E. (2003). Using mutant alleles to determine the structure and function of leucine-rich repeat receptor-like kinases. Curr Opin Plant Biol **6,** 507-516.

Djamei, A., Pitzschke, A., Nakagami, H., Rajh, I., and Hirt, H. (2007). Trojan horse strategy in Agrobacterium transformation: abusing MAPK defense signaling. Science **318,** 453-456.

Dodds, P.N., and Rathjen, J.P. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. Nat Rev Genet **11,** 539-548.

Dunning, F.M., Sun, W., Jansen, K.L., Helft, L., and Bent, A.F. (2007). Identification and mutational analysis of Arabidopsis FLS2 leucine-rich repeat domain residues that contribute to flagellin perception. Plant Cell **19,** 3297-3313.

Ellis, J.G., Dodds, P.N., and Lawrence, G.J. (2007). Flax rust resistance gene specificity is based on direct resistance-avirulence protein interactions. Annu Rev Phytopathol **45,** 289-306.

Erbs, G., Silipo, A., Aslam, S., De Castro, C., Liparoti, V., Flagiello, A., Pucci, P., Lanzetta, R., Parrilli, M., Molinaro, A., Newman, M.A., and Cooper, R.M. (2008). Peptidoglycan and muropeptides from pathogens Agrobacterium and Xanthomonas elicit plant innate immunity: structure and activity. Chem Biol **15,** 438-448.

Espinosa, A., Guo, M., Tam, V.C., Fu, Z.Q., and Alfano, J.R. (2003). The Pseudomonas syringae type III-secreted protein HopPtoD2 possesses protein tyrosine phosphatase activity and suppresses programmed cell death in plants. Mol Microbiol **49,** 377-387.

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D., Daniels, M.J., and Parker, J.E. (1999). EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. Proc Natl Acad Sci U S A **96,** 3292-3297.

Felix, G., and Boller, T. (2003). Molecular sensing of bacteria in plants. The highly conserved RNA-binding motif RNP-1 of bacterial cold shock proteins is recognized as an elicitor signal in tobacco. J Biol Chem **278,** 6201-6208.

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., Grosskopf, D.G., Regenass, M., and Boller, T. (1991a). Rapid changes of protein phosphorylation are involved in transduction of the elicitor signal in plant cells. Proc Natl Acad Sci U S A **88,** 8831-8834.

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.

Felix, G., Grosskopf, D.G., Regenass, M., Basse, C.W., and Boller, T. (1991b). Elicitor-induced ethylene biosynthesis in tomato cells: characterization and use as a bioassay for elicitor action. Plant Physiol **97,** 19-25.

Feng, F., Yang, F., Rong, W., Wu, X., Zhang, J., Chen, S., He, C., and Zhou, J.M. (2012). A Xanthomonas uridine 5'-monophosphate transferase inhibits plant immune kinases. Nature **485,** 114-118.

Ferrari, S., Plotnikova, J.M., De Lorenzo, G., and Ausubel, F.M. (2003). Arabidopsis local resistance to Botrytis cinerea involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. Plant J **35,** 193-205.

Feys, B.J., Wiermer, M., Bhat, R.A., Moisan, L.J., Medina-Escobar, N., Neu, C., Cabral, A., and Parker, J.E. (2005). Arabidopsis SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. Plant Cell **17,** 2601-2613.

Fletcher, J.C., Brand, U., Running, M.P., Simon, R., and Meyerowitz, E.M. (1999). Signaling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. Science **283,** 1911-1914.

Flor, H.H. (1971). Current status of the gene-for-gene concept Annu Rev Phytopathol **9,** 275-296.

Flury, P., Klauser, D., Schulze, B., Boller, T., and Bartels, S. (2013). The anticipation of danger: microbe-associated molecular pattern perception enhances AtPep-triggered oxidative burst. Plant Physiol **161,** 2023-2035.

Fradin, E.F., Abd-El-Haliem, A., Masini, L., van den Berg, G.C., Joosten, M.H., and Thomma, B.P. (2011). Interfamily transfer of tomato Ve1 mediates Verticillium resistance in Arabidopsis. Plant Physiol **156,** 2255-2265.

Fradin, E.F., Zhang, Z., Juarez Ayala, J.C., Castroverde, C.D., Nazar, R.N., Robb, J., Liu, C.M., and Thomma, B.P. (2009). Genetic dissection of Verticillium wilt resistance mediated by tomato Ve1. Plant Physiol **150,** 320-332.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. (1993). Requirement of salicylic Acid for the induction of systemic acquired resistance. Science **261,** 754-756.

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.

Garcia-Brugger, A., Lamotte, O., Vandelle, E., Bourque, S., Lecourieux, D., Poinssot, B., Wendehenne, D., and Pugin, A. (2006). Early signaling events induced by elicitors of plant defenses. Mol Plant Microbe Interact **19,** 711-724.

Geldner, N., Hyman, D.L., Wang, X., Schumacher, K., and Chory, J. (2007). Endosomal signaling of plant steroid receptor kinase BRI1. Genes Dev **21,** 1598-1602.

Gimenez-Ibanez, S., Ntoukakis, V., and Rathjen, J.P. (2009a). The LysM receptor kinase CERK1 mediates bacterial perception in Arabidopsis. Plant Signal Behav **4,** 539-541.

Gimenez-Ibanez, S., Hann, D.R., Ntoukakis, V., Petutschnig, E., Lipka, V., and Rathjen, J.P. (2009b). AvrPtoB targets the LysM receptor kinase CERK1 to promote bacterial virulence on plants. Curr Biol **19,** 423-429.

Glawischnig, E. (2007). Camalexin. Phytochemistry **68,** 401-406.

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

Glazebrook, J., Chen, W., Estes, B., Chang, H.S., Nawrath, C., Metraux, J.P., Zhu, T., and Katagiri, F. (2003). Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. Plant J **34,** 217-228.

Glazebrook, J., Zook, M., Mert, F., Kagan, I., Rogers, E.E., Crute, I.R., Holub, E.B., Hammerschmidt, R., and Ausubel, F.M. (1997). Phytoalexin-deficient mutants of Arabidopsis reveal that PAD4 encodes a regulatory factor and that four PAD genes contribute to downy mildew resistance. Genetics **146,** 381-392.

Gohre, V., and Robatzek, S. (2008). Breaking the barriers: microbial effector molecules subvert plant immunity. Annu Rev Phytopathol **46,** 189-215.

Gohre, V., Spallek, T., Haweker, H., Mersmann, S., Mentzel, T., Boller, T., de Torres, M., Mansfield, J.W., and Robatzek, S. (2008). Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. Curr Biol **18,** 1824-1832.

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., Felix, G., and Boller, T. (1999). A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana. Plant J **18,** 277-284.

Gou, X., He, K., Yang, H., Yuan, T., Lin, H., Clouse, S.D., and Li, J. (2010). Genome-wide cloning and sequence analysis of leucine-rich repeat receptor-like protein kinase genes in Arabidopsis thaliana. BMC Genomics **11,** 19.

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.

Granado, J., Felix, G., and Boller, T. (1995). Perception of Fungal Sterols in Plants (Subnanomolar Concentrations of Ergosterol Elicit Extracellular Alkalinization in Tomato Cells). Plant Physiol **107,** 485-490.

Gray, W.M., Muskett, P.R., Chuang, H.W., and Parker, J.E. (2003). Arabidopsis SGT1b is required for SCF(TIR1)-mediated auxin response. Plant Cell **15,** 1310-1319.

Greenberg, J.T., and Yao, N. (2004). The role and regulation of programmed cell death in plant-pathogen interactions. Cell Microbiol **6,** 201-211.

Halkier, B.A., and Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. Annu Rev Plant Biol **57,** 303-333.

Hanania, U., and Avni, A. (1997). High affinity binding site for ethylene-inducing xylanase elicitor on Nicotiana tabacum membranes. Plant J **12,** 113-120.

Hann, D.R., and Rathjen, J.P. (2007). Early events in the pathogenicity of Pseudomonas syringae on Nicotiana benthamiana. Plant J **49,** 607-618.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature **410,** 1099-1103.

He, J.X., Gendron, J.M., Yang, Y., Li, J., and Wang, Z.Y. (2002). The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in Arabidopsis. Proc Natl Acad Sci U S A **99,** 10185-10190.

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., Powell, R.A., Yang, H., Yuan, T., Guo, Z., and Li, J. (2008). Receptor-like protein kinases, BAK1 and BKK1, regulate a light-dependent cell-death control pathway. Plant Signal Behav **3,** 813-815.

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.

He, P., Shan, L., Lin, N.C., Martin, G.B., Kemmerling, B., Nurnberger, T., and Sheen, J. (2006). Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in Arabidopsis innate immunity. Cell **125,** 563-575.

He, S.Y., Nomura, K., and Whittam, T.S. (2004). Type III protein secretion mechanism in mammalian and plant pathogens. Biochim Biophys Acta **1694,** 181-206.

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.

Hovmoller, M.S., Yahyaoui, A.H., Milus, E.A., and Justesen, A.F. (2008). Rapid global spread of two aggressive strains of a wheat rust fungus. Mol Ecol **17,** 3818-3826.

Howe, G.A., and Jander, G. (2008). Plant immunity to insect herbivores. Annu Rev Plant Biol **59,** 41-66.

Hu, H., Xiong, L., and Yang, Y. (2005). Rice SERK1 gene positively regulates somatic embryogenesis of cultured cell and host defense response against fungal infection. Planta **222,** 107-117.

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., and Ryan, C.A. (2007). Endogenous peptide defense signals in Arabidopsis differentially amplify signaling for the innate immune response. Proc Natl Acad Sci U S A **104,** 10732-10736.

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.

Huffaker, A., Dafoe, N.J., and Schmelz, E.A. (2011). ZmPep1, an ortholog of Arabidopsis elicitor peptide 1, regulates maize innate immunity and enhances disease resistance. Plant Physiol **155,** 1325-1338.

Inohara, N., and Nunez, G. (2003). NODs: intracellular proteins involved in inflammation and apoptosis. Nat Rev Immunol **3,** 371-382.

Jackson, R.W., Mansfield, J.W., Ammouneh, H., Dutton, L.C., Wharton, B., Ortiz-Barredo, A., Arnold, D.L., Tsiamis, G., Sesma, A., Butcher, D., Boch, J., Kim, Y.J., Martin, G.B., Tegli, S., Murillo, J., and Vivian, A. (2002). Location and activity of members of a family of virPphA homologues in pathovars of Pseudomonas syringae and P. savastanoi. Mol Plant Pathol **3,** 205-216.

Jaillais, Y., Hothorn, M., Belkhadir, Y., Dabi, T., Nimchuk, Z.L., Meyerowitz, E.M., and Chory, J. (2011). Tyrosine phosphorylation controls brassinosteroid receptor activation by triggering membrane release of its kinase inhibitor. Genes Dev **25,** 232-237.

Jambunathan, N., Siani, J.M., and McNellis, T.W. (2001). A humidity-sensitive Arabidopsis copine mutant exhibits precocious cell death and increased disease resistance. Plant Cell **13,** 2225-2240.

Janjusevic, R., Abramovitch, R.B., Martin, G.B., and Stebbins, C.E. (2006). A bacterial inhibitor of host programmed cell death defenses is an E3 ubiquitin ligase. Science **311,** 222-226.

Jehle, A.K., Furst, U., Lipschis, M., Albert, M., and Felix, G. (2013a). Perception of the novel MAMP eMax from different Xanthomonas species requires the Arabidopsis receptor-like protein ReMAX and the receptor kinase SOBIR. Plant Signal Behav **8**.

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

Jeworutzki, E., Roelfsema, M.R., Anschutz, U., Krol, E., Elzenga, J.T., Felix, G., Boller, T., Hedrich, R., and Becker, D. (2010). Early signaling through the Arabidopsis pattern recognition receptors FLS2 and EFR involves Ca-associated opening of plasma membrane anion channels. Plant J **62,** 367-378.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M., and Glazebrook, J. (1999). Arabidopsis thaliana PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. Proc Natl Acad Sci U S A **96,** 13583-13588.

Johnson, K.L., and Ingram, G.C. (2005). Sending the right signals: regulating receptor kinase activity. Curr Opin Plant Biol **8,** 648-656.

Johnson, L.N., Noble, M.E., and Owen, D.J. (1996). Active and inactive protein kinases: structural basis for regulation. Cell **85,** 149-158.

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

Joshi, B.N., Sainani, M.N., Bastawade, K.B., Deshpande, V.V., Gupta, V.S., and Ranjekar, P.K. (1999). Pearl millet cysteine protease inhibitor. Evidence for the presence of two distinct sites responsible for anti-fungal and anti-feedent activities. Eur J Biochem **265,** 556-563.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E., and Shibuya, N. (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. Proc Natl Acad Sci U S A **103,** 11086-11091.

Kamoun, S. (2007). Groovy times: filamentous pathogen effectors revealed. Curr Opin Plant Biol **10,** 358-365.

Karlova, R., Boeren, S., Russinova, E., Aker, J., Vervoort, J., and de Vries, S. (2006). The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 protein complex includes BRASSINOSTEROID-INSENSITIVE1. Plant Cell **18,** 626-638.

Kemmerling, B., Schwedt, A., Rodriguez, P., Mazzotta, S., Frank, M., Qamar, S.A., Mengiste, T., Betsuyaku, S., Parker, J.E., Mussig, C., Thomma, B.P., Albrecht, C., de Vries, S.C., Hirt, H., and Nurnberger, 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. (2005a). 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, M.G., da Cunha, L., McFall, A.J., Belkhadir, Y., DebRoy, S., Dangl, J.L., and Mackey, D. (2005b). Two Pseudomonas syringae type III effectors inhibit RIN4-regulated basal defense in Arabidopsis. Cell **121,** 749-759.

Kim, T.W., Guan, S., Sun, Y., Deng, Z., Tang, W., Shang, J.X., 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.

Kim, Y.J., Lin, N.C., and Martin, G.B. (2002). Two distinct Pseudomonas effector proteins interact with the Pto kinase and activate plant immunity. Cell **109,** 589-598.

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.

Klarzynski, O., Plesse, B., Joubert, J.M., Yvin, J.C., Kopp, M., Kloareg, B., and Fritig, B. (2000). Linear beta-1,3 glucans are elicitors of defense responses in tobacco. Plant Physiol **124,** 1027-1038.

Kobayashi, M., Ohura, I., Kawakita, K., Yokota, N., Fujiwara, M., Shimamoto, K., Doke, N., and Yoshioka, H. (2007). Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. Plant Cell **19,** 1065-1080.

Koch, E., and Slusarenko, A. (1990). Arabidopsis is susceptible to infection by a downy mildew fungus. Plant Cell **2,** 437-445.

Korves, T., and Bergelson, J. (2004). A novel cost of R gene resistance in the presence of disease. Am Nat **163,** 489-504.

Kroj, T., Rudd, J.J., Nurnberger, T., Gabler, Y., Lee, J., and Scheel, D. (2003). Mitogen-activated protein kinases play an essential role in oxidative burst-independent expression of pathogenesis-related genes in parsley. J Biol Chem **278,** 2256-2264.

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.

Krupa, A., Preethi, G., and Srinivasan, N. (2004). Structural modes of stabilization of permissive phosphorylation sites in protein kinases: distinct strategies in Ser/Thr and Tyr kinases. J Mol Biol **339,** 1025-1039.

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.

Kvitko, B.H., Park, D.H., Velasquez, A.C., Wei, C.F., Russell, A.B., Martin, G.B., Schneider, D.J., and Collmer, A. (2009). Deletions in the repertoire of Pseudomonas syringae pv. tomato DC3000 type III secretion effector genes reveal functional overlap among effectors. PLoS Pathog **5,** e1000388.

Lacombe, S., Rougon-Cardoso, A., Sherwood, E., Peeters, N., Dahlbeck, D., van Esse, H.P., Smoker, M., Rallapalli, G., Thomma, B.P., Staskawicz, B., Jones, J.D., and Zipfel, C. (2010). Interfamily transfer of a plant pattern-recognition receptor confers broad-spectrum bacterial resistance. Nat Biotechnol **28,** 365-369.

Laluk, K., Luo, H., Chai, M., Dhawan, R., Lai, Z., and Mengiste, T. (2011). Biochemical and genetic requirements for function of the immune response regulator BOTRYTIS-INDUCED KINASE1 in plant growth, ethylene signaling, and PAMP-triggered immunity in Arabidopsis. Plant Cell **23,** 2831-2849.

Lamb, C., and Dixon, R.A. (1997). The Oxidative Burst in Plant Disease Resistance. Annu Rev Plant Physiol Plant Mol Biol **48,** 251-275.

Lecourieux, D., Ranjeva, R., and Pugin, A. (2006). Calcium in plant defence-signalling pathways. New Phytol **171,** 249-269.

Lee, H., Chah, O.K., and Sheen, J. (2011). Stem-cell-triggered immunity through CLV3p-FLS2 signalling. Nature **473,** 376-379.

Lee, S.W., Han, S.W., Sririyanum, M., Park, C.J., Seo, Y.S., and Ronald, P.C. (2009). A type I-secreted, sulfated peptide triggers XA21-mediated innate immunity. Science **326,** 850-853.

Lehti-Shiu, M.D., Zou, C., Hanada, K., and Shiu, S.H. (2009). Evolutionary history and stress regulation of plant receptor-like kinase/pelle genes. Plant Physiol **150,** 12-26.

Leon-Reyes, A., Spoel, S.H., De Lange, E.S., Abe, H., Kobayashi, M., Tsuda, S., Millenaar, F.F., Welschen, R.A., Ritsema, T., and Pieterse, C.M. (2009). Ethylene modulates the role of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 in cross talk between salicylate and jasmonate signaling. Plant Physiol **149,** 1797-1809.

Leslie, M.E., Lewis, M.W., Youn, J.Y., Daniels, M.J., and Liljegren, S.J. (2010). The EVERSHED receptor-like kinase modulates floral organ shedding in Arabidopsis. Development **137,** 467-476.

Levine, A., Pennell, R.I., Alvarez, M.E., Palmer, R., and Lamb, C. (1996). Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. Curr Biol **6,** 427-437.

Lewis, M.W., Leslie, M.E., Fulcher, E.H., Darnielle, L., Healy, P.N., Youn, J.Y., and Liljegren, S.J. (2010). The SERK1 receptor-like kinase regulates organ separation in Arabidopsis flowers. Plant J **62,** 817-828.

Li, D., Wang, L., Wang, M., Xu, Y.Y., Luo, W., Liu, Y.J., Xu, Z.H., Li, J., and Chong, K. (2009). Engineering OsBAK1 gene as a molecular tool to improve rice architecture for high yield. Plant Biotechnol J **7,** 791-806.

Li, J. (2011). Direct involvement of leucine-rich repeats in assembling ligand-triggered receptor-coreceptor complexes. Proc Natl Acad Sci U S A **108,** 8073-8074.

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.

Libault, M., Wan, J., Czechowski, T., Udvardi, M., and Stacey, G. (2007). Identification of 118 Arabidopsis transcription factor and 30 ubiquitin-ligase genes responding to chitin, a plant-defense elicitor. Mol Plant Microbe Interact **20,** 900-911.

Liebrand, T.W., van den Berg, G.C., Zhang, Z., Smit, P., Cordewener, J.H., America, A.H., Sklenar, J., Jones, A.M., Tameling, W.I., Robatzek, S., Thomma, B.P., and Joosten, M.H. (2013). Receptor-like kinase SOBIR1/EVR interacts with receptor-like proteins in plant immunity against fungal infection. Proc Natl Acad Sci U S A **110,** 10010-10015.

Liew, F.Y., Xu, D., Brint, E.K., and O'Neill, L.A. (2005). Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol **5,** 446-458.

Lin, N.C., and Martin, G.B. (2007). Pto- and Prf-mediated recognition of AvrPto and AvrPtoB restricts the ability of diverse pseudomonas syringae pathovars to infect tomato. Mol Plant Microbe Interact **20,** 806-815.

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.

Lindeberg, M., Cunnac, S., and Collmer, A. (2009). The evolution of Pseudomonas syringae host specificity and type III effector repertoires. Mol Plant Pathol **10,** 767-775.

Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J., Fan, F., Wang, J., Jin, C., Chang, J., Zhou, J.M., and Chai, J. (2012). Chitin-induced dimerization activates a plant immune receptor. Science **336,** 1160-1164.

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.

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

Lu, D., Wu, S., He, P., and Shan, L. (2010a). Phosphorylation of receptor-like cytoplasmic kinases by bacterial flagellin. Plant Signal Behav **5**.

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L., and He, P. (2010b). 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.

Ludwig, A.A., Saitoh, H., Felix, G., Freymark, G., Miersch, O., Wasternack, C., Boller, T., Jones, J.D., and Romeis, T. (2005). Ethylene-mediated cross-talk between calcium-dependent protein kinase and MAPK signaling controls stress responses in plants. Proc Natl Acad Sci U S A **102,** 10736-10741.

Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B., and Ton, J. (2011). Callose deposition: a multifaceted plant defense response. Mol Plant Microbe Interact **24,** 183-193.

Mackey, D., and McFall, A.J. (2006). MAMPs and MIMPs: proposed classifications for inducers of innate immunity. Mol Microbiol **61,** 1365-1371.

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.

Mantelin, S., Peng, H.C., Li, B., Atamian, H.S., Takken, F.L., and Kaloshian, I. (2011). The receptor-like kinase SlSERK1 is required for Mi-1-mediated resistance to potato aphids in tomato. Plant J **67,** 459-471.

Mao, G., Meng, X., Liu, Y., Zheng, Z., Chen, Z., and Zhang, S. (2011). Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in Arabidopsis. Plant Cell **23,** 1639-1653.

Mathur, J., Molnar, G., Fujioka, S., Takatsuto, S., Sakurai, A., Yokota, T., Adam, G., Voigt, B., Nagy, F., Maas, C., Schell, J., Koncz, C., and Szekeres, M. (1998). Transcription of the Arabidopsis CPD gene, encoding a steroidogenic cytochrome P450, is negatively controlled by brassinosteroids. Plant J **14,** 593-602.

Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S.Y. (2006). Plant stomata function in innate immunity against bacterial invasion. Cell **126,** 969-980.

Mersmann, S., Bourdais, G., Rietz, S., and Robatzek, S. (2010). Ethylene signaling regulates accumulation of the FLS2 receptor and is required for the oxidative burst contributing to plant immunity. Plant Physiol **154,** 391-400.

Meyers, B.C., Kozik, A., Griego, A., Kuang, H., and Michelmore, R.W. (2003). Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis. Plant Cell **15,** 809-834.

Mithofer, A., and Boland, W. (2008). Recognition of herbivory-associated molecular patterns. Plant Physiol **146,** 825-831.

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.

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.

Moscatiello, R., Mariani, P., Sanders, D., and Maathuis, F.J. (2006). Transcriptional analysis of calcium-dependent and calcium-independent signalling pathways induced by oligogalacturonides. J Exp Bot **57,** 2847-2865.

Mucyn, T.S., Clemente, A., Andriotis, V.M., Balmuth, A.L., Oldroyd, G.E., Staskawicz, B.J., and Rathjen, J.P. (2006). The tomato NBARC-LRR protein Prf interacts with Pto kinase in vivo to regulate specific plant immunity. Plant Cell **18,** 2792-2806.

Mueller, K., Bittel, P., Chinchilla, D., Jehle, A.K., Albert, M., Boller, T., and Felix, G. (2012a). Chimeric FLS2 receptors reveal the basis for differential flagellin perception in Arabidopsis and tomato. Plant Cell **24,** 2213-2224.

Mueller, K., Chinchilla, D., Albert, M., Jehle, A.K., Kalbacher, H., Boller, T., and Felix, G. (2012b). Contamination Risks in Work with Synthetic Peptides: flg22 as an Example of a Pirate in Commercial Peptide Preparations. Plant Cell **24,** 3193-3197.

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.

Muskett, P., and Parker, J. (2003). Role of SGT1 in the regulation of plant R gene signalling. Microbes Infect **5,** 969-976.

Mussig, C., Fischer, S., and Altmann, T. (2002). Brassinosteroid-regulated gene expression. Plant Physiol **129,** 1241-1251.

Naito, K., Taguchi, F., Suzuki, T., Inagaki, Y., Toyoda, K., Shiraishi, T., and Ichinose, Y. (2008). Amino acid sequence of bacterial microbe-associated molecular pattern flg22 is required for virulence. Mol Plant Microbe Interact **21,** 1165-1174.

Nakashita, H., Yasuda, M., Nitta, T., Asami, T., Fujioka, S., Arai, Y., Sekimata, K., Takatsuto, S., Yamaguchi, I., and Yoshida, S. (2003). Brassinosteroid functions in a broad range of disease resistance in tobacco and rice. Plant J **33,** 887-898.

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

Navarro, L., Zipfel, C., Rowland, O., Keller, I., Robatzek, S., Boller, T., and Jones, J.D. (2004). The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. Plant Physiol **135,** 1113-1128.

Nekrasov, V., Li, J., Batoux, M., Roux, M., Chu, Z.H., Lacombe, S., Rougon, A., Bittel, P., Kiss-Papp, M., Chinchilla, D., van Esse, H.P., Jorda, L., Schwessinger, B., Nicaise, V., Thomma, B.P., Molina, A., Jones, J.D., and Zipfel, C. (2009). Control of the pattern-recognition receptor EFR by an ER protein complex in plant immunity. EMBO J **28,** 3428-3438.

Noguchi, T., Fujioka, S., Choe, S., Takatsuto, S., Yoshida, S., Yuan, H., Feldmann, K.A., and Tax, F.E. (1999). Brassinosteroid-insensitive dwarf mutants of Arabidopsis accumulate brassinosteroids. Plant Physiol **121,** 743-752.

Nolan, K.E., Kurdyukov, S., and Rose, R.J. (2011). Characterisation of the legume SERK-NIK gene superfamily including splice variants: Implications for development and defence. BMC Plant Biol **11**.

Nomura, K., Melotto, M., and He, S.Y. (2005). Suppression of host defense in compatible plant-Pseudomonas syringae interactions. Curr Opin Plant Biol **8,** 361-368.

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.

Ntoukakis, V., Schwessinger, B., Segonzac, C., and Zipfel, C. (2011). Cautionary notes on the use of C-terminal BAK1 fusion proteins for functional studies. Plant Cell **23,** 3871-3878.

Nuhse, T.S., Peck, S.C., Hirt, H., and Boller, T. (2000). Microbial elicitors induce activation and dual phosphorylation of the Arabidopsis thaliana MAPK 6. J Biol Chem **275,** 7521-7526.

Nuhse, T.S., Bottrill, A.R., Jones, A.M., and Peck, S.C. (2007). Quantitative phosphoproteomic analysis of plasma membrane proteins reveals regulatory mechanisms of plant innate immune responses. Plant J **51,** 931-940.

Nurnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev **198,** 249-266.

Odell, J.T., Nagy, F., and Chua, N.H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature **313,** 810-812.

Oh, M.H., Wang, X., Wu, X., Zhao, Y., Clouse, S.D., and Huber, S.C. (2010). Autophosphorylation of Tyr-610 in the receptor kinase BAK1 plays a role in brassinosteroid signaling and basal defense gene expression. Proc Natl Acad Sci U S A **107,** 17827-17832.

Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D., and Daniels, M.J. (1996). Characterization of eds1, a mutation in Arabidopsis suppressing resistance to Peronospora parasitica specified by several different RPP genes. Plant Cell **8,** 2033-2046.

Pearce, G., Strydom, D., Johnson, S., and Ryan, C.A. (1991). A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. Science **253,** 895-897.

Peterson, R.K.D., and Higley, L.G. (2001). Biotic stress and yield loss. RCR Press, Boca Raton, Florida, USA, ISBN 0849311454, 9780849311451.

Petnicki-Ocwieja, T., Schneider, D.J., Tam, V.C., Chancey, S.T., Shan, L., Jamir, Y., Schechter, L.M., Janes, M.D., Buell, C.R., Tang, X., Collmer, A., and Alfano, J.R. (2002). Genomewide identification of proteins secreted by the Hrp type III protein secretion system of Pseudomonas syringae pv. tomato DC3000. Proc Natl Acad Sci U S A **99,** 7652-7657.

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.

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

Pretorius, Z.A., Singh, R.P., Wagoire, W.W., and Payne, T.S. (2000). Detection of Virulence to Wheat Stem Rust Resistance Gene Sr31 in Puccinia graminis. f. sp. tritici in Uganda. Plant Dis. **84,** 203-203.

Qi, Y., Tsuda, K., Glazebrook, J., and Katagiri, F. (2011). Physical association of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) immune receptors in Arabidopsis. Mol Plant Pathol **12,** 702-708.

Radutoiu, S., Madsen, L.H., Madsen, E.B., Felle, H.H., Umehara, Y., Gronlund, M., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and Stougaard, J. (2003). Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. Nature **425,** 585-592.

Ramonell, K.M., Zhang, B., Ewing, R.M., Chen, Y., Xu, D., Stacey, G., and Somerville, S. (2002). Microarray analysis of chitin elicitation in Arabidopsis thaliana. Mol Plant Pathol **3,** 301-311.

Rate, D.N., Cuenca, J.V., Bowman, G.R., Guttman, D.S., and Greenberg, J.T. (1999). The gain-of-function Arabidopsis acd6 mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. Plant Cell **11,** 1695-1708.

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

Robatzek, S., Bittel, P., Chinchilla, D., Kochner, P., Felix, G., Shiu, S.H., and Boller, T. (2007). Molecular identification and characterization of the tomato flagellin receptor LeFLS2, an orthologue of Arabidopsis FLS2 exhibiting characteristically different perception specificities. Plant Mol Biol **64,** 539-547.

Rojo, E., Sharma, V.K., Kovaleva, V., Raikhel, N.V., and Fletcher, J.C. (2002). CLV3 is localized to the extracellular space, where it activates the Arabidopsis CLAVATA stem cell signaling pathway. Plant Cell **14,** 969-977.

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.

Russinova, E., Borst, J.W., Kwaaitaal, M., Cano-Delgado, A., Yin, Y., Chory, J., and de Vries, S.C. (2004). Heterodimerization and endocytosis of Arabidopsis brassinosteroid receptors BRI1 and AtSERK3 (BAK1). Plant Cell **16,** 3216-3229.

Ryan, C.A., Huffaker, A., and Yamaguchi, Y. (2007). New insights into innate immunity in Arabidopsis. Cell Microbiol **9,** 1902-1908.

Salmeron, J.M., Oldroyd, G.E., Rommens, C.M., Scofield, S.R., Kim, H.S., Lavelle, D.T., Dahlbeck, D., and Staskawicz, B.J. (1996). Tomato Prf is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the Pto kinase gene cluster. Cell **86,** 123-133.

Santiago, J., Henzler, C., and Hothorn, M. (2013). Molecular mechanism for plant steroid receptor activation by somatic embryogenesis co-receptor kinases. Science **341,** 889-892.

Schmelzer, E. (2002). Cell polarization, a crucial process in fungal defence. Trends Plant Sci **7,** 411-415.

Schmidt, E.D., Guzzo, F., Toonen, M.A., and de Vries, S.C. (1997). A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development **124,** 2049-2062.

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., and Ronald, P.C. (2012). Plant innate immunity: perception of conserved microbial signatures. Annu Rev Plant Biol **63,** 451-482.

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., and Zipfel, C. (2011). Activation of plant pattern-recognition receptors by bacteria. Curr Opin Microbiol **14,** 54-61.

Shah, J., Tsui, F., and Klessig, D.F. (1997). Characterization of a salicylic acid-insensitive mutant (sai1) of Arabidopsis thaliana, identified in a selective screen utilizing the SA-inducible expression of the tms2 gene. Mol Plant Microbe Interact **10,** 69-78.

Shan, L., He, P., Li, J., Heese, A., Peck, S.C., Nurnberger, T., Martin, G.B., and Sheen, J. (2008). Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. Cell Host Microbe **4,** 17-27.

Shibuya, N., Kaku, H., Kuchitsu, K., and Maliarik, M.J. (1993). Identification of a novel high-affinity binding site for N-acetylchitooligosaccharide elicitor in the membrane fraction from suspension-cultured rice cells. FEBS Lett **329,** 75-78.

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., Minami, E., Okada, K., Yamane, H., Kaku, H., and Shibuya, N. (2010). Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. Plant J **64,** 204-214.

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.

Shiu, S.H., and Bleecker, A.B. (2001a). Plant receptor-like kinase gene family: diversity, function, and signaling. Sci STKE **2001,** re22.

Shiu, S.H., and Bleecker, A.B. (2001b). Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. Proc Natl Acad Sci U S A **98,** 10763-10768.

Shiu, S.H., and Bleecker, A.B. (2003). Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in Arabidopsis. Plant Physiol **132,** 530-543.

Smith-Becker, J., Marois, E., Huguet, E.J., Midland, S.L., Sims, J.J., and Keen, N.T. (1998). Accumulation of salicylic acid and 4-hydroxybenzoic acid in phloem fluids of cucumber during systemic acquired resistance is preceded by a transient increase in phenylalanine ammonia-lyase activity in petioles and stems. Plant Physiol **116,** 231-238.

Smith, K.D., Andersen-Nissen, E., Hayashi, F., Strobe, K., Bergman, M.A., Barrett, S.L., Cookson, B.T., and Aderem, A. (2003). Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol **4,** 1247-1253.

Staskawicz, B.J., Dahlbeck, D., and Keen, N.T. (1984). Cloned avirulence gene of Pseudomonas syringae pv. glycinea determines race-specific incompatibility on Glycine max (L.) Merr. Proc Natl Acad Sci U S A **81,** 6024-6028.

Stuiver, M.H., and Custers, J.H. (2001). Engineering disease resistance in plants. Nature **411,** 865-868.

Suarez-Rodriguez, M.C., Adams-Phillips, L., Liu, Y., Wang, H., Su, S.H., Jester, P.J., Zhang, S., Bent, A.F., and Krysan, P.J. (2007). MEKK1 is required for flg22-induced MPK4 activation in Arabidopsis plants. Plant Physiol **143,** 661-669.

Sun, W., Cao, Y., Jansen Labby, K., Bittel, P., Boller, T., and Bent, A.F. (2012). Probing the Arabidopsis flagellin receptor: FLS2-FLS2 association and the contributions of specific domains to signaling function. Plant Cell **24,** 1096-1113.

Sun, Y., Han, Z., Tang, J., Hu, Z., Chai, C., Zhou, B., and Chai, J. (2013a). Structure reveals that BAK1 as a co-receptor recognizes the BRI1-bound brassinolide. Cell Res **23,** 1326-1329.

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

Sun, Y., Fan, X.Y., Cao, D.M., Tang, W., He, K., Zhu, J.Y., He, J.X., Bai, M.Y., Zhu, S., Oh, E., Patil, S., Kim, T.W., Ji, H., Wong, W.H., Rhee, S.Y., and Wang, Z.Y. (2010). Integration of brassinosteroid signal transduction with the transcription network for plant growth regulation in Arabidopsis. Dev Cell **19,** 765-777.

Szekeres, M., Nemeth, K., Koncz-Kalman, Z., Mathur, J., Kauschmann, A., Altmann, T., Redei, G.P., Nagy, F., Schell, J., and Koncz, C. (1996). Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in Arabidopsis. Cell **85,** 171-182.

Takai, R., Isogai, A., Takayama, S., and Che, F.S. (2008). Analysis of flagellin perception mediated by flg22 receptor OsFLS2 in rice. Mol Plant Microbe Interact **21,** 1635-1642.

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.

Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H.S., Han, B., Zhu, T., Zou, G., and Katagiri, F. (2003). Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen Pseudomonas syringae. Plant Cell **15,** 317-330.

Thomma, B.P., Nurnberger, T., and Joosten, M.H. (2011). Of PAMPs and effectors: the blurred PTI-ETI dichotomy. Plant Cell **23,** 4-15.

Thomma, B.P., Eggermont, K., Penninckx, I.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P., and Broekaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proc Natl Acad Sci U S A **95,** 15107-15111.

Thordal-Christensen, H. (2003). Fresh insights into processes of nonhost resistance. Curr Opin Plant Biol **6,** 351-357.

Tian, D., Traw, M.B., Chen, J.Q., Kreitman, M., and Bergelson, J. (2003). Fitness costs of R-gene-mediated resistance in Arabidopsis thaliana. Nature **423,** 74-77.

Torii, K.U. (2004). Leucine-rich repeat receptor kinases in plants: structure, function, and signal transduction pathways. Int Rev Cytol **234,** 1-46.

Torres, M.A., and Dangl, J.L. (2005). Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol **8,** 397-403.

Torres, M.A., Jones, J.D., and Dangl, J.L. (2005). Pathogen-induced, NADPH oxidase-derived reactive oxygen intermediates suppress spread of cell death in Arabidopsis thaliana. Nat Genet **37,** 1130-1134.

Trewavas, A.J., and Malho, R. (1998). Ca2+ signalling in plant cells: the big network! Curr Opin Plant Biol **1,** 428-433.

Trujillo, M., Ichimura, K., Casais, C., and Shirasu, K. (2008). Negative regulation of PAMP-triggered immunity by an E3 ubiquitin ligase triplet in Arabidopsis. Curr Biol **18,** 1396-1401.

Tsuda, K., Sato, M., Glazebrook, J., Cohen, J.D., and Katagiri, F. (2008). Interplay between MAMP-triggered and SA-mediated defense responses. Plant J **53,** 763-775.

Van der Biezen, E.A., and Jones, J.D. (1998). Plant disease-resistance proteins and the gene-for-gene concept. Trends Biochem Sci **23,** 454-456.

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

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H., and Ryals, J. (1994). Salicylic Acid Is Not the Translocated Signal Responsible for Inducing Systemic Acquired Resistance but Is Required in Signal Transduction. Plant Cell **6,** 959-965.

Veronese, P., Nakagami, H., Bluhm, B., Abuqamar, S., Chen, X., Salmeron, J., Dietrich, R.A., Hirt, H., and Mengiste, T. (2006). The membrane-anchored BOTRYTIS-INDUCED KINASE1 plays distinct roles in Arabidopsis resistance to necrotrophic and biotrophic pathogens. Plant Cell **18,** 257-273.

Viprey, V., Del Greco, A., Golinowski, W., Broughton, W.J., and Perret, X. (1998). Symbiotic implications of type III protein secretion machinery in Rhizobium. Mol Microbiol **28,** 1381-1389.

Volko, S.M., Boller, T., and Ausubel, F.M. (1998). Isolation of new Arabidopsis mutants with enhanced disease susceptibility to Pseudomonas syringae by direct screening. Genetics **149,** 537-548.

Wan, J., Zhang, X.C., Neece, D., Ramonell, K.M., Clough, S., Kim, S.Y., Stacey, M.G., and Stacey, G. (2008). A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in Arabidopsis. Plant Cell **20,** 471-481.

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., Li, X., Meisenhelder, J., Hunter, T., Yoshida, S., Asami, T., and Chory, J. (2005a). Autoregulation and homodimerization are involved in the activation of the plant steroid receptor BRI1. Dev Cell **8,** 855-865.

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, X., Goshe, M.B., Soderblom, E.J., Phinney, B.S., Kuchar, J.A., Li, J., Asami, T., Yoshida, S., Huber, S.C., and Clouse, S.D. (2005b). Identification and functional analysis of in vivo phosphorylation sites of the Arabidopsis BRASSINOSTEROID-INSENSITIVE1 receptor kinase. Plant Cell **17,** 1685-1703.

Wang, Y., Bao, Z., Zhu, Y., and Hua, J. (2009). Analysis of temperature modulation of plant defense against biotrophic microbes. Mol Plant Microbe Interact **22,** 498-506.

Wang, Y., Li, J., Hou, S., Wang, X., Li, Y., Ren, D., Chen, S., Tang, X., and Zhou, J.M. (2010). A Pseudomonas syringae ADP-ribosyltransferase inhibits Arabidopsis mitogen-activated protein kinase kinases. Plant Cell **22,** 2033-2044.

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., Seto, H., Fujioka, S., Yoshida, S., and Chory, J. (2001). BRI1 is a critical component of a plasma-membrane receptor for plant steroids. Nature **410,** 380-383.

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.

Warren, R.F., Henk, A., Mowery, P., Holub, E., and Innes, R.W. (1998). A mutation within the leucine-rich repeat domain of the Arabidopsis disease resistance gene RPS5 partially suppresses multiple bacterial and downy mildew resistance genes. Plant Cell **10,** 1439-1452.

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

Wildermuth, M.C., Dewdney, J., Wu, G., and Ausubel, F.M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defense. Nature **414,** 562-565.

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 Nurnberger, 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.

Wu, G., Wang, X., Li, X., Kamiya, Y., Otegui, M.S., and Chory, J. (2011). Methylation of a phosphatase specifies dephosphorylation and degradation of activated brassinosteroid receptors. Sci Signal **4,** ra29.

Xiang, T., Zong, N., Zhang, J., Chen, J., Chen, M., and Zhou, J.M. (2011). BAK1 is not a target of the Pseudomonas syringae effector AvrPto. Mol Plant Microbe Interact **24,** 100-107.

Xiang, T., Zong, N., Zou, Y., Wu, Y., Zhang, J., Xing, W., Li, Y., Tang, X., Zhu, L., Chai, J., and Zhou, J.M. (2008). Pseudomonas syringae effector AvrPto blocks innate immunity by targeting receptor kinases. Curr Biol **18,** 74-80.

Xu, W., Huang, J., Li, B., Li, J., and Wang, Y. (2008). Is kinase activity essential for biological functions of BRI1? Cell Res **18,** 472-478.

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.

Yamaguchi, Y., Huffaker, A., Bryan, A.C., Tax, F.E., and Ryan, C.A. (2010). PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in Arabidopsis. Plant Cell **22,** 508-522.

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.

Yang, S., Yang, H., Grisafi, P., Sanchatjate, S., Fink, G.R., Sun, Q., and Hua, J. (2006). The BON/CPN gene family represses cell death and promotes cell growth in Arabidopsis. Plant J **45,** 166-179.

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.

Yoshida, S., and Parniske, M. (2005). Regulation of plant symbiosis receptor kinase through serine and threonine phosphorylation. J Biol Chem **280,** 9203-9209.

Zhang, J., Shao, F., Li, Y., Cui, H., Chen, L., Li, H., Zou, Y., Long, C., Lan, L., Chai, J., Chen, S., Tang, X., and Zhou, J.M. (2007a). A Pseudomonas syringae effector inactivates MAPKs to suppress PAMP-induced immunity in plants. Cell Host Microbe **1,** 175-185.

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, W., Fraiture, M., Kolb, D., Loffelhardt, 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, X.C., Wu, X., Findley, S., Wan, J., Libault, M., Nguyen, H.T., Cannon, S.B., and Stacey, G. (2007b). Molecular evolution of lysin motif-type receptor-like kinases in plants. Plant Physiol **144,** 623-636.

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.

Zhu, Y., Qian, W., and Hua, J. (2010). Temperature modulates plant defense responses through NB-LRR proteins. PLoS Pathog **6,** e1000844.

Zipfel, C. (2008). Pattern-recognition receptors in plant innate immunity. Curr Opin Immunol **20,** 10-16.

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.

Zuo, J., Niu, Q.W., and Chua, N.H. (2000). Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J **24,** 265-273.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Delphine Chinchilla and Prof. Dr. Thomas Boller for the opportunity to complete my PhD studies at the Botanical Institute of the University of Basel. I'm grateful to Dr. Delphine Chinchilla, in whose laboratory I carried out my research project, for her support and advice, with respect to the planning of experiments and the writing of this thesis. Special thanks to Dr. Sebastian Bartels who helped me to improve my scientific writing and supported me to finish my PhD manuscript. In addition I would like to thank Prof. Dr. Jürg Felix for his dedication as co-referee for my thesis.

I wish to thank Dr. Ana Domínguez-Ferreras and Dr. Dagmar Hann for sharing data and their scientific knowledge with me and for all their support, suggestions and help over the past four years. I very much enjoyed our discussions in the lab and outside work hours. I would like to thank them also for reading and correcting the manuscript of this thesis.

I am also grateful to Dr. Sebastian Bartels, Dr. Franck Vazquez, Dr. Etienne Bucher and Dr. Pierre-Emanuel Courty for their ideas as well as their scientific and technical support.

It was a pleasure to work with all my former and present colleagues at the Botanical Institute with whom I always had a good time. I enjoyed the atmosphere, their friendship and support over the years. Furthermore, I thank Erika Roth and Maura Ellenberg for their kind and helpful assistance in administrative issues and Vaclav Mandak, Giacomo Busco, Kurt Ineichen, André Rzepecki and PD Dr. Jürg Oetiker for their support in technical and computer issues.

Finally I wish to express my utmost gratefulness to my family, especially my husband Thomas Heusser and our two children, Sarolt and Botond and my parents Ágnes Horváth and Csaba Kiss for the continuous encouragement, support and patience during my studies.

CURRICULUM VITAE

Personal details

Name: Márta Kiss-Papp
Date of birth: 13 June 1982
Nationality: Hungarian
Civil status: married
Children: two

Education

2008-2014 **PhD-studies in Plant Sciences**, Botanical Institute of the University of Basel, Switzerland
Focus: Molecular plant biology, plant immunology

2002-2008 **Master's degree of Biological Sciences,** Université Louis Pasteur, Strasbourg, France
Focus: Plant physiology, molecular and cell biology

2001-2002 **Certificate in French *language***, *Université Populaire Européenne, Strasbourg, France*

1997-2001 **High school diploma**, Berzsenyi Dániel Gimnázium, Budapest, Hungary
Main subjects: biology, chemistry, physic

Research experiences

09/2008-12/2013
PhD project: **The role of BAK1 in the regulation of plant innate immunity: functional and genetic characterization of BAK1 overexpression in *Arabidopsis thaliana*** (Section of Plant Physiology, Botanical Institute, Basel, Switzerland)
Supervisor: Dr. Delphine Chinchilla

10/2007-06/2008
Master's thesis: **Functional characterization of two genes, *CAS1* and *CPI* in the biosynthetic pathway of sterols in *Arabidopsis thaliana*** (Isoprenoids Department, Institut de Biologie Moleculaire des Plantes, CNRS, Strasbourg, France)
Supervisor: Dr. Huber Schaller

07/2007-08/2007
Genetic QTL mapping of a barley population for their drought stress resistance (Quantitative Genetics and Gene Mapping Group, Department of Plant Molecular Biology, Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, Hungary)
Supervisor: Dr. András F. Bálint

07/2006-08/2006
Study of transposable elements of *Saccharomyces cerevisiae* genome (Laboratoire Génétique Moléculaire Génomique Microbiologie, CNRS, Université de Strasbourg, Strasbourg, France)
Supervisor: Dr. Claudine Bleykasten-Grosshans

Academic experiences

2008-2012

Part-time teaching at the University of Basel. Practical courses in Plant Physiology (4th semester students), practical courses in Plant Molecular Biology (8th semester students).

Publications

Nekrasov, V., Li, J., Batoux, M., Roux, M., Chu, Z.H., Lacombe, S., Rougon, A., Bittel, P., Kiss-Papp, M., Chinchilla, D., van Esse, H.P., Jorda, L., Schwessinger, B., Nicaise, V., Thomma, B.P., Molina, A., Jones, J.D., Zipfel, C. (2009). Control of the pattern-recognition receptor EFR by an ER protein complex in plant immunity. Embo J **21**, 3428-3438.

Conference contributions

12th International Symposium on Plant Protein Phosphorylation, Tübingen, Germany
Poster: Kiss-Papp M., Dominguez-Ferréras A., Beeler S., Boller T., Chinchilla D. (2011). The role of BAK1 in the regulation of plant innate immunity

SWISSPLANT'11, Meiringen, Switzerland
Poster: Kiss-Papp M., Beeler S., Boller T., Chinchilla D. (2011). Overexpression of the receptor-like kinase BAK1 induces constitutive defense responses in Arabidopsis

PSC PhD Symposium 2010, Zürich, Switzerland
"Plants meeting future challenges – food and energy for the society in a changing environment"
Poster: Kiss-Papp M., Beeler S., Boller T., Chinchilla D. (2010). Overexpression of BRI1 - ASSOCIATED RECEPTOR KINASE 1 (BAK1) induces constitutive defense responses in Arabidopsis in absence of elicitors