Modulation of multiple BAK1-dependent signaling pathways by two atypical receptor-like kinases

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Table of content

1. Introduction .......................................................................................................................... 1
   1.1. Expansion of RLKs in the land plant kingdom ................................................................. 1
   1.2. Innate immunity in insects and mammals ....................................................................... 2
   1.3. PTI and ETI, the two layers of plant immunity .............................................................. 4
       1.3.1. Plant MAMPs and the defense activation ................................................................. 5
       1.3.2. MAMP perception systems in plants ....................................................................... 7
       1.3.3. Effector-triggered susceptibility or how to shut down host defense machineries to become pathogenic ...................................................................................... 9
       1.3.4. Effector-triggered Immunity and cell death control ................................................. 10
   1.4. BAK1, a multiple co-receptor involved in development and immunity .......................... 12
       1.4.1. BAK1 is the co-receptor of BRI1 ........................................................................... 13
       1.4.2. BAK1 has a BL-independent role in cell death control .......................................... 15
       1.4.3. BAK1, a central regulator of innate immunity ......................................................... 16
       1.4.4. BAK1 is a target of the AvrPto effector .................................................................. 17
       1.4.5. BIK1 differentially regulates BAK1-dependent pathways ....................................... 18
       1.4.6. Is BAK1 a component of crosstalks between receptors signaling? ......................... 19
       1.4.7. BIR1 and BON1 interact with BAK1 and negatively control cell death .................. 19
   1.5. Aim of the thesis ............................................................................................................. 20

2. Material and Methods ........................................................................................................... 21
   2.1. Material ........................................................................................................................... 21
       2.1.1. Chemicals ............................................................................................................... 21
       2.1.2. Media ..................................................................................................................... 21
       2.1.3. Antibiotics ............................................................................................................. 21
       2.1.4. Bacterial strains .................................................................................................... 22
       2.1.5. Primers .................................................................................................................. 22
       2.1.6. T-DNA insertion lines ........................................................................................... 22
       2.1.7. Antibodies ............................................................................................................ 23
   2.2. Plant material and method .............................................................................................. 23
       2.2.1. Plant growth condition ........................................................................................... 23
       2.2.2. Transient transformation of *N. benthamiana* by *Agrobacterium tumefaciens* ....... 24
       2.2.3. Stable transformation of *Arabidopsis thaliana* by *Agrobacterium tumefaciens* ... 24
       2.2.4. Generation of knockdown lines for *BIR2* ............................................................... 24
2.3. Bacterial transformation methods ............................................................ 25
  2.3.1. Transformation of E. coli DH5α ......................................................... 25
  2.3.2. Transformation of Agrobacterium tumefaciens .................................... 25

2.4. DNA analysis ............................................................................................... 25
  2.4.1. Bacterial plasmid extraction ............................................................... 25
  2.4.2. Plant genomic DNA extraction ............................................................ 26
  2.4.3. Restriction enzyme digestion of DNA ................................................ 26
  2.4.4. Polymerase Chain Reaction (PCR) ..................................................... 26
  2.4.5. DNA agarose gel electrophoresis ........................................................ 27
  2.4.6. Purification of DNA fragment from agarose sample ......................... 27
  2.4.7. Gateway BP reaction ......................................................................... 27
  2.4.8. Gateway LR reaction ......................................................................... 27
  2.4.9. DNA sequencing .................................................................................. 28
  2.4.10. Quantitative Real-time PCR .............................................................. 28

2.5. RNA analysis ............................................................................................... 28
  2.5.1. RNA extraction .................................................................................... 28
  2.5.2. DNAse treatment of RNA ................................................................. 29
  2.5.3. Reverse transcription .......................................................................... 29

2.6. Protein analysis ............................................................................................ 29
  2.6.1. Extraction of proteins from plant material ........................................... 29
  2.6.2. Protein concentration measurement .................................................... 29
  2.6.3. SDS-PAGE .......................................................................................... 30
  2.6.4. Western blot ......................................................................................... 30
  2.6.5. Coomassie Brilliant Blue staining ...................................................... 30
  2.6.6. Co-immunoprecipitation experiment .................................................. 30
  2.6.7. Immunoprecipitation of BIR2 followed by MS analysis ..................... 31

2.7. Phenotypical analysis .................................................................................. 31
  2.7.1. Seeds sterilization ................................................................................. 31
  2.7.2. Hypocotyl length assay ....................................................................... 31
  2.7.3. MAMP-induced growth inhibition ..................................................... 31
  2.7.4. Oxidative burst .................................................................................... 32
  2.7.5. MAMP-induced transcriptional changes ............................................. 32
  2.7.6. Infection with Alternaria brassicicola .................................................. 32
  2.7.7. Trypan blue staining ........................................................................... 32
  2.7.8. Callose deposition measurement ........................................................ 33
  2.7.9. Hormone measurement ...................................................................... 33
2.8. Microscopy ......................................................................................... 34
  2.8.1. Light and epifluorescence microscopy .......................................... 34
  2.8.2. Confocal microscopy .................................................................... 34
  2.8.3. FRET-FLIM experiment ................................................................. 34
  2.8.4. Split-YFP experiment ................................................................... 35
2.9. Statistical analysis ............................................................................... 35

3. Results ................................................................................................... 36
  3.1. The BIR family, a new family of BAK1 interactors .............................. 36
    3.1.1. Identification of BIR proteins as potential BAK1 interactors ........... 36
    3.1.2. Interaction analysis by Co-IP in N. benthamiana ............................ 37
    3.1.3. Interaction of BIR proteins with BAK1 in BiFC experiment .......... 38
    3.1.4. BIR family interacts with BAK1 in FRET-FLIM experiment .......... 39
    3.1.5. BIR2, BIR3 and BIR4 interact with BAK1 kinase domain in Y2H .... 40
    3.1.6. BIR proteins interact also with SERK1 ......................................... 41
    3.1.7. The BIR protein family, a family of atypical receptor-like kinases ... 42
    3.1.8. Phylogenetic study of the BIR family .......................................... 45
    3.1.9. Expression pattern of BIRs upon biotic pathogen treatments .......... 46
  3.2. The BAK1-interacting receptor 2 (BIR2) .............................................. 48
    3.2.1. Production of a BIR2 antibody .................................................... 48
    3.2.2. Phenotypical analysis of bir2 mutants and amiR-BIR2 lines ............ 48
    3.2.3. BIR2 localizes to the plasma membrane ..................................... 51
    3.2.4. Complementation of bir2-1 and bir2-2 mutants ............................. 63
    3.2.5. BIR2 overexpression negatively affects BL and elf18 signaling ..... 65
    3.2.6. BIR2 influences BAK1-dependent complex formation ................ 67
    3.2.7. Does BIR2 influence the activity/phosphorylation level of BAK1? .... 70
    3.2.8. Identification of potential BIR2 interactors by co-immunoprecipitation followed by mass spectrometry analysis ........................................ 71
  3.3. The BAK1-Interacting Receptor 3 ...................................................... 74
    3.3.1. Localization of BIR3 ................................................................... 74
    3.3.2. Phenotypical analysis of BIR3-deficient T-DNA lines .................... 75
    3.3.3. Phenotypical analysis of BIR3 overexpressing lines ..................... 78
    3.3.4. BIR3 heteromerizes with BIR2 and BIR1 in N. benthamiana ........ 84

4. Discussion .............................................................................................. 86
  4.1. BIRs interact constitutively with BAK1 at the plasma membrane ....... 86
4.2. BIR2 and BIR3 function as pseudokinases ......................................................... 87
4.3. Differential regulation of BIRs gene expression .................................................. 88
4.4. Knocking out BIR2 differentially affects BAK1-related pathways ................. 89
4.5. BIR2 negatively controls MAMP signaling directly at the BAK1-FLS2 complex formation ................................................................................................................ 90
4.6. BIR2 negatively regulates BAK1 at basal state ................................................... 91
4.7. Cell death controlled by BIR2 and BAK1 .............................................................. 93
4.8. Are there more components in the BAK1-BIR2-FLS2 regulon? ...... 95
4.9. BIR3, another negative regulator of BAK1 ........................................................ 96
4.10. Do BIR2 and BIR3 act together? ...................................................................... 97
4.11. Ancestral function of BAK1 and BIRs ................................................................. 98
4.12. Conclusion ........................................................................................................... 99

5. Summary .................................................................................................................. 100
6. Zusammenfassung .................................................................................................. 101

7. References ............................................................................................................... 102

8. Appendix .................................................................................................................. 132
8.1. Abbreviations ....................................................................................................... 132
8.2. Primers list ............................................................................................................ 134
List of figures

Figure 1-1. Quantitative responses in plant defense follow a zigzag model. Adapted from (Jones and Dangl, 2006) .............................................................................................................5
Figure 3-1. LC-ESI-MS/MS results of BAK1-GFP co-immunoprecipitated proteins ..........37
Figure 3-2. BIR family members interact with BAK1 in Co-IP experiments .......................38
Figure 3-3. BIR family members interact with BAK1 in BiFC experiments in N. benthamiana. ........................................................................................................................................39
Figure 3-4. BIR family members interact with BAK1 in FRET-FLIM experiment ..............40
Figure 3-5. BIR proteins also interact with SERK1 ............................................................41
Figure 3-6. BIR2, BIR3 and BIR4 present atypical kinase domains .................................44
Figure 3-7. Phylogenetic tree of Arabidopsis BIR proteins family and their related RLKs in different species ..............................................................................................................46
Figure 3-8. Relative expression of BIR1 to 4 (Microarray data from AtGenExpress). ........47
Figure 3-9. Characterization of amiR-BIR2 lines ..............................................................50
Figure 3-10. Subcellular localization of the BIR2-YFP fusion protein ...............................51
Figure 3-11. BIR2-deficient Col-0 lines are hyperresponsive to elf18 ...............................53
Figure 3-12. BIR2-deficient Ws-0 lines are hyperresponsive to elf18 ...............................54
Figure 3-13. BIR2-deficient lines are hyperresponsive to flg22 ........................................55
Figure 3-14. BIR2-deficient lines are hyperresponsive to elf18 ........................................57
Figure 3-15. bir2 mutants show enhanced Alternaria brassicicola-induced cell death ........59
Figure 3-16. bir2 mutant show stronger defense responses against Pto DC3000 ..........59
Figure 3-17. bir2 and amiR-BIR2 lines restrict Pto DC3000 growth ...............................60
Figure 3-18. Knockout and knockdown of BIR2 has no significant impact on BL responses ..................................................................................................................................62
Figure 3-19. Expression of the coding region of BIR2 under its own promoter fully complements all observed phenotypes ...............................................................................64
Figure 3-20. BIR2 overexpression negatively affects BL and elf18 signaling ....................66
Figure 3-21. BAK1 dissociates from BIR2 upon several ligands treatments ....................68
Figure 3-22. BIR2 controls BAK1-FLS2 interaction levels independently of SA ............70
Figure 3-23. BIR2 knockdown doesn’t affect BAK1 phosphorylation state .......................71
Figure 3-24. IP followed by LC-MS/MS analysis led to the identification of BIR2-interacting candidates ..................................................................................................................73
Figure 3-25. Subcellular localization of the BIR3-YFP fusion protein ...............................74
Figure 3-26. bir3 mutant lines show decreased BIR3 transcripts ....................................75
Figure 3-27. BIR3 has no significant impact on BL responses ........................................76
Figure 3-28. bir3 mutants are hypersensitive to MAMP treatment .................................77
Figure 3-29. bir3 mutation have no effect on Alternaria-induced cell death ....................78
Figure 3-30. Morphological phenotype of 35-BIR3 lines .................................................79
List of figures and tables

Figure 3-31. BIR3 overexpression compromises BL signaling ..................................................80
Figure 3-32. BIR3 overexpression leads to elf18 insensitivity .....................................................81
Figure 3-33. BIR3 overexpression suppresses cell death control .................................................82
Figure 3-34. Overexpression of BIR3 leads to strong reduction in BAK1-FLS2 interaction
        after flg22 treatment .............................................................................................................83
Figure 3-35. BIR1, BIR2 and BIR3 can heteromerize in vivo .......................................................85
Figure 4-1. Schematic representation of BIR2 regulation ............................................................93

List of tables

Table 1. Media used in this study ....................................................................................................21
Table 2. Antibiotics used in this study ..........................................................................................22
1. Introduction

Plants, unlike animals, are sessile organisms and cannot escape from environmental threats. Detection of biotic signals and production of a proper immune response is absolutely critical to survive. Plant immunity, similarly to what is observed in the animal kingdom, relies on the detection of conserved microbial structures called microbe-associated molecular patterns (MAMPs) that are recognized by pattern recognition receptors (PRRs). Several plant PRRs have been identified so far and they mostly belong to the receptor-like kinases (RLK) subfamily, a family of receptors that expanded specifically in the plant kingdom. The best-studied case of MAMP recognition is the perception of flagellin by the leucine-rich repeat (LRR)-RLK flagellin-sensing 2 (FLS2). To fully respond to flagellin perception, FLS2 interacts with Brassinosteroid-insensitive 1 (BRI1)-associated kinase 1 (BAK1), a LRR-RLK with a small extracellular domain. BAK1 is a general regulator of RLKs and a central part of the plant defense machinery but serves also additional roles in plant development by interacting with a number of different ligand-binding RLKs. It was first identified as co-receptor of BRI1. Interestingly, besides its crucial role in MAMP and BL signaling, BAK1 also functions as a positive regulator in cell death containment. As fine-tuning of the responses is necessary to balance the trade-off between growth and defense, the mechanisms how the multifunctional BAK1 co-receptor regulates and is regulated to define specificity between several signals and pathways have become a crucial question in the last years.

1.1. Expansion of RLKs in the land plant kingdom

In plants, the two most common detection systems exists comprising receptor histidine (His) kinases and receptor-like serine (Ser)/threonin (Thr) kinases (RLKs). RLKs form a large monophyletic family related to the Drosophila Pelle and the human IRAK (interleukin-1 receptor-associated kinase) family. In Arabidopsis, around 400 RLKs present a similar organization as animal receptor tyrosine kinases (RTKs) with a variable extracellular domain, a transmembrane domain and an intracellular Ser/Thr kinase domain while the other members of the family lacking extracellular and transmembrane domains are named receptor-like
cytoplasmic kinases (RLCK). Plants present a huge amount of RLKs compared to the other kingdom and it is hypothesized that this large expansion in the number of RLKs occurred while plant adapted to terrestrial life, most likely through extensive genome and gene duplication (Shiu and Bleecker, 2001a; Shiu and Bleecker, 2003). Furthermore, plants also contain receptor-like proteins (RLPs) lacking intracellular kinase domain. RLKs and RLPs present a big diversity in the extracellular domains exhibiting a variety of motifs including LRR, similar to TLRs, forming the largest subfamily, lysine motif (LysM), lectin domain and many others, suggesting that they can perceive a wide range of extracellular signals (Shiu and Bleecker, 2001b; Shiu and Bleecker, 2003). An increasing amount of RLKs, especially from the LRR-RLK subfamily, have been characterized acting in a range of processes generally through ligand binding. *Arabidopsis* brassinosteroid-insensitive 1 (BRI1) (He et al., 2000; Wang et al., 2001), HAESA (Jinn et al., 2000; Kumpf et al., 2013) and CLAVATA1 (CLV1) (Clark et al., 1993; Clark et al., 1997) regulate developmental processes such as brassinolide perception, floral organ abscission and meristem differentiation respectively. S-receptor kinase (SRK) from *Brassicaceae* is involved in self-incompatibility (Stein et al., 1991b; Goring and Rothstein, 1992; Stein and Nasrallah, 1993). Some are involved in plant-microorganism symbiosis such as *Medicago* nodulation receptor kinase (NORK) (Endre et al., 2002), and *Lotus* SYMRK (Stracke et al., 2002) that after cleavage of its malectin-like domain (MLD) forms a complex with Nod factor receptor 5 (NFR5) (Antolin-Llovera et al., 2014) and. Interestingly, an increasing amount of receptors appears to be involved in defense signaling like Xa21 from rice (Song et al., 1995) or FLS2 (Felix et al., 1999; Gómez-Gómez et al., 1999; Gómez-Gómez and Boller, 2000; Gomez-Gomez and Boller, 2002) suggesting that the huge number of RLKs in plants provided an expansion in the defense-related receptors possibly to compensate the lack of adaptive immunity.

1.2. **Innate immunity in insects and mammals**

Living organisms are constantly confronting parasitic infections. To face them, higher organisms evolved several strategies referred to as the immune system. A prerequisite for defense is the recognition of the invading parasite followed by the activation of a signal transduction finally leading to effective defense responses
that will stop the propagation of the parasite. In vertebrates, two types of immunity protect the host against infections named innate and adaptive immunity. The adaptive immune system relies on T- and B-lymphocytes. They both use highly specific antigen receptors like immunoglobulin or T-cell receptors that are de novo produced in each organism. This immune system is therefore highly specific. The concept of innate immunity in animals only arose in the late 80’s with Charles Janeway’s ground-breaking reflexions (Janeway, 1989). In contrast to adaptive immunity, the innate immune system detects conserved invariant patterns found in most of the organisms of a given class, referred as PAMP or MAMP, through receptors that are found in the germplasm, named PRRs (Medzhitov and Janeway, 1998b; Medzhitov and Janeway, 2000). Innate immunity provides a basal resistance but in the same time is also necessary to initiate adaptive immunity. Indeed, adaptive immunity only takes place when the pathogen has first been detected by a PRR (Medzhitov et al., 1997; Medzhitov and Janeway, 1998a; Medzhitov, 2007).

While several classes of PRR exist in animals, the most studied and the first class of PRRs to be identified is the one containing the *Drosophila* Toll and the human Toll-like receptors (TLRs) (Lemaitre et al., 1996; Medzhitov et al., 1997). The Toll receptor was identified in a mutant screen as a key component in dorso-ventral differentiation in the early *Drosophila* embryo (Anderson et al., 1985a; Anderson et al., 1985b; Hashimoto et al., 1988) through the perception of the extracellular processed peptide signal Spätzle (Stein et al., 1991a; Morisato and Anderson, 1994; Schneider et al., 1994). In adult *Drosophila*, upon perception of the cytokine Spätzle, Toll activates antifungal response by controlling the production of the antifungal peptide Drosomycin (Fehlbaum et al., 1994; Lemaitre et al., 1996). Humans encode eleven functional TLRs (TLR1-11) that mediate inflammatory responses mainly through the induction of NF-κB family after detection of a large set of MAMPs. Localized at the plasma membrane, TLR1, TLR2, TLR4, TLR5 and TLR6 recognize several lipoproteins, lipopolysaccharides or flagellin (Poltorak et al., 1998; Chow et al., 1999; Lien et al., 1999; Schwandner et al., 1999; Ozinsky et al., 2000; Poltorak et al., 2000; Hayashi et al., 2001; Buwitt-Beckmann et al., 2006) while TLR3, TLR7, TLR8 and TLR9 act in the endocytic compartment to recognize nucleic acid MAMPs from bacteria and viruses like
single-stranded (ss) or double-stranded (ds) RNA and unmethylated CpG-DNA (Hemmi et al., 2000; Alexopoulou et al., 2001; Bauer et al., 2001; Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004). Furthermore, TLR11 and TLR13 from mice recognize profilin-like molecule from \textit{Toxoplasma gondii} and a conserved 23S ribosomal sequence respectively (Yarovinsky et al., 2005; Oldenburg et al., 2012).

Toll and TLRs consist of an extracellular LRR domain, a transmembrane domain and an intracellular Toll-Interleukin 1 (TIR) domain. In \textit{Drosophila}, Toll interacts with the adaptor \textit{DmMyD88} (Myeloid differentiation primary response gene 88) to transduce the signal through Tube/Pelle, Cactus and Dorsal. In mammals, TLRs interact with several adaptors, as for example Myd88, and the signaling is transduced through Interleukin-1 receptor-associated kinases (IRAK), IκB and NFκB (Medzhitov et al., 1998; Chtarbanova and Imler, 2011). In an evolutionary independent manner, plants have evolved similar detection systems consisting of an extracellular LRR domain and an intracellular kinase domain.

1.3. PTI and ETI, the two layers of plant immunity

Pathogenic and non-pathogenic microbes are constantly challenging plants that must recognize the intruders and activate a battery of defense responses in order to stop their propagation. While animals use innate immunity as well as adaptive immunity, plants only rely on innate immunity to achieve efficient disease resistance. Nevertheless, plants have evolved two distinct ways of pathogen recognition and defense response activation. The first layer of immunity, PTI, also called basal or horizontal immunity depends on the recognition of conserved microbial molecular patterns by PRRs (Boller and Felix, 2009; Zipfel, 2009; Zipfel and Robatzek, 2010; Schwessinger and Ronald, 2012). This recognition activates a battery of defense responses usually sufficient to defend against non-adapted microbes. Arms race brought microbes to evolve the ability of transferring effectors in the host cell and to become pathogenic by shutting down PTI (Feng and Zhou, 2012; Wawra et al., 2012). Co-evolution pressures led to the detection of effectors used by microorganism to shut down PTI by intracellular plant receptors called (NB-LRRs) or R proteins. This mechanism, called ETI or R gene-mediated resistance, relies on a highly specific direct or indirect recognition of an effector
by an R protein in a gene-for-gene specific manner (Maekawa et al., 2011; Bonardi et al., 2012; Bonardi and Dangl, 2012). ETI leads to stronger defense responses, than MTI, often accompanied by cell death formation. It can be illustrated by the zigzag model proposed by Jones and Dangl (2006).

Figure 1-1. Quantitative responses in plant defense follow a zigzag model. Adapted from (Jones and Dangl, 2006)

First, plants detect microbe-associated molecular patterns (MAMPs, red diamonds) via PRRs triggering MAMP-triggered immunity (MTI). Successful pathogens produce and secrete into the plant cell effectors that interfere with MTI enabling pathogen nutrition and dispersal. This phase is called effector-triggered susceptibility (ETS). In the next phase, an effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI), an amplified version of MTI. ETI often reaches the threshold of HR induction. As an ongoing arm race, pathogen isolates are selected for loss of the red effector. They gained new effectors (in blue) that can help pathogens to suppress ETI. Selection favours new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI.

1.3.1. Plant MAMPs and the defense activation

Plants developed systems to sense the presence of microbes such as bacteria, fungi or oomycetes at the plasma membrane. Similar to innate immunity in animals, this first layer of immunity, named PTI, also called basal or horizontal
immunity depends on the recognition of MAMPs by PRRs. Well-studied MAMPs detected by plants include proteinaceous flagellin, EF-Tu, the bacterial cold shock protein (Felix and Boller, 2003), oomycete transglutaminase (Brunner et al., 2002), necrosis- and ethylene-inducing peptide 1 (Nep1)-like proteins (Qutob et al., 2006), harpin (El-Maarouf et al., 2001), the enigmatic MAMP activity from Xanthomonads (eMAX) from *Xanthomonas* (Jehle et al., 2013), SCLEROTINIA CULTURE FILTRATE ELICITOR1 (SCFE1) (Zhang et al., 2013b), fungal polygalacturonases (D’Ovidio et al., 2004; Zhang et al., 2013a), the ethylene-induced xylanase from the saprophytic ascomycete *Trichoderma viride* (Bailey et al., 1990; Avni et al., 1994; Rotblat et al., 2002; Boller and Felix, 2009), polysaccharidic MAMPs like lipooligosaccharides (LPS) (Silipo et al., 2005), peptidoglycan (PGN) (Gust et al., 2007), chitin (Felix et al., 1993) and its derivate chitosan (Bohland et al., 1997), and lipophilic MAMPs as for example arachidonic acid (Tjamos and Kucacute, 1982) and ergosterol (Granado et al., 1995; Cardinale et al., 2000). Endogenous plant molecules released upon catabolic action of the microbes, named damage- or danger-associated molecular patterns (DAMPs), can also be sensed. They include cutin monomers (Kauss et al., 1999), cell wall oligosaccharides (Denoux et al., 2008) and the 23-aa Atpep1 peptide (Huffaker et al., 2006). M/DAMPs perception triggers a standard set of defense responses regardless of the type of microorganism detected and are usually sufficient to defend against non-adapted microbes. M/DAMP detection triggers opening of ion channels that enable H⁺ and Ca²⁺ influx as well as K⁺, Cl⁻ and NO₃⁻ efflux leading to membrane depolarization and increase of extracellular pH (Blume et al., 2000; El-Maarouf et al., 2001; Boller and Felix, 2009). Ca²⁺ influx from the apoplast to the cytoplasm leads to activation of calcium-dependant proteins such as Ca²⁺-dependant protein kinases (CDPKs) (Boudsocq et al., 2010). Reactive-oxygen species (ROS), ethylene and nitric oxide (NO) production (Apel and Hirt, 2004; Asai et al., 2008; Yoshioka et al., 2009) are also characteristic of PTI responses. These responses are followed by rapid changes in protein phosphorylation necessary for a proper signal transduction (Felix et al., 1991). Indeed, M/DAMP triggers the activation of the mitogen-activated protein (MAP) kinase (MPK) MPK3, MPK4 and MPK6 (Asai et al., 2002; Wan et al., 2004; Pitzschke et al., 2009). It leads to the activation of some of the largest classes of plant transcription factors
(TFs), the WRKYs (Eulgem and Somssich, 2007) followed by strong transcriptional reprogramming (Zhang et al., 2002; Navarro et al., 2004; Boudsocq et al., 2010). Furthermore, application of MAMPs in efficient concentration leads to growth inhibition exemplifying a shift of the balance from growth to defense. Together with genetic tools, most of these responses were and are still used to identify and characterize M/DAMP as well as their receptors.

1.3.2. MAMP perception systems in plants

Flagellin, the proteinaceous subunit that builds the long filament of the bacterial flagellum, is the most-studied plant MAMP (Gomez-Gomez and Boller, 2002; Boller and Felix, 2009). Interestingly, animals also sense flagellin, via TLR5 (Hayashi et al., 2001), but through a different epitope suggesting that plant and animals evolved the same perception system via convergent evolutionary mechanisms (Smith et al., 2003). In plants, the most conserved epitope of flagellin act as the active MAMP (Felix et al., 1999). This 22-amino acid peptide called flg22 is sufficient to trigger defense response at subnanomolar concentrations. Some bacterial species either pathogenic or symbiotic present mutations in this peptide showing that this epitope was under selective pressure to evade their perception by plants (Felix et al., 1999). The 28-LRR repeats RLK flagellin-sensing 2 (FLS2) has been identified as receptor for flagellin perception in a forward genetic screen using seedling growth inhibition upon flg22 as a read out (Gómez-Gómez et al., 1999; Gómez-Gómez and Boller, 2000; Zipfel et al., 2004). Binding of radioactive flg22 peptide to the receptor confirmed FLS2 as the true flg22 receptor (Chinchilla et al., 2006). Another LRR-RLK, named EFR, belonging to the same subfamily as FLS2 was found to activate defense responses upon perception of the abundant cytoplasmic MAMP Elongation Factor Tu (EF-Tu) and its active epitope elf18 in the Brassicaceae genus (Kunze et al., 2004; Zipfel et al., 2006). Interestingly, rice can detect a different epitope from EF-Tu, called EFa50 (Furukawa et al., 2014), suggesting that Brassicaceae and rice evolutionary converged to detect the same protein as a MAMP (Furukawa et al., 2014).

Several MAMPs are perceived through receptor-like proteins (RLPs). The tomato LRR-RLP LeEIX1 and LeEIX2 bind Trichoderma cell wall-derived xylanase (Bailey et al., 1990; Ron and Avni, 2004) with LeEIX1 acting in the intracellular
signaling. Furthermore, *Arabidopsis* ReMAX/RLP1 (Jehle et al., 2013), responsiveness to Botrytis polygalacturonase-1 (RBPG1)/RLP42 (Zhang et al., 2013a) and RLP30 (Zhang et al., 2013b) perceive eMAX (Jehle et al., 2013), fungal endopolygalacturonases (Zhang et al., 2013a) and SCFE1 (Zhang et al., 2013b) respectively. Some RLPs are also considered as race-specific receptors and detect the Avr products of avirulence genes. For example, Verticilium1 (Ve1) detects the fungal Avirulence on Verticilium 1 tomato (Ave1) (de Jonge et al., 2012). Recent studies on SCFE1 show that responses to SCFE1 were completely impaired not only in *rlp30* mutants but also in *suppressor of bir1 (sobir1)/evershed (evr)* mutants (Zhang et al., 2013b). Secondly, RLP30 interacts directly with SOBIR1 and transient expression of RLP30 only confers sensitivity to SCFE1 when co expressed with SOBIR1 (Zhang et al., 2013b). Furthermore, SOBIR1 is necessary for Ve1-mediated resistance and acts by stabilizing Ve1 proteins in vivo (Liebrand et al., 2013b). Strikingly, SOBIR1/LRR-RLP interactions are ligand-independent and constitutive. SOBIR1 acts as a scaffold for RLPs as well as coreceptor to transduce the signal into the cytoplasm (Liebrand et al., 2013a).

Plants also detect polysaccharidic MAMPs from fungal cell walls and from the bacterial cell envelope via LysM motifs (Gust et al., 2012). Interestingly, these motifs are only found in the extracellular domain of plant RLPs and RLKs but are not present in animals. The LysM motif RLK CERK1 has been identified as the receptor for fungal chitin (Miya et al., 2007). In rice, it is thought to act together with the LysM containing protein CEBiP (Kaku et al., 2006) while homologues of CEBiP in *Arabidopsis* are not involved in chitin perception. Another LysM-RLK closely related to CERK1, LYK4, plays a positive role in chitin perception and in resistance to biotrophic and necrotrophic pathogens (Wan et al., 2012). Furthermore, perception of peptidoglycan (Gust et al., 2007) occurs through a tripartite module consisting of CERK1 together with two LysM-domain and GPI (glycosylphosphatidylinositol) -anchored proteins LYM1 and LYM3 (Willmann et al., 2011).

Genetic engineering with PRR might be a potent strategy for plant improvement. Indeed, tobacco, that lacks EFR become more resistant to *Ralstonia solanacearum* when exogenous EFR is expressed confirming that interspecies transfer of PRR might increase plant disease resistance (Lacombe et al., 2010).
1.3.3. **Effector-triggered susceptibility or how to shut down host defense machineries to become pathogenic**

To become pathogenic and enable parasitism, microorganisms produce effectors that are secreted in the apoplast or directly inside the host cell (Bozkurt et al., 2012; Feng and Zhou, 2012). Every pathogen secretes a collection of effectors that generally act by modifying plant physiology in order to favour growth and spread of the pathogen. These changes in favour of the pathogen are collectively called effector-triggered susceptibility and work through simultaneous perturbation of several host processes. Similarly to RNA viruses using suppressors of silencing, like P19 from the Tomato Bushy Stunt Virus (TBSV) trapping virus derived siRNA (Lakatos et al., 2004), to circumvent the plant RNA silencing machinery (Voinnet, 2005), bacteria, fungi and oomycetes secrete effectors to shut down PTI (Feng and Zhou, 2012). They can work upstream of MAMP recognition, target PRRs directly and/or act downstream of PRR to block signalisation events (He et al., 2006; Shan et al., 2008; Feng and Zhou, 2012). For example, the two LysM effectors ECP6 and Slp1, from *Cladosporium fulvum* and *Magnaporthe oryzae* respectively, compete with CERK1 for chitin binding to avoid fungal recognition (de Jonge et al., 2010; Mentlak et al., 2012). The C-terminus of AvrPtoB from *Pseudomonas syringae*, sharing homologies with U-box E3 ubiquitin ligases, ubiquitinates FLS2 and CERK1 to facilitate their degradation (Gohre et al., 2008; Gimenez-Ibanez et al., 2009). The *P. syringae* Type III secretion system (T3SS) HopAI1 and HopF2 both act at the level of MAPK cascade signaling. While HopAI1 dephosphorylates MAP kinases MPK3 and MPK6 preventing their rephosphorylation (Zhang et al., 2007), HopF2 inhibits MAP kinase kinase 5 (M KK5) through its ADP-ribosyltransferase activity (Wang et al., 2010). Effectors can also highjack specific cellular plant machineries. For example, HopT1-1 suppresses the slicing activity of Argonaute1 (AGO1) resulting in shutting off the miRNA pathway to promote bacterial growth (Navarro et al., 2008). TAL effectors from *Xanthomonas* spp. and *Ralstonia solanacearum* mimic eukaryotic transcription factors (TFs) by coded repeats targeting specific sequences of the host promoter (Boch et al., 2009; Moscou and Bogdanove, 2009; Scholze and Boch, 2011). Taken together, the plethora of pathogenic effectors provides an impressive in-
sight on how co-evolution let prokaryotes adapt to eukaryotic defense mecha-
nisms.

1.3.4. **Effector-triggered Immunity and cell death control**

As counteraction to ETS, plants evolved the ability to detect secreted effectors through intracellular resistance (R) proteins (Flor, 1956; Doubly et al., 1960). Most R proteins encode nucleotide-binding LRR (NB-LRR) also called NLR because of their similarities to the animal NOD-like receptor family (Meyers et al., 1999; Maekawa et al., 2012). TIR-NB-LRR and coiled-coil (CC)-NB-LRR proteins represent the two main families of NLRs. Their activation leads to a strong defense response called Hypersensitive Response (HR) (Beers and McDowell, 2001) generally coming along with SA accumulation, gene expression reprograming and localized cell death at the site of infection limiting propagation of pathogens that are following a biotrophic lifestyle. Interestingly, defense responses largely overlap between ETI and PTI in terms of genes involved but the differences might appear more at the amplitude and timing of upregulation (Navarro et al., 2004). Perception of effectors by NLRs can be direct but mostly appears as an indirect phenomenon. In the case of direct perception, activation of NLRs occurs upon direct binding of the effector to the LRR part of the NLR, like in the recognition of AVRa10 by mildew resistance locus A 10 (MLA10) in barley (Ellis et al., 2007). Indirect recognition can be explained by the „guard model” when modifications on a host target caused by an effector can be sensed (Jones and Dangl, 2006; van der Hoorn and Kamoun, 2008). For example, the two membrane-localized CC-NB-LRRs resistance to Pseudomonas syringae pv. maculicola 1 (RPM1) and Resistance to P. syringae 2 (RPS2) recognize either phosphorylation or cleavage of the host protein RPM1-interacting protein 4 (RIN4) by the bacterial effectors AvrRpm1 and the cysteine protease AvrRpt2 respectively. Recognition of RIN4 modification leads to effective defense activation (Leister et al., 1996; Mackey et al., 2002; Axtell et al., 2003; Axtell and Staskawicz, 2003; Mackey et al., 2003; Belkhadir et al., 2004). In both direct and indirect perception, activation of NLRs occurs through conformational changes and ADP to ATP exchange (Takken and Goverse, 2012). While the identification of downstream signaling components has been mostly unsuccessful likely due to
redundancy in the signaling pathways, it is well documented that Enhanced Disease Susceptibility 1 (EDS1) is necessary for TIR-NB-LRRs signaling while CC-NB-LRRs rely on Non-Race Specific Disease Resistance 1 (NDR1) (Parker et al., 1996; Aarts et al., 1998; Day et al., 2006).

Certain gain-of-function mutations leading to constitutive activation of NLRs such as in *suppressor of npr1-5-based salicylic acid insensitivity 4 (ssi4)* (Shirano et al., 2002), *sensitive to low humidity 1 (slh1)* (Noutoshi et al., 2005), *resistance to Potato Virux X (Rx)* (Bendahmane et al., 2002) and *uni-1D* (Igari et al., 2008) show constitutive cell death formation and higher disease resistance. Furthermore, knockdown lines for *RIN4* present typical high SA contents, *PR1* expression and a typical dwarfism while the *rin4* knockout line is embryonic lethal (Belkhdar et al., 2004). These phenotypes are completely reverted in the *rin4rps2rpm1* triple mutant suggesting that loss of *RIN4* leads to strong *RPM1* or *RPS2*-dependent defense response activation in the absence of pathogens (Belkhdar et al., 2004). Knockout mutants for *BONZAI1 (BON1)*, a gene required for growth homeostasis at varying temperatures, shows temperature sensitive lesion mimicking resulting in stunted growth and constitutive defense responses at 22°C, similar to the *rin4* knockdown line, but not affected anymore at 28°C (Hua et al., 2001; Yang et al., 2006; Lee and McNellis, 2009). The TIR-NB-LRR *SNC1 (suppressor of npr1-1, constitutive 1)* is responsible for the 22°C phenotype of *bon1-1* as mutation in *SNC1* revert the *bon1* mutant phenotype (Yang and Hua, 2004; Li et al., 2007; Wang et al., 2011). Interestingly, BON1 controls *SNC1* gene expression levels but it has not been demonstrated yet if *SNC1* is guarding BON1 (Yang and Hua, 2004; Li et al., 2007). Several other mutants in genes that are not R genes have been shown to present gradual phenotypes including dwarfism and runaway cell death phenotypes like *mitogen-activated protein kinase 4 (mpk4)* (Petersen et al., 2000), *mapk/erk kinase kinase 1 (mekk1)* (Ichimura et al., 2006; Nakagami et al., 2006), *mitogen-activated protein kinase mkk 1 (mkk1)mkk2* (Gao et al., 2008), *suppressor of SA insensitive 1 (ssi1)* (Shah et al., 1999), *ssi2* (Shah et al., 2001), *constitutively activated cell death1 (cad1)* (Morita-Yamamuro et al., 2005), *lesions simulating disease resistance response (lsd)* mutants (Dietrich et al., 1994), *constitutive expression of PR (cpr)* mutants (Clarke et al., 2000), *the accelerated cell death (acd)* mutants...
(Greenberg and Ausubel, 1993; Greenberg et al., 1994). Recently, a genetic study shed light on the cell death mechanism occurring in the MEKK1-MKK1/MKK2-MPK4 signaling cascade (Gao et al., 2008; Kong et al., 2012). This MAPK pathway negatively regulates MEKK2 that activates SUMM2, a CC-NB-LRR, when MPK4 downregulation is lost (Kong et al., 2012; Zhang et al., 2012; Su et al., 2013). Furthermore, inactivation of MPK4 by the HopAI effector leads to SUMM2-mediated immunity (Zhang et al., 2012). We can generally hypothesize that some of the genes are guarded in a similar way as shown for RIN4 or MPK4 and some negatively control cell death through downstream components of R gene.

Interestingly, approximately 2% of intraspecific crosses in Arabidopsis thaliana leads to F1 progeny presenting similar runaway cell death phenotypes (Bomblies et al., 2007). These phenotypes are due to incompatibilities between alleles that arose in evolutionarily divergent populations leading to reproductive isolation (Bomblies and Weigel, 2007). For example, an allele of Strubbelig Receptor Family 3 (SRF3) drives incompatibility between European and Asian Arabidopsis accessions (Alcázar et al., 2010). SRF3 appears to be under high selective pressure and guarded against effectors by a cluster of R genes. Allelic divergences impair interaction between guard and guardee and mimic the presence of the targeting effectors (Alcázar et al., 2010). It is postulated that, generally, molecular incompatibilities leading to cell death occur between R genes and their regulatory components (Bomblies et al., 2007; Bomblies and Weigel, 2007; Alcázar et al., 2010; Bomblies, 2010).

1.4. BAK1, a multiple co-receptor involved in development and immunity

Since its discovery as the BRI1 co-receptor, BAK1/somatic embryogenesis receptor kinase 3 (SERK3) together with its close homologues from the SERK family have been found to play pleiotropic roles in light signaling (Whippo and Hangarter, 2005), MAMP (Chinchilla et al., 2007; Heese et al., 2007; Roux et al., 2011) and DAMP signaling (Postel et al., 2010; Schulze et al., 2010), cell death control (He et al., 2007; Kemmerling et al., 2007), somatic embryogenesis, male sporogenesis (Albrecht et al., 2005), and tapetum formation (Colcombet et al., 2010).
2005; Chinchilla et al., 2009). BAK1 acts as co-receptor of BRI1 in brassinosteroid (BR) signaling (Li et al., 2002; Nam and Li, 2002), FLS2 (Chinchilla et al., 2007) and EFR (Roux et al., 2011) in MAMP signaling and PEPR1 (Schulze et al., 2010) in DAMP signaling where it generally phosphorylates receptors to enhance the signal transduction citations. Furthermore, it also negatively controls cell death through a mechanism that still remains obscure (He et al., 2007; Kemmerling et al., 2007). The role of BAK1 in the different pathways as well as the regulation of complex formation involving BAK1 will be discussed in this chapter.

1.4.1. **BAK1 is the co-receptor of BRI1**

BRs are steroid hormones that regulate various plant developmental features like germination, cell expansion and division, photomorphogenesis, senescence and disease resistance (Clouse et al., 1996; Krishna, 2003). Mutations in genes involved in brassinolide (BL) perception, signaling or biosynthesis provoke strong dwarfism, dark green leaves, reduced apical dominance, delayed senescence, and male sterility, as well as altered vascular patterning, inability to properly respond to light signals and variation in responses to biotic and abiotic stresses (Clouse, 1996; Li et al., 1996; Szekeres et al., 1996; Li and Chory, 1997). The receptor for BL perception is the LRR-RLK BR-Insensitive 1 (BRI1) (Li and Chory, 1997; He et al., 2000; Wang et al., 2001). It was identified as a BR-insensitive dwarf mutant. Its 25-LRR extracellular domain has been crystallized recently and present a typical twisted shape in which BR binds into a pocket formed by LRR 21 and 22 (Hothorn et al., 2011; She et al., 2011). BRI1 contains an autoinhibitory C-terminal tail that is released upon ligand binding (Wang et al., 2005). Furthermore, BRI1 phosphorylates the BRI1 KINASE INHIBITOR 1 (BKI1) on a tyrosine in a reiterated [KR][KR] membrane-targeting motif leading to release of BKI1 from the membrane upon BL perception (Wang and Chory, 2006; Jaillais et al., 2011b). Release of the repressor enables heteromerization of BRI1 and sequential transphosphorylation with BAK1 and its homologues to form active signaling complexes (Li et al., 2002; Nam and Li, 2002; Karlova et al., 2006; Wang et al., 2008; Yun et al., 2009). This activation enables BRI1 to phosphorylate two classes of RLCKs: BR-Signaling Kinases (BSKs) (Tang et al., 2008) and Consti-
tutive Differential Growth1 (CDG1) (Kim et al., 2011). They activate the PP1-type phosphatase BRI1 Suppressor 1 (BSU1) that will subsequently deactivate the Brassinosteroid-Insensitive 2 kinase (BIN2) by dephosphorylating a conserved phospho-tyrosine residue (Kim et al., 2009). The transcription factor Brassinazole-Resistant 1 (BZR1) that is not phosphorylated by BIN2 anymore at high BL concentrations, is dephosphorylated by a PP2A phosphatase (He et al., 2002; Tang et al., 2011). This leads to dissociation of BZR1 from 14-3-3 proteins, shuttling of BZR1 to the nucleus, binding to DNA and regulation of gene expression (He et al., 2005; Gampala et al., 2007; Ryu et al., 2007; Sun et al., 2010). Besides BZR1, BES1 also regulate BL-induced gene expression (Yin et al., 2005). Both BES1 and BZR1 have atypical basic helix-loop-helix (bHLH) DNA-binding domains and bind to E-box and/or BRRE (BR Response Element) to regulate BR target gene expression (He et al., 2005; Yin et al., 2005).

The key step for BRI1 activation is its heteromerization with SERK family members. Structural studies revealed that the extracellular domain (ECD) of BRI1 presents a highly twisted superhelical solenoid structure and that BL binds between the 70-amino acid island domain and the LRR concave surface (Hothorn et al., 2011; She et al., 2011). Based on previous models, BRI1 binding of BL leads to rearrangement in the kinase domain and recruitment of SERK proteins as enhancers of downstream signaling (Wang et al., 2008). Interestingly, triple serk1bak1bkk1 mutants are completely insensitive to BL comparable to bri1 null alleles. Overexpression of BRI1 fails to promote growth in this triple mutant background pointing to an absolute requirement of SERK proteins for proper BL responses (Gou et al., 2012). Beside genetic requirement of SERKs in BL signaling, several studies on BRI1 and BAK1 point mutations support the involvement of BAK1 not only in BL signaling but also in BL perception. The bri1-112 allele, a mutation in the LRR 25, is still able to bind BL but remains functionally inactive suggesting that binding of BL might not be the only requirement for signaling (Friedrichsen et al., 2000; Wang et al., 2001). Furthermore, the elg mutant presenting a D122N substitution in LRR3 of BAK1 is upregulated in BL signaling because of higher BAK1-BRI1 interaction (Halliday et al., 1996; Whippo and Hangarter, 2005; Jaillais et al., 2011a; Chung et al., 2012). Extracellular domains of BRI1 and SERK1 are present together with BL as heterodimers where the N-
terminal cap of SERK1 simultaneously binds BRI1 on LRR 25 and the island domain. The BL molecule stabilizes the SERK1-BL-BRI1 complex and acts as a molecular glue (Santiago et al., 2013; Sun et al., 2013a). Taken together, extensive genetic and structural studies revealed the importance of BAK1 and other SERKs not only in signaling but also in BL perception.

1.4.2. **BAK1 has a BL-independent role in cell death control**

Following a reverse genetic approach using microarray analysis, Kemmerling et al. could show that BAK1 is nicely upregulated upon treatment with several *P. syringae* strains as well as MAMPs (Kemmerling et al., 2007). *bak1* mutant alleles exhibit loss of cell death control accompanied by bigger lesion and higher disease index upon treatment with *Alternaria brassicicola* while several other mutants affecting BL signaling showed no alteration in cell death control (Kemmerling et al., 2007). Furthermore, exogenous treatment with BL rescued the growth phenotype of *bak1* mutants while the cell death phenotype remained (Kemmerling et al., 2007). This points to an independent control of cell death and BL pathways in *bak1* mutants. A complementary study showed that *bak1/bkk1* double mutants exhibit a strong dwarfism due to runaway cell death and strong increase of salicylic acid signaling (He et al., 2007). Double and triple mutants analysis confirmed that inside the SERK family only BAK1 and BKK1 are involved in cell death control (Albrecht et al., 2008). Overexpressing of *NahG*, a bacterial gene encoding a salicylate hydroxylase converting SA in an inactive catechol molecule (Delaney et al., 1994) rescued the cell death phenotype, showing that SA at least partially controls the *bak1/bkk1* phenotype (He et al., 2007). Furthermore, *bak1/bkk1* seedlings grown in the dark did not show any necrosis compared to seedlings grown in long day conditions, suggesting that light influences the cell death formation in this mutant (He et al., 2008). While moderate overexpression of BAK1 can rescue the cell death phenotype (Kemmerling et al., 2007), strong overexpression provokes runaway cell death that can be rescued by simultaneous overexpression of BRI1 (Belkhadir et al., 2012). It suggests that BAK1 needs a docking receptor such as BRI1 to be kept in check and to avoid detrimental effects like cell death activation. In conclusion, BAK1 appears to be an important cell death regulator.
1.4.3. BAK1, a central regulator of innate immunity

Beside its role in BL signaling and cell death control, BAK1 and its homologues are essential for MAMP signaling. A forward genetic screen for flg22 insensitivity in a RLK mutants population led to the identification of BAK1 as a key component of flg22 signaling (Chinchilla et al., 2007). bak1 mutants treated with flg22 exhibit strongly reduced responses like ROS, FRK1 gene expression or growth inhibition. Co-immunoprecipitation experiments confirmed the association of BAK1 and FLS2 in a flg22-dependent manner (Chinchilla et al., 2007; Heese et al., 2007). The BAK1-FLS2 interaction occurs within one second after flg22 elicitation suggesting a close vicinity of the receptor and its co-receptor in the membrane (Schulze et al., 2010). BAK1 also rapidly associates with EFR upon elf18 application (Roux et al., 2011), the kinase domain of BAK1 interacts with PEPR1 kinase domain in yeast two-hybrid assay (Postel et al., 2010) and a rice homologue of SERKs OsSERK2 heteromerizes with XA21 to mediate resistance against Xanthomonas oryzae pv. oryzae (Xoo) (Monaghan and Zipfel, 2012; Chen et al., 2014). In addition to BAK1, several other SERKs oligomerize with FLS2 and EFR in a ligand-dependent manner correlating with the contrasted importance of BAK1 in FLS2- and EFR-mediated pathways (Roux et al., 2011). Loss of cell death control and downregulation of BL signaling kept the study of MAMP-specific BAK1-mediated function difficult because of the pleiotropic effects. However, the identification of the hypoactive bak1-5 allele only affected in MAMP pathway, revealed the involvement of BAK1 and SERK4/BKK1 in disease resistance against the biotrophic oomycete Hyaloperonospora arabidopsidis (Schwessinger et al., 2011). Furthermore, bak1-5bkk1 double mutant are almost insensitive to fts22, elf18 and Atpep1 demonstrating a major role for BAK1 and its close homologue BKK1 in those pathways (Schwessinger et al., 2011). bak1 mutants were also shown to be downregulated in response to other MAMPs like LPS, PGN or INF1 in bak1 mutants lines suggesting that BAK1 heteromerizes with additional PRRs (Heese et al., 2007; Shan et al., 2008; Belkhadir et al., 2012). While BAK1 was generally thought to act as an adaptor protein enhancing flg22 responses a recent structural study showed that similarly to what is ob-
served in the BRI1-BL-SERK1 complex, BAK1 acts as a true co-receptor by binding the C-terminus of the FLS2-bound flg22 (Sun et al., 2013b).

Beside its role in RLK-mediated immunity, BAK1 emerges to be a crucial component of RLP-mediated immunity as well. Indeed, in addition to the requirement of SOBIR1, Cf- and Ve1-mediated immunity need also BAK1 homologues in tomato (Liebrand et al., 2013b). BAK1 was also found to interact constitutively with LeEIX1 regulating LeEIX2-dependent responses in tomato (Bar et al., 2010; Bar et al., 2011). Furthermore, SCFE1 responses are strongly impaired in bak1-5 mutant (Zhang et al., 2013b). While no direct interaction between BAK1 and SOBIR1 has been detected yet (Gao et al., 2009), the two highly conserved RLKs (Liebrand et al., 2013a) appear to be key components in RLP-mediated defense similarly. This is reminiscent to regulation of abscission by SERK1 and SOBIR1 possibly through direct interaction with HAESA (HAE) and HAESA-LIKE2 (HSL2) (Leslie et al., 2010; Lewis et al., 2010). Interestingly, ReMAX and RBPG1 signal independently of BAK1 demonstrating that BAK1 involvement in RLP-mediated immunity is not obligatory (Jehle et al., 2013; Zhang et al., 2013a). Furthermore, it is still unclear whether SOBIR1 act as a scaffold protein or/and signal transducer for RLPs. Therefore, BAK1 might still be the key signaling component in RLP-mediated defense responses (Liebrand et al., 2013a).

1.4.4. BAK1 is a target of the AvrPto effector

Virulent pathogens use a sophisticated system to overcome host defense by injecting a battery of effectors into the host cell via a T3SS, targeting many key components of the MTI pathway. AvrPto is an effector secreted by the bacterium Pseudomonas syringae pv. tomato DC3000. AvrPto has been shown to interact in vitro with FLS2 and EFR and to strongly downregulate responses to flg22 and elf18 (Xiang et al., 2008). Interestingly, transgenic Arabidopsis lines expressing a 35S-AvrPto construct exhibit a bri1-5 mutant-like morphology as well as insensitivity to flg22 and elf18 (Shan et al., 2008). It points to a negative regulation of BAK1 by AvrPto. While several manuscripts (Xiang et al., 2008; Xiang et al., 2011) using co-immunoprecipitation as main assay privilege a higher AvrPto-FLS2 affinity compared to AvrPto-BAK1 suggesting that AvrPto acts by binding several ligand-binding receptors rather than targeting the multiple co-receptor
BAK1, the initial manuscript describing the negative effect of AvrPto on MAMP signaling (Shan et al., 2008) rather favours BAK1 as main target of AvrPto. In the future, further experiments, defining precisely the affinity of AvrPto for different kinases, will shed light on the mechanism of action of AvrPto. The AvrPto-BAK1 interaction provides a brilliant example where an effector efficiently downregulates several MAMP pathways by targeting a single, multi-functional co-receptor.

1.4.5. BIK1 differentially regulates BAK1-dependent pathways

Botrytis-induced kinase 1 (BIK1) is a RLCK that has first been found to positively regulate defense to Botrytis cinerea and Alternaria brassicicola via upregulation of SA pathways together with a downregulation of JA pathways (Veronese et al., 2006). BIK1 belongs to the large PBS1-like family (PBL). BIK1 interacts directly with FLS2 and BAK1. Upon flg22 application, BIK1 is phosphorylated by BAK1 and subsequently phosphorylates BAK1 and FLS2 (Lu et al., 2010b; Lu et al., 2010a; Zhang et al., 2010). This step is thought to be critical to enhance flg22 signaling downstream of FLS2. Partial insensitivity of bik1 mutants to flg22 corroborates this hypothesis. BIK1 also plays a positive role in PEPR1 and ethylene pathways by directly interacting with PEPR1 and probably PEPR2 (Liu et al., 2013; Tintor et al., 2013). Remarkably, CERK1 also interacts with BIK1 exemplifying a partial overlap of LysM-RLK and LRR-RLK immune signaling (Zhang and Zhou, 2010). Several effectors like Pseudomonas AvrPphB or Xanthomonas AvrAC can target BIK1 and PBL proteins in order to shut down plant immune responses (Zhang et al., 2010; Feng et al., 2012). A recent study also reports the involvement of BIK1 in BL signaling. BIK1 interacts with and is phosphorylated by BRI1 in a BAK1-independent manner (Lin et al., 2013). Interestingly, BIK1 does not play a positive regulatory role in the BL pathway but acts rather as a repressor since bik1 mutants show higher responses to BL. In conclusion, BIK1 has a positive role together with BAK1 in MAMP signaling and in the cell death control pathway but plays an antagonistic role in the BL pathway in a BAK1-independent manner.
1.4.6. Is BAK1 a component of crosstalks between receptors signaling?

Upon flg22 treatment, plants shift their metabolism from usual growth to defense signaling leading to strong growth arrest. Considering that BAK1 plays a key role in BL and flg22 pathways, the idea that flg22 and BL modulate themselves through BAK1 appears as an attractive hypothesis. Interestingly, when Col-0 plants were co-treated with BL and flg22, flg22 had no effect on BL responses while BL strongly reduces general flg22 responses like ROS production and gene expression (Albrecht et al., 2012). Overexpression of dwarf4 (DWF4), a gene involved in BL biosynthesis also leads to highly reduced flg22 signaling (Belkhadir et al., 2012). At this level, it can be hypothesized that BL perception titrates out BAK1 molecules available for FLS2. However, co-treated samples have similar amounts of BAK1 interacting with BRI1 and FLS2 compared to single-treated samples and chitin responses are similarly downregulated by BL treatment, pointing to a role of BL downstream of the FLS2-BAK1-BIK1 module (Albrecht et al., 2012). On the other hand, Arabidopsis plants overexpressing BRI1 show strong reduction in flg22 responses that can be overcome by simultaneous overexpression of BAK1 suggesting that BAK1 seems to be the limiting factor for proper flg22 signaling (Belkhadir et al., 2012). However, recent reports indicate that the trade-off between BL and MAMP occurs at the transcriptional level through the interaction of BZR1 and WRKY40 (Lozano-Duran et al., 2013) and through the bHLH transcription factor HBI1 (Fan et al., 2014; Malinovsky et al., 2014) confirming that BL negatively affects MAMP signaling through a BAK1-independent mechanism.

1.4.7. BIR1 and BON1 interact with BAK1 and negatively control cell death

While the mode of action of BAK1 generally involves interaction with receptors to form active signaling units and phosphorylation of receptors to activate the signal transduction, no potential ligand-binding receptors involved in cell death control have been found yet. Thus, mechanisms how BAK1 controls cell death re-
remains obscure. The BAK1-interacting receptor 1 (BIR1) has been identified as a negative regulator of cell death and interacts in a constitutive manner with BAK1 and its homologues SERK1, SERK2 and BKK1 in the absence of a ligand (Gao et al., 2009). bir1 mutants present severe dwarfism and die after the cotyledon stage at 22°C. Change to 28°C conditions enable the plant to survive and to produce seeds but they still remain very small. bir1 mutants accumulate very high amounts of SA, present high PR1 expression and mutation in eds1 and pad4 partially revert those phenotypes. Surprisingly, the bir1 mutant does not show any alteration in flg22 response (Gao et al., 2009). A suppressor screen on bir1 knockout lines identified mutations in the LRR-RLK SOBIR1 that revert the cell death phenotype suggesting that BIR1 acts in an R gene-mediated pathway as well as an yet undescribed SOBIR1-dependent pathway (Gao et al., 2009). BIR1 and BAK1 interact with BON1 (Wang et al., 2011). Together with its close homologue BON3, BON1 controls growth homeostasis and cell death via negative regulation of multiple R-genes including SNC1 (Hua et al., 2001; Li et al., 2009). Interestingly, bak1-1 allele in the Ws-0 background, natural mutant of SNC1, still present defects in cell death control suggesting that there is an additional SNC1-independent pathway. In conclusion, the interaction of BAK1, BIR1 and BON1 suggests that they work together in one pathway to negatively control cell death through an R gene-dependent mechanism.

1.5. **Aim of the thesis**

BAK1 is involved in multiple cellular processes namely MAMP and BL signaling and cell death control. BAK1 functions via dimerization and sequential phosphorylation with the ligand-binding receptors BRI1, FLS2, EFR and PEPR1. However, no ligand-binding receptor has been found yet to act in the cell death pathway. The thesis focuses on the characterization of two BAK1-interacting receptors BIR2 and BIR3. Both proteins belong to a small LRR-RLK subfamily that contains also BIR1 (Gao et al., 2009) and BIR4. The aim of this thesis was to understand what is the function of BIR2 and how it regulates BAK1-dependent processes to get a better understanding on the regulation of the multifunctional BAK1 and its associated signaling components.
2. Material and Methods

2.1. Material

2.1.1. Chemicals

All chemicals were ordered from Biorad (München), Fluka (Buchs, CH), Merck (Darmstadt), Carl Roth (Karlsruhe), Duchefa (Haarlem, NL) or Sigma (Steinheim). Organic solvants were delivered by Brenntag Chemiepartner GmbH NL (Plochingen) and Merck. The ingredients used for culture media were ordered from Invitrogen (Carlsbad, USA), Merck, Sigma-Aldrich (Saint-Louis, USA) and Duchefa (Haarlem, NL). Enzyme used for nucleic acids studies were ordered from Invitrogen, Stratagene (La Jolla, USA), New England Biolabs (Beverly, USA). membranes for blotting were purchased from GE Healthcare (Freiburg) and Roche (Basel, Switzerland). The flg22 peptide was synthesized by Peptide Specialties Laboratories (Heidelberg), and the elf18 and pep1 peptides were a kind gift from Georg Felix and Sebastian Bartels respectively.

2.1.2. Media

The different media used are presented in the following table.

Table 1. Media used in this study

<table>
<thead>
<tr>
<th>Medium</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>10 g/l Bacto-Trypton; 5g/l Bacto-Yeast; 5 g/l NaCl</td>
</tr>
<tr>
<td>King’s B</td>
<td>20g/l Glycerol; 40 g/l Proteose-Peptone3; 10 ml/l K2HPO4; 10 ml/l MgSO4</td>
</tr>
<tr>
<td>½ MS</td>
<td>2.2 g/l MS-Salt (Sigma, Duchefa); set pH 5.7 with KOH</td>
</tr>
<tr>
<td>SOC</td>
<td>2.0 g/l Trypton; 0.5 g/l Yeast extract; 10 mM NaCl; 2,5 mM KCl; 10 mM MgCl2; 10 mM MgSO4; 20 mM Glucose; set at pH 7 with NaOH</td>
</tr>
</tbody>
</table>

2.1.3. Antibiotics

Media were supplemented with antibiotics at concentration listed in the following table.
Table 2. Antibiotics used in this study

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>50 µg/µl</td>
</tr>
<tr>
<td>Cycloheximid</td>
<td>50 µg/µl</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>25 µg/µl</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50 µg/µl</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>50 µg/µl (from 25 mg/ml stock in Methanol)</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>100 or 200 µg/µl</td>
</tr>
<tr>
<td>Tetracyclin</td>
<td>12.5 µg/µl (from 12.5 mg/ml stock in Ethanol)</td>
</tr>
</tbody>
</table>

2.1.4. **Bacterial strains**

2.1.4.1. *Escherichia coli* strain DH5α

The *E. coli* strain DH5α (F-(Φ80lacZΔM15) Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK−, mK+) phoA supE44 λ− thi-1 gyrA96 relA1) was used for cloning of the different constructs.

2.1.4.2. **Agrobacterium strain**

The *Agrobacterium tumefaciens* strain GV3101 (T-DNA− vir+ rif−, pMP90 gen+) was used for transient and stable plant transformation.

2.1.5. **Primers**

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

2.1.6. **T-DNA insertion lines**

T-DNA insertion mutants used for *BIR2* functional studies are *bir2*-1 (GK-793F12) (Rosso et al., 2003) and *bir2*-2 (tilling line from WISC β-Pool). Mutant in *BIR3* are *bir3*-1 (SALK_132078) and *bir3*-2 (SALK_116632). As control in the
study, we also used bak1-1 (CS6125), bak1-4 (SALK_116202) (Kemmerling et al., 2007), cerk1-2 (N409189) (Miya et al., 2007) and bri1-5 (CS6126) (Noguchi et al., 1999) mutant lines.

2.1.7. Antibodies

Polyclonal anti-BAK1 (Mazzotta, 2012) and anti-BIR2 antibodies were generated by immunizing guinea pigs with the respective synthetic peptides (BAK1: DSTSQIENEYPSGPR and BIR2: C+DDFPLIFDTQENEKV) derived from the C-termini of BAK1 and BIR2 by Eurogentec (Seraing, Belgium). Antibodies (final bleed) were affinity purified against the respective peptide. Test and preparation of the BIR2 antibody are detailed in the result part. Anti-FLS2 antibodies were a gift from Delphine Chinchilla’s lab (Chinchilla et al., 2007). If not indicated otherwise, antibodies were diluted in blocking solution to the following dilutions: α-GFP (Acris), 1:5,000; α-FLAG (Sigma), 1:2,000; α-c-myc (Sigma), 1:10,000; α-BAK1, 1:2,000; α-FLS2, 1:250; α-BIR2, 1:4,000; α-phosphoserine/threonin (α-phospho), 1:1000.

2.2. Plant material and method

2.2.1. Plant growth condition

2.2.1.1. Arabidopsis thaliana growth condition

Arabidopsis thaliana plants were grown on steam-sterilized GS90-soil (Gebr. Patzer GmbH) mixed with Vermiculite in a growth chamber (8 h light, 16 h dark, 22°C, 110 µmolm⁻²s⁻¹, 50-60% humidity).

2.2.1.2. Nicotiana benthamiana growth condition

Nicotiana (N.) benthamiana plants were grown on a mixture of soil and sand supplemented with 0.1% (v/v) Confidor in the greenhouse (13 h light, 11 h darkness).
2.2.2. Transient transformation of *N. benthamiana* by *Agrobacterium tumefaciens*  

*Agrobacterium tumefaciens* GV3101 or C58C1 carrying the indicated constructs were grown 36 h at 28°C in LB medium supplemented with appropriate antibiotics. Cultures were pelleted and resuspended in 10 mM MgCl$_2$ to OD$_{600}$ = 1. *Agrobacterium* cells were mixed in a 1:1 ratio with a strain containing the suppressor of silencing P19 (Voinnet et al., 2003). The mixture was then infiltrated with a needleless syringe into 3-week-old *N. benthamiana* leaves. The presence of proteins was tested by Western blot or fluorescence microscopy, 2 to 3 days (d) after inoculation.

2.2.3. Stable transformation of *Arabidopsis thaliana* by *Agrobacterium tumefaciens*  

*A. thaliana* plants were stably transformed by the floral dip method (Clough and Bent, 1998). 500 ml liquid LB medium supplemented with the appropriate antibiotics was inoculated with a preculture of selected agrobacteria and cultivated for further 18 - 24 h at 28°C. The cells were pelleted for 10 min at 5000 x g and resuspended in fresh 5 % (w/v) sucrose solution at a density of 0.8 (OD$_{600}$nm). After addition of 0.02 % (v/v) Silwet L-77, young *Arabidopsis* inflorescences were dipped into the bacterial suspension. Afterwards the plants were incubated at 100 % humidity for 24 h and allowed to set seeds. The transformants were selected for Basta (glufosinate-ammonium) or Hygromycin resistance.

2.2.4. Generation of knockdown lines for *BIR2*  

The artificial microRNA (amiRNA)-mediated gene silencing method was used to specifically knockdown *BIR2* in Col-0 background. The four primers necessary for the cloning (see) were computationally generated by the Web microRNA Designer and the cloning was performed in four PCR-steps as described (WMD; http://wmd.weigelworld.org). The *BIR2*-specific amiRNA was introduced into the pCR8 vector via T/A cloning and subsequently cloned in the pB2GW7 by LR re-
action. *Agrobacterium tumefaciens* GV3101 were used to stably transform *Arabidopsis thaliana* Col-0 plants by the floral dip method. Analysis of BIR2 expression downregulation was tested by quantitative RT-PCR using primers listed in Table and by Western blot using α-BIR2 antibodies. Two independent lines that we called amiR-BIR2 were used for functional studies.

### 2.3. Bacterial transformation methods

#### 2.3.1. Transformation of *E. coli* DH5α

1 µl of plasmid was added to heat-shock competent *E. coli* DH5α cells. After 30 minutes (min) incubation on ice, cells were heated up during 1 min at 42°C. Then, 450 µl SOC medium was added and the mix shaken during 45 min-1 hour (h) at 37°C. The transformed cells were selected on solid LB agar plates containing appropriate antibiotics after overnight incubation at 37°C.

#### 2.3.2. Transformation of *Agrobacterium tumefaciens*

1 µl of plasmid was added to 40 µl of electro-competent bacteria cells. The mixture was transferred into an electroporation cuvette and electroporated. After electroporation, cells were mixed with 400 ml of LB medium and incubated at 28°C for 2 h. The transformed cells were selected on LB agar plates containing appropriate antibiotics after 48 h incubation at 28°C.

### 2.4. DNA analysis

#### 2.4.1. Bacterial plasmid extraction

##### 2.4.1.1. Extraction of bacterial plasmid by alkaline lysis

A bacterial pellet from a 2 mL overnight culture was resuspended in 100 µl lysis buffer (50 mM Tris/HCl pH 8.0, 50 mM EDTA pH 8.0, 15 % Saccharose), lysed with 200 µl alkaline SDS-buffer (200 mM NaOH, 1 % (w/v) SDS) for maximum 5 min and finally 150 µl Potassium acetate buffer (3 M Potassium acetate, 11.5 % (v/v) acetic acid) was added. The mix was centrifuged and the aqueous phase containing plasmid DNA was precipitated with 0.6 Vol Isopropanol. The
pellet was washed with 70 % ethanol and dissolved in TE-buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) or deionized water.

2.4.1.2. Miniprep kit preparation

For preparation of pure plasmid needed for sequencing, a Qiaquick miniprep kit (Qiagen) was preferred to a standard miniprep. Preparation was performed following the manufacturer protocol.

2.4.1.3. Midiprep kit preparation

Preparation of high amounts of purified plasmid was performed with the Midiprep kit (GE Healthcare) following the manufacturer protocol.

2.4.2. Plant genomic DNA extraction

Genomic DNA from Arabidopsis leaf tissue was isolated according to Edward's isolation protocol (Green and Sambrook, 2012). One grinded leaf was homogenized in 200 µl Edwards buffer (200 mM Tris/HCl pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% (w/v) SDS) at room temperature. Samples were centrifuged for 10 min at 13000 rpm. 200 µl isopropanol were added to the supernatant and thoroughly mixed. DNA was precipitated at RT for 5 min and centrifuged for 10 min at 4 °C with 14000 rpm. The pellet was washed with 70% (v/v) EtOH and centrifuged for 5 min at RT at 14000 rpm. Supernatant was then discarded and the air-dried pellet was dissolved in 50 µl H2O.

2.4.3. Restriction enzyme digestion of DNA

Restriction reaction was performed with 1 U enzyme/µg DNA at 37°C for 2 h according to the manufacturer’s conditions.

2.4.4. Polymerase Chain Reaction (PCR)

PCR reactions were carried out with a lab-made Taq polymerase while a high fidelity Pfu Polymerase (Fermentas) was used for cloning PCR according to the manufacturer instructions. The 20 µL PCR mix (1 x reaction buffer, 200 µM dNTPs, 0.2 µM of each primer, 1 U DNA polymerase, > 1µg DNA template) was subjected to a denaturation step at 95°C during 3 min. Then, the thermocycler
performed up to 40 cycles containing one denaturation step of 30 s at 95°C, one annealing step of 30 s between 45°C and 68°C depending on the primers, one elongation step at 72°C and 1min/kb. Finally, the sample was subjected to a 10 min final elongation step at 72°C.

2.4.5. DNA agarose gel electrophoresis

DNA electrophoresis was performed on a 0.8-1.5 % agarose gel in 1x TAE-buffer (40 mM Tris/acetate pH 8.0, 2 mM EDTA pH 8.0) at 60-120 V. A 1kb ladder (Fermentas) was used as size marker. Ethidium bromide (0.5 µg/ml) present in the gel helps visualisation of DNA by a UV-Transilluminator (Infinity-3026 WL/26 MX, Peqlab).

2.4.6. Purification of DNA fragment from agarose sample

After migration, DNA band was excised from the gel with a scalpel and DNA was extracted with the QIAquick gel extraction kit (Qiagen) prior to the manufacturer instructions.

2.4.7. Gateway BP reaction

100 fmol of the insert and 100 fmol of pDONR201 vector were mixed together overnight with 2 µl BP recombinase mix (Invitrogen). After fast vortexing, the mix was incubated overnight at 25°C. The reaction was stopped by adding 1 µl of Proteinase K (Invitrogen) and the samples were incubated for 15 min at 37°C. 2 µl of the reaction volume was used to transform E. coli DH5α.

2.4.8. Gateway LR reaction

A reaction mix containing 50 to 150 ng of the entry vector, 200 to 250 ng of the destination vector and TE buffer pH 8.0 up to a final volume of 4 µl, and 1 µl of the LR Clonase II mix (Invitrogen) was incubated overnight at 25°C. The reaction was stopped by adding 1 µl of Proteinase K (Invitrogen) and the samples were incubated for 15 min at 37°C. 2 µl of the reaction volume was used to transform E. coli DH5α.
2.4.9. DNA sequencing

30 to 100 ng/µl of purified plasmid were sequenced by the GATC biotech AG and the sequence analysis was performed using CLC DNA Workbench (CLC Bio) software.

2.4.10. Quantitative Real-time PCR

For quantitative Real-time PCR (qPCR), cDNA preparation was diluted 5 times with sterile water and 1 µl of diluted cDNA was used in a 20 µl reaction. RT-qPCR amplifications and measurements were performed with the iQ5 Multicolour Real Time PCR detection system (Biorad). RT-qPCR amplifications were monitored using the ABsolute SYBR Green Fluorescein Mix (Thermo Scientific) and gene expression data were quantified using the 2−ΔΔCT method (Livak and Schmittgen, 2001). All quantifications were made in duplicates on RNA samples obtained from three independent experiments, each performed with a pool of two leaves or 3-6 seedlings.

2.5. RNA analysis

2.5.1. RNA extraction

1 ml Trizol (Invitrogen) was added to 200 mg of ground plant material, mixed by vortexing and incubated 10 minutes at room temperature. After addition of 200 µL Chloroform, samples were vortexed intensively at least 10 s, incubated 10 min at room temperature and centrifuged during 10 min at 12000 x g. The aqueous upper phase was taken in another tube and RNA was precipitated with 1 volume of isopropanol. After centrifugation at 4°C for 10 min at 14000 rpm, the RNA pellet was washed with 70 % Ethanol, air-dried and dissolved in 40 µL of DNAse free water. RNA concentration was quantified using a Nanodrop 2000 (Peqlab Biotechnologie GmbH).
2.5.2. **DNAse treatment of RNA**

1 x DNAse buffer and 1 U RNAse free DNAsel (Invitrogen) was added to 5 µg of RNA in a 20 µL mix. The mix was then incubated at 37°C for 1 h. RNA was purified with a phenol/chloroform/isoamyl alcohol mix and precipitated. After a washing step with 70% ethanol, the pellet was dissolved in 10 µL DEPC-treated water.

2.5.3. **Reverse transcription**

2-5 µg RNA in 10 µL H2O was denatured at 70°C for 10 min. 10 µL of Reverse transcription (RT) mix (1 x RT buffer, 6 µM oligo-dT, 0.5 mM dNTPs, 5 U reverse transcriptase M-MuLV RT RevertAid (Fermentas)) was immediately added. The mix was incubated 90 min at 42°C and the enzyme was then deactivated at 70°C for 10 min.

2.6. **Protein analysis**

2.6.1. **Extraction of proteins from plant material**

Total protein was extracted with a buffer containing a detergent enabling extraction of membrane bound proteins (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1%(v/v) Nonidet P40 and 1 tablet of protease inhibitor cocktail/10 mL (Roche)). Grounded *A. thaliana* or *N. benthamiana* tissue was solubilised 30 min on ice in the extraction buffer. The extract was purified by centrifugation (10 min, 4°C and 14000 rpm). Protein concentration was then measured with the Bradford method (2.6.2) and the extract was either directly loaded on a gel or used for a co-immunoprecipitation experiment.

2.6.2. **Protein concentration measurement**

The protein concentration was quantified using the Bradford method (Bradford, 1976) and Roti-Quant solution (Carl Roth). Standard curve was calculated using bovine serum albumin (BSA).
2.6.3. SDS-PAGE

SDS-Polyacrylamide gel electrophoresis (PAGE) was performed as previously described (Sambrook and Russell, 2006). Protein extracts were loaded on SDS-PAGE with 8 % cross-linking and electrophoresis was performed in 1 x SDS running buffer (25mM Tris base, 192 mM Glycine, 0.1 % (w/v) SDS) between 100 and 150 V for 30 to 60 min depending on the protein size.

2.6.4. Western blot

Proteins were transferred after SDS-PAGE onto a PVDF membrane (Roche) in 1 x transfer buffer (25mM Tris base, 192 mM Glycine) using a Mini Trans-Blot® Electrophoretic Transfer Cell (BioRad) for one hour at 4°C at 110 V. Unspecific binding sites were blocked by incubation of the membrane for 1 h at RT with 5 % (w/v) fat free milk powder in 1 x TBST (150 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.1 % (v/v) Tween 20). After adding the primary antibody to the blocking solution, membrane was incubated overnight at 4°C. After 3 x 5 minutes washing with 1 x TBST or 1 x PBST, the membrane was incubated with a secondary antibody for 1 h. The signal of a horseradish peroxidase-coupled secondary antibody was detected using the ECL Kit (GE Healthcare) according to the manufacturer’s instructions.

2.6.5. Coomassie Brilliant Blue staining

Proteins present in a gel or on a membrane after Western blotting were colored with staining solution (0.125 % (w/v) Coomassie blue R-250, 50 % (v/v) MeOH; 10 % (v/v) acetic acid). After incubation for 10 min at RT the unspecific stain was washed in destaining solution (50 % (v/v) MeOH, 10 % (v/v) acetic acid).

2.6.6. Co-immunoprecipitation experiment

Leaves or seedlings were ground in liquid nitrogen, and 1 mL extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, Nonidet P40 1%) per g of tissue powder was added. Samples were clarified by centrifugation at 4°C and 13,000 rpm for 10 min. After 3 washing steps with the extraction buffer, 15 µL protein A agarose
beads (Roche) were incubated for 1 h with purified α-BIR2, purified α-BAK1 or α-YFP antibodies. Supernatants containing equal protein amounts were incubated for 4 h at 4°C with the beads. Beads were washed 3 times with 200 µL of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl before adding 2x loading buffer and heating at 95°C for 10 min.

2.6.7. Immunoprecipitation of BIR2 followed by MS analysis

For the co immunoprecipitation experiment followed by Mass spectrometry analysis, 10 g of 12 day-old seedlings grown 5 d on ½ MS agar plates and 7 d in liquid ½ MS supplied with 1% sucrose, were used and 10 mL extraction buffer was added. After denaturation with the Laemmli buffer, the protein content was quantified with a 2-D Quant kit (GE Healthcare).

2.7. Phenotypical analysis

2.7.1. Seeds sterilization

*Arabidopsis* seeds were sterilized by chlorine gas treatment. Seeds were transferred to Eppendorf tubes and placed in a desiccator. In a glass placed in the desiccator, 2 ml of 37 % HCl were added to 50 ml of 12 % sodium hypochlorite solution forming the chlorine gaz. The lid of the desiccator was immediately closed and vacuum was generated to get an airtight seal. The seeds were incubated for 4 h.

2.7.2. Hypocotyl length assay

Surface sterilized seedlings were grown on plates with or without the indicated hormones or inhibitors at 22°C on vertical half strength MS-agar plates for 6 d in the dark and 7 d in long day light condition. Photographs of the seedlings were taken. Roots and hypocotyls lengths were measured using ImageJ software.

2.7.3. MAMP-induced growth inhibition

Surface sterilized seeds were sown on ½ MS media containing 0.8% agar, incubated for 2 d in the dark at 4°C and allowed to germinate for 5 d at 22°C. Seedlings were transferred to ½ MS liquid media containing 1% sucrose in a sterile 24-
well plate and treated with 100 µM elf18 or flg22 for 7 days. Six pools of 8 seedlings were then weighted.

2.7.4. Oxidative burst

Leaf discs were excised from 6 week-old Arabidopsis plants and incubated in water overnight. The following day, the discs were transferred to a solution of 20 µM luminol L-012 (Wako Pure Chemical Industries) and 10 µg/mL (w/v) horseradish peroxidase in a 96-well plate treated with 100 nM flg22 or elf18. The plates were analysed for a period of at least 30 min using a multiplate reader Centro LB 900 (Berthold Technologies). For each data point, at least 9 replicates were measured.

2.7.5. MAMP-induced transcriptional changes

Arabidopsis seedlings grown 7 days on ½ MS plates in long-day conditions were transferred to ½ liquid MS medium supplemented with 1 % (w/v) sucrose and incubated overnight. After addition of the indicated MAMP, seedlings were incubated with gentle shaking and harvested after 4 h followed by RNA extraction. FRK1 transcripts level was tested by qRT-PCR.

2.7.6. Infection with Alternaria brassicicola

Alternaria brassicicola spores used for the infection assays were obtained as published previously (Thomma et al., 1999). Leaves of 5 week-old Arabidopsis plants were drop-inoculated with two 5 µl droplets of spore solution (5x10^5 Spores/ml). Two leaves per plant and a minimum of 8 plants per line were infected. Plants of different lines were randomly distributed in the tray and incubated at 100% relative humidity. Fungal infection was scored and disease index was defined as previously published (Kemmerling et al., 2007).

2.7.7. Trypan blue staining

Cell death and fungal mycelium in Alternaria-infected tissue were stained with trypan blue as described previously (Kemmerling et al., 2007). Upon infection, leaf material was placed in a trypan blue solution (8% (v/v) lactic acid; 8% (v/v)
glycerol; 8% (v/v) Aqua-Phenol; 66% (v/v) EtOH; 0.36% (w/v) trypan blue) and placed at 100°C during 1-2 min. Leaves were destained in 1% chloral hydrate solution for 2 h and overnight with gentle shaking. Stained material was examined under a light microscope (Nikon Microscope eclipse 80i).

2.7.8. Callose deposition measurement

To visualize callose deposition, plants were MAMP-infiltrated for 24 h, subsequently fixed with a 1% (v/v) glutaraldehyde, 5mM citric acid, 90mM Na2HPO4 solution and stained with aniline blue. Visualization was performed using UV-epifluorescence microscopy. Signal intensities were measured using ImageJ software by measuring the mean signal intensity from 10 replicate fields of 2500 square pixels per data point.

2.7.9. Hormone measurement

Salicylate contents were measured as previously described (Lenz et al., 2011).

2.7.10. Bacterial growth assays

*Pseudomonas* strain DC3000 culture was diluted with 10 mM MgCl2 to a density of 1 x 10^4 cfu/ml (OD_{600} of 0.2 corresponds to 10^8 cfu/ml) and was then infiltrated with a needleless syringe into the leaf apoplast. Two leaves per plant and four plants were infected per genotype per time point. The growth of bacteria was determined after 0 and 2 d after infection. For the quantification, infected leaves were harvested, washed for one minute in 70% (v/v) EtOH and one minute in water. Two leaf discs/leaf with a diameter of 5 mm have been excised, ground and homogenized in 200 µl of 10 mM MgCl2. 10 µl of each homogenate were then plated undiluted and in different dilutions onto LB agar plates. After 48 h incubation at 28°C, the growth of bacteria was determined by colony counting, and subsequently mean values and standard errors were determined.
2.8. Microscopy

2.8.1. Light and epifluorescence microscopy

Trypan blue staining was visualized with light microscopy while callose deposition and split-YFP experiments were visualized with epifluorescence microscopy. Both approaches were carried out with the same microscope (Microscope eclipse 80i, Nikon) and a 20 x / 0.50 objective (Plan Fluor). Image acquisition was done with a digital camera (Digital-Sight DS-U1) and the images were processed with the Lucia Image software.

2.8.2. Confocal microscopy

Visualization of YFP-tagged proteins fluorescence was performed using a confocal laser-scanning microscope (TCS SP2, Leica) and a 63 x water immersion objective. Images acquisition and processing was performed with the LCS Lite software.

Concave plasmolysis has been induced by incubation of the leaves in 850 mM NaCl for 1 min.

2.8.3. FRET-FLIM experiment

FLIM measurements were performed on a Biorad Radiance 2100 MP system (Hercules, USA) in combination with a Nikon TE 300 inverted microscope (Tokyo, Japan) (Russinova et al., 2004; Bucherl et al., 2010). Two photon excitation pulses are generated by a Ti-Sapphire Mira Laser (Coherent), pumped by a 5 W Verdi laser, resulting in excitation pulses of 200 fs at a repetition of 76 Mhz. A 60x/1.2 water immersion objective was used. Donor protein and acceptor proteins were cloned in the pH7CWG2 and pB7YWG2 vectors respectively (Karimi et al., 2002). Donor fluorescence (CFP) emission was selected using a 480DF30 band pass filter and detected by a Hamamatsu R3809U MCP (Hamamatsu, Japan) photomultiplier with a time resolution of 50 ps. Fluorescence images of 64x64 pixel size were acquired using the B&H SPC 830 module (Becker & Hickl, Germany) and were analysed according to previous work (Bucherl et al., 2010).
2.8.4. Split-YFP experiment

*BIR* family coding regions were cloned into the vector pUBC-cYFP (Grefen et al., 2010) to form C-terminal fusions with the C-terminal part of YFP, whereas the *BAK1* cDNA was cloned into pUBC-nYFP (Grefen et al., 2010) to generate a C-terminal fusion with the N-terminal part of YFP. The constructs were transiently expressed in *N. benthamiana* leaves as described above and the YFP fluorescence has been visualized 48 h post infiltration by epifluorescence microscopy.

2.9. Statistical analysis

Statistical significance between two groups has been checked by using a two-tailed unpaired Student’s t test. For multiple comparisons, the one-way ANOVA method was performed combined with the Tukey’s honest significant difference (HSD) test. Significant differences are indicated with different letters (*p < 0.01*). For multivariate analysis, we applied a two-way ANOVA analysis to the original data. If statistical differences within and between the two parameters were detected and no correlation between the two parameters was calculated, we applied Tukey’s HSD test. Asterisks represent significant differences (*p < 0.05; **p < 0.01; ***p < 0.001).
3. Results

3.1. The BIR family, a new family of BAK1 interactors

3.1.1. Identification of BiR proteins as potential BAK1 interac-
tors

In her PhD thesis released in 2012, Sara Mazzotta performed a Co-IP fol-
lowed by mass spectrometry (MS) analysis on Arabidopsis thaliana expressing
BAK1-GFP fusion protein under the constitutive 35S promoter (Figure 3-1)
(Mazzotta, 2012). The experiment led to the identification of a number of potential
BAK1 interactors. Presence of 14-3-3 proteins, patellins, Rab GTPases and
calnexins was found in the candidate list. 14-3-3 proteins were shown on one
hand to interact directly with BRI1 and on the other hand to control BR-related
BZR transcription factor activity (Karlova et al., 2006; Gampala et al., 2007;
Jaspert et al., 2011). Patellins are components of the plasma membrane known
to play a role in endocytosis and cell plate formation, and Arabidopsis Patellin-2 is
known to be phosphorylated upon salt stress (Hsu et al., 2009). Calnexins are
important proteins that function in ER-quality control where it controls the proper
folding of proteins, including BRI1 (Hong et al., 2008). Presence of these potential
interactors was not surprising since they are commonly found to be interacting
with LRR-RLK and provide a proof for the reliability of the experiment. Interest-
ingly, the two interactors with the best peptide coverage are two predicted LRR-
RLKs. We named them BAK1-interacting receptor 2 (BIR2) and 3 (BIR3) for their
sequence similarity to BIR1 (Gao et al., 2009). According to the published classi-
fication (Shiu and Bleecker, 2001b, a), the two RLKs, together with another
orthologue, BIR4, belong to the same subfamily as BIR1 - the LRR Xa subfamily.
This subfamily stands phylogenetically between the BRI1/BRL (Li and Chory,
1997; Cano-Delgado et al., 2004; Zhou et al., 2004) and the PSKR1/PSY1/EMS1
subfamilies (Matsubayashi et al., 2002; Amano et al., 2007; Jia et al., 2008). Al-
though the four BIR members are closely related to BRI1 and the PSKRs, they
have a structure similar to the SERK family members with a short extracellular
4,5 repeat LRR domain, a transmembrane domain and an intracellular kinase domain (Boller and Felix, 2009). The short extracellular domain suggests that BIRs act as regulatory proteins rather than as ligand-binding receptors.

**Figure 3-1. LC-ESI-MS/MS results of BAK1-GFP co-immunoprecipitated proteins.**
MaxQuant analysis results of BAK1-GFP immunoprecipitates from plants treated with *A. brassicicola* for 12h compared with water-treated seedlings. Log$_2$ protein abundance ratio of *Alternaria brassicicola* and mock-treated values (X-axis) are compared with the log$_{10}$ IBAQ intensity values (Y-axis). Representative proteins are indicated by the name directly after the corresponding circle.

### 3.1.2. Interaction analysis by Co-IP in *N. benthamiana*

BIR2 and BIR3 have been identified as interactors of BAK1 by Co-IP-MS analysis. To confirm the interactions of BIR2 and BIR3 with BAK1 and to test the possible interaction between BAK1 and BIR4, myc-tagged BAK1 were transiently expressed in *N. benthamiana* together with each of the BIR family proteins fused to YFP. Immunoprecipitation of tagged BIR proteins with YFP antibodies was per-
formed. BAK1-myc was co-immunoprecipitated with all BIRs (Figure 3-2) providing the evidence that BAK1 interacts with all members of the BIR family.

![Diagram](image)

**Figure 3-2. BIR family members interact with BAK1 in Co-IP experiments.**

(A) Co-immunoprecipitation of BAK1-FLAG with BIR2-YFP transiently expressed in *N. benthamiana* leaves. Total proteins (input) were subjected to immunoprecipitation (IP) with anti-YFP antibodies followed by immunoblot analysis with anti-FLAG antibodies to detect BAK1 and anti-YFP antibodies to detect BIR2. (B) Co-immunoprecipitation of BAK1-myc with BIR1, BIR3 or BIR4 fused to YFP transiently expressed in *N. benthamiana* leaves under the control of the 35S promoter. Total proteins were subjected to immunoprecipitation with anti-YFP antibody followed by immunoblot analysis with anti-myc and anti-GFP antibodies to detect BAK1 and the different BIR proteins, respectively.

### 3.1.3. Interaction of BIR proteins with BAK1 in BiFC experiment

We also performed bimolecular fluorescence complementation (BiFC) experiments. BAK1 and BIR proteins fused to the C-terminal and N-terminal part of YFP respectively, were transiently co-expressed under the control of an *ubiquitin10 (UBI10)* promoter (Grefen et al., 2010) in *N. benthamiana*. The potential fluorescence was visualized by epifluorescence microscopy. Fluorescence was observed at the plasma membrane for the four BAK1-BIR combinations but not in
the negative control where BAK1-nYFP was co-expressed with an empty cYFP tag (Figure 3-3). This experiment provides strong evidence that BAK1 can interact with all members of the BIR family in planta when full-length proteins are expressed.

![Image of fluorescence microscopy](image)

**Figure 3-3. BIR family members interact with BAK1 in BiFC experiments in *N. benthamiana*.

Bimolecular fluorescence complementation analysis was performed with full-length proteins fused to the N- or the C-terminal part of YFP and expressed under an ubiquitin promoter as indicated. Fluorescence was visualized by epifluorescence microscopy.

### 3.1.4. BIR family interacts with BAK1 in FRET-FLIM experiment

Another line of evidence for interaction of BAK1 and BIR family members was shown using FRET-FLIM. Reduction in fluorescence lifetime indicates close vicinity of proteins fused to donor and acceptor fluorophores, respectively. BAK1-CFP was used as the donor combined with all BIR family members fused to YFP as acceptors. Constructs were expressed in *A. thaliana* protoplasts, and the fluorescence of both constructs was verified by confocal microscopy. A significant reduction of the lifetime was observed for all interactors revealing that all BIRs localize in a close vicinity to BAK1 (Figure 3-4). Although, BIR1 to BIR4 are interacting with BAK1, there are differences in FRET efficiency among the interactors varying from 5,7% for BIR1, 10,9% for BIR4, 13,8% for BIR2 and 15,4% for BIR3. Taken together, BIR1 shows the weakest interaction with BAK1 while BIR2 and BIR3 exhibit the strongest.
Results

Figure 3-4. BIR family members interact with BAK1 in FRET-FLIM experiment.

FRET imaged by FLIM in Arabidopsis protoplasts transiently expressing BAK1-CFP or BIRs fused to YFP for 16 h. Mean fluorescence lifetime values (t) and lifetime distribution are presented as pseudocolor images according to the scale shown on the right with blue representing normal lifetime indicating no interaction (2.5 ns) and light green showing energy transfer between closely co-localized proteins (2.0 ns).

3.1.5. BIR2, BIR3 and BIR4 interact with BAK1 kinase domain in Y2H

We also used the yeast two-hybrid system. BAK1 and BIR kinase domains were cloned in the pGBK7T and pGADT7 vector, respectively. PJ69-4a yeast strain transformed with BAK1-KD and BIR-KD construct was subjected to selective growth assays on the SC/-Leu/-Trp or SC/-Leu/-Trp/-Ade/-His selection medium. Growth was observed on the selective medium for the BAK1-BIR2, BAK1-BIR3 and BAK1-BIR4 combinations (Figure 3-5). Interestingly, no growth was observed for the BAK1-BIR1 combination suggesting that the extracellular domains of BAK1 and BIR1 are required for the interaction. Taken together, these results confirm the interaction of BAK1 kinase domain with BIR2, BIR3 and BIR4 kinase domains and suggest that the extracellular domains of BIR1 and BAK1 are necessary for BAK1-BIR1 interaction.
3.1.6. **BIR proteins interact also with SERK1**

As mentioned in the introduction, BAK1 belongs to a larger family, called the SERK family. This subfamily contains four other homologues of BAK1/SERK3 named SERK1, SERK2, SERK4 and SERK5. It is known as well, that FLS2, EFR and BRI1 interact not only with BAK1 upon ligand induction but also with SERK1, SERK2 and SERK4/BKK1 (Albrecht et al., 2008; Roux et al., 2011; Gou et al., 2012). We were interested to see if BIRs are only specifically interacting with BAK1 or if they also have affinity to other SERKs. As a start, each BIR protein tagged with YFP was co-expressed together with myc-tagged SERK1 in *N. benthamiana*. All BIR proteins can interact with SERK1 (Figure 3-6). These results show that indeed all BIR proteins can interact not just with BAK1 but also with its relative SERK1. It will be interesting in the future to address the interaction between BIRs and SERK2, and BIRs and SERK4, the close homologue of BAK1 that also regulate cell death formation.
Figure 3-6. BIR proteins also interact with SERK1
Co-immunoprecipitation of SERK1-myc with BIR-YFP fusion proteins transiently expressed in N. benthamiana leaves. Total proteins (input) were subjected to immunoprecipitation (IP) with anti-YFP antibodies followed by immunoblot analysis with anti-myc antibodies to detect SERK1-myc and anti-YFP antibodies to detect BIR-YFP proteins.

3.1.7. The BIR protein family, a family of atypical receptor-like kinases

Although the BIR proteins belong to the LRRX family because of their homology in the kinase domain, they only carry a short 4,5 extracellular LRR domain and therefore look similar to BAK1 and the SERK family members. However, in contrast to BAK1, they do not contain the proline-rich region in the outer juxtamembrane region characteristic for SERK family proteins but instead they have a second conserved cysteine pair that BAK1 is lacking. Interestingly, extracellular domain size is not the only difference found between BIRs and the other LRR X members. Indeed, key residues important for kinase activity are mutated in BIRs, especially in BIR2, BIR3 and BIR4 (Figure 3-7). Strubbelig (SUB) is a LRR-RLK involved in the formation and shape of several organs by influencing cell morphogenesis and presents also mutation in key residues like a glutamine (Q) instead
of an arginine (R) in the HRD motif, and a serine (S) instead of a phenylalanine (F) in the DFG motif (Chevalier et al., 2005). It has been shown experimentally that SUB kinase does not exhibit any activity and that the kinase function is not needed in planta (Chevalier et al., 2005). Therefore, SUB has not been described as a kinase but as a pseudokinase or atypical kinase (Boudeau et al., 2006; Castells and Casacuberta, 2007). A comparison of kinase domains of BAK1, SUB, and BIR1 to BIR4 is presented in Figure 3-7. In the glycine-rich loop, BIR1 contains only the two last glycines and BIR2-4 only the last one. In the HRD and DFG motif, BIR1 present only an asparagine instead of the aspartic residue in the HRD motif presenting the features of non-RD kinases like FLS2. BIR2 to 4 exhibit more drastic mutations. R and the aspartic acid (D) in the HRD motif as well as F in the DFG motif are mutated. No autophosphorylation activity or substrate phosphorylation activity could be detected for the BIR2-4 proteins (Mazzotta, 2012) while BIR1 kinase domain exhibits autophosphorylation activity in the presence of Mn$^{2+}$ (Wang et al., 2011). Moreover, analysis of BIR2 crystal structure shows that BIR2 is indeed an inactive pseudokinase with an occluded ATP binding domain and the inability to bind ATP (Blaum et al., 2014). BIR1 is still active but presents weak activity while for BIR2-4 no activity was detectable as shown for SUB. Further in vitro and in planta experiments as well as crystal structures of the other BIR proteins will provide further evidence about their kinase inactivity to confirm them as pseudokinases.
Figure 3-7. BIR2, BIR3 and BIR4 present atypical kinase domains.

(A) An alignment of *Arabidopsis* BIR1-4, BAK1 and STRUBBELIG kinase domains reveals that BIR2 as STRUBBELIG lacks important catalytic residues (highlighted by boxes, glycine-rich loop, RD and DFG motif) in different subunits (roman numbers) of the kinase domain. (B) Phylogenetic tree of the LRRXα subfamily plus STRUBBELIG and BAK1 created using the maximum likelihood method. Alignment has been done with MUSCLE, phylogeny with the PhyML software and the tree rendering with TreeDyn (Phylogeny.fr software). The legend represents the branch length.
3.1.8. Phylogenetic study of the BIR family

To explore the relation between Arabidopsis BIR proteins, a phylogenetic analysis was performed. Full-length protein sequences were used to produce a phylogenetic tree using the maximum likelihood method. We observed that BIR2 to 4 are more closely related to each other than BIR1 (Figure 3-7). To extend the evolutionary considerations of our study, orthologues of BIR proteins from Physcomitrella patens (3), Selaginella moellendorffii (3), Oryza sativa (3), Zea mays (4), Medicago truncatula (3) and Populus trichocarpa (7) were found using the database of the Phytozome.net website. A phylogenetic tree of BIR homologues and the Arabidopsis BAK1 has been created using PhyML and TreeDyn (http://phylogeny.fr) (Figure 3-8). Interestingly, BIRs proteins form two main clades. The first clade contains the Arabidopsis BIR1 and 15 homologues. P. patens and S. moellendorffii homologues stand in this clade but form a small little sub-clade suggesting that they belong to the BIR1 clade but that they are still evolutionary quite far. This observation is understandable since mosses and ferns are more ancient evolutionary than angiosperms. The second clade contains BIR2 to BIR4 and 8 orthologues. Interestingly, RLKs forming the BIR1 clade mainly present an intact DFG motif as well as HRN motif where the kinases forming the second clade present mutated DFG and HRD motifs. While all BIRs are able to interact with BAK1, BIR2 to 4 diverged evolutionary from BIR1 with the apparition of mutations in their kinase domain. Potentially, these duplication events led to the arousal of new functions.
Results

Figure 3.8. Phylogenetic tree of Arabidopsis BIR proteins family and their related RLKs in different species.

Phylogenetic relationship of BIR1 to 4 and related RLKs from other species. The entire protein sequences were aligned using ClustalW2. The tree was created using PhyML and TreeDyn (http://phylogeny.fr). Red numbers represent the branch support values. Conservation of DFG, and RD motif and Glycine-rich loop are shown for each gene. Branch support values are presented in red and the legend represents the branch length.

3.1.9. Expression pattern of BIRs upon biotic pathogen treatments

Expression pattern of the four BIR members under different stimuli has been checked using microarray data from the Bio-Analytic Resource for Plant Biology (BAR) (http://bar.utoronto.ca). First, we observed that BIR1 to BIR3 are expressed in all tissues while BIR4 is only weakly expressed (data not shown). We checked the expression pattern of BIRs upon biotic stimuli such as microbes and
MAMPs. Upon application of all four different MAMPs, \textit{BIR2} is strongly upregulated. \textit{BIR1} expression slightly increases upon NPP1, \textit{hrpZ} and LPS at 2 hours but is slightly reduced after 4 hours. These results suggest that \textit{BIR2} might play a role in MAMP signaling. Furthermore, treatment with avirulent or non-pathogenic bacteria strains leads to \textit{BIR1} and \textit{BIR2} expression upregulation (Figure 3-9). Interestingly, this upregulation doesn’t appear upon treatment with virulent strains suggesting that effectors negatively regulate \textit{BIR1} and \textit{BIR2} expression. Surprisingly, \textit{BIR3} follows an opposite regulation. Indeed, \textit{BIR3} expression is clearly downregulated upon MAMP and bacteria application. \textit{BIR3} might play a different possibly opposite role than \textit{BIR1} and \textit{BIR2} in disease resistance.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3-9.png}
\caption{Relative expression of \textit{BIR1} to 4 (Microarray data from AtGenExpress). Relative expression presented after bacterial infection (A) and MAMP treatment (B). Results are means ± SE (n = 3).}
\end{figure}
3.2. The BAK1-interacting receptor 2 (BIR2)

Out of the two newly identified RLKs, BIR2 expression is upregulated upon MAMP and biotrophic pathogen treatment suggesting its involvement in innate immune processes. Its function was addressed in order to unravel its involvement in immune responses and in BAK1-dependent pathways.

3.2.1. Production of a BIR2 antibody

In order to detect native BIR2 protein, we aim to produce a polyclonal BIR2 antibody. The LRR-RLK family contains about 235 members, and the shared homology between the members is quite strong. Nevertheless, the very C-terminal area of LRR-RLK is known to be more variable. Therefore, the antibody was raised against the C-terminal peptide of BIR2 (C+DDFPLIFDTQENEKV). The specificity of the final bleed was tested on Ws-0 and bir2-2 a null mutant for BIR2. Protein extracts of both genotypes were used in a Western blot assay and membranes were incubated with the antibodies at a concentration of 1/2000 (Figure 3-10 B). It could specifically detect BIR2 at a size of about 67 kDa, the expected size for BIR2. This antibody is therefore a very good tool for BIR2 protein studies.

3.2.2. Phenotypical analysis of bir2 mutants and amiR-BIR2 lines

3.2.2.1. Characterization of bir2-1 and bir2-2 T-DNA insertion lines

Two available T-DNA insertion lines bir2-1 (GK-793F12) and bir2-2 (tilling line from WISC β-Pool) were used for the functional characterization of BIR2. Protein and transcript levels were checked in both lines and Col-0 and Ws-0 was used as controls. Western blot performed with the α-BIR2 antibody shows that the bir2-2 allele does not contain any BIR2 proteins but bir2-1 shows residual protein levels (Figure 3-10). Quantitative (q)RT-PCR analysis confirmed this result also on the RNA level (Figure 3-10). No BIR2 transcripts were detectable in bir2-2 and BIR2 expression in bir2-1 was reduced to about 50% compared to Col-0. Taken together, it shows that bir2-2 is a null mutant while bir2-1 is only a knockdown line concerning transcript and protein levels.
3.2.2.2. Characterization of BIR2 artificial microRNA lines

Since the bir2-1 mutant allele in the Col-0 background still contains half of the BIR2 transcripts, knockdown lines using an artificial microRNA system (Schwab et al., 2006) were produced (Chapter 2.2.4). BIR2 protein and transcript levels were checked by Western blot and qRT-PCR. Expression levels of two independent lines expressing a BIR2-specific amiRNA named amiR-BIR2 lines at the T3 generation are presented (Figure 3-10). No residual BIR2 proteins were detectable in both amiR-BIR2 lines and quantification by qRT-PCR provides the evidence that amiR-BIR2 lines contain less than 3% of BIR2 transcripts, showing that BIR2 expression is strongly silenced in these lines. Furthermore, to verify the specificity of the amiRNA construct, levels of BIR1 and BIR3 transcripts have been tested by qRT-PCR. Results from the qPCR showed that expression of BIR1 is slightly enhanced in bir2-1 and in both amiR-BIR2 lines. BIR3 expression is slightly reduced in these lines while BIR1 expression is slightly enhanced but the differences are weak and not significant. We considered this variation of expression to be a secondary effect due to BIR2 downregulation rather than a direct effect of the artificial microRNA construct.
Figure 3-10. Characterization of amiR-BIR2 lines

(A) BIR2 gene expression in Col-0 and two independent amiR-BIR2 lines was measured by quantitative RT-PCR analysis, normalized to EF1a expression, and plotted relative to expression in Col-0. Results are means ± SE (n = 3). (B) Western blot analysis with an α-BIR2 antibody detects protein levels of BIR2 in Col-0, bir2-1, two independent amiR-BIR2 lines, Ws-0 and bir2-2 plants. Coomassie brilliant blue (CBB) staining of total protein reveals equal loading. (C) BIR1 and (D) BIR3 gene expression in Col-0 and two independent amiR-BIR2 lines was measured by quantitative RT-PCR analysis, normalized to EF1a expression, and plotted relative to expression in Col-0. Results are means ± SE (n = 3).
3.2.3. BIR2 localizes to the plasma membrane

Since BIR2 is an LRR-RLK containing a transmembrane domain, membrane localization is expected. To address its localization, protoplasts (Russinova et al., 2004) and stable transgenic Arabidopsis lines expressing BIR2-YFP under the CaMV (Cauliflower Mosaic Virus) 35S promoter were produced. The localization was visualized by laser scanning confocal microscopy. Without treatment, the fluorescence localizes at the periphery of the cell. Plasmolysis was performed to retract the plasma membrane from the cell wall and confirmed the plasma membrane localization of BIR2-YFP (Figure 3-11).

![Figure 3-11. Subcellular localization of the BIR2-YFP fusion protein](image)

A 35S::BIR2-YFP construct was expressed in Arabidopsis protoplasts (A) and stable Arabidopsis plants (B). The localization was visualized by laser scanning confocal microscopy. The pictures show an intersection at the equatorial zone of the protoplasts and plant epidermal cells. Plasmolysis was triggered by addition of 850 mM NaCl. Arrows indicate YFP-labelled plasma membrane retracted from the cell wall (B, lower panel).
3.2.3.1. **BIR2 negatively controls elf18 responses**

One major genetic polymorphism between Col-0 and Ws-0 concerns the *FLS2* locus. Compared to Col-0, Ws-0 contains a point mutation in the *FLS2* locus leading to the presence of an early stop codon. Since Ws-0 is a natural *fls2* mutant, we decided first to focus on elf18 responses, in order to test the influence of BIR2 on BAK1-dependent MAMP signaling. Different assays were performed to compare elf18 responses in *bir2* mutants and amiR-*BIR2* lines compared to the respective WT controls Col-0 and Ws-0 (Figure 3-12; Figure 3-13). All lines tested show a stronger growth inhibition of seedlings upon 100 nM elf18 compared to controls. ROS production was also tested upon 100 nM elf18 in *bir2* mutants and amiR-*BIR2* lines. All tested lines exhibit significantly higher ROS production than Col-0 or Ws-0. Significantly higher *FRK1* expression and higher callose deposition was also shown in BIR2-deficient lines compared to wild type plants (Figure 3-12; Figure 3-13). Irrespective of the residual expressed protein, *bir2-1* mutant shows the strongest phenotype in most assays. Taken together, all mutant and amiR-*BIR2* lines exhibit higher response to elf18 in four independent assays showing that BIR2 is a negative regulator of the EFR pathway.
Results

Figure 3-12. BIR2-deficient Col-0 lines are hyperresponsive to elf18

(A) Fresh-weight ratio of 12-day-old Col-0, bir2-1 and amiR-BIR2 lines seedlings grown for 7 days in presence or not of 100 nM elf18. The bar graph represents the average fresh-weight ratio. Statistical analysis was performed on 6 pools of 8 seedlings (n=48). (B) FRK1 gene expression analysis in Col-0, bak1-4, bir2-1, two independent amiR-BIR2 lines monitored by qRT-PCR. cDNAs were produced from pools of three 10-day-old seedlings treated or not with 100 nM elf18. Expression values were normalized to EF1a, expressed as a ratio to Col-0 untreated samples and presented as fold induction. Bars represent mean ratios ± SE. (C) Oxidative burst triggered by 100 nM elf18 on leaf discs from 6-week-old Col-0, bak1-4, bir2-1, two independent amiR-BIR2 lines. Mean and standard error out of 9 replicates are represented on the graph. (D) Callose deposition visualized in 5-week-old leaves of Col-0, bak1-4, bir2-1, two independent amiR-BIR2 lines, Ws-0 and bir2-2 infiltrated with 0,1% BSA; 0,1 M NaCl containing or not 100 nM elf18. Representative staining picture out of six biological replicates is presented. Numbers represent relative staining intensity level means ± SE (n = 6). Asterisks represent significant differences to wild type (*p<0.5; **p<0.01; ***p<0.001; Student’s t-test)
**Results**

Figure 3-13. *BIR2*-deficient Ws-0 lines are hyperresponsive to elf18

(A) Fresh-weight ratio of 12-day-old Ws-0 and *bir2-2* seedlings grown for 7 days in presence or not of 100 nM elf18. The bar graph represents the average fresh-weight ratio. Statistical analysis was performed on 6 pools of 8 seedlings (n=48). (B) FRK1 gene expression analysis in Ws-0 and *bir2-2* lines monitored by quantitative RT-PCR. cDNA were produced from pools of three 10-day-old seedlings treated or not with 100 nM elf18. Expression values were normalized to *EF1α*, expressed as a ratio to Col-0 untreated samples and presented as fold induction. Bars represent mean ratios ± SE. (C) Oxidative burst triggered by 100 nM elf18 on leaf discs from 6-week-old Ws-0 and *bir2-2*. Mean and Standard error out of 9 replicates are represented on the graph. (D) Callose deposition visualized in 5-week-old leaves of Ws-0 and *bir2-2* infiltrated with 0.1% BSA; 0.1 M NaCl containing or not 100 nM elf18. Representative staining picture out of six biological replicates is presented. Numbers represent staining intensity level means ± SE (n = 6). Asterisks represent significant differences to wild type (*p<0.5; **p<0.01; ***p<0.001; Student’s t-test).
3.2.3.2. **BIR2** negatively regulates flg22-triggered responses

Secondly, the response to flg22 has been tested in Col-0 background lines. Responses to flg22 were checked in **bir2-1**, amiR-**BIR2** lines and Col-0. In both oxidative burst and flg22-induced growth inhibition assays, **bir2-1** and amiR-**BIR2** exhibit significantly stronger responses than the Col-0 control (Figure 3-14). These assays confirm that **BIR2**-deficient lines are hyperresponsive to both flg22 and elf18, two MAMPs that are depending on BAK1 and more generally on SERK receptors.

![Figure 3-14](image)

**Figure 3-14. BIR2-deficient lines are hyperresponsive to flg22**

(A) Fresh-weight ratio of 12-d-old Col-0, **bir2-1** and amiR-**BIR2** seedlings grown for 7 days with the indicated concentrations of flg22. Growth is represented relative to the mock-treated wild type. Results are means ± SE (n=6). Asterisks represent significant differences to wild type (**p<0.001; Student’s t-test). (B) ROS production measured over a period of 30 min represented as RLU in Col-0, **bir2-1** and two independent amiR-**BIR2** lines on leaf discs after elicitation with 100 nM flg22. Results are mean ± SE (n = 9). All experiments were repeated at least three times with similar results.
3.2.3.3. **BIR2 supresses fungal-induced cell death**

As previously published, BAK1 is not only involved in MAMP and BL signaling but also plays a positive role in cell death control (He et al., 2007; Kemmerling et al., 2007). We noticed that senescence of leaves and cotyledons occurs earlier in *bir2* mutants compared to WT control (data not shown). We inoculated *bir2* mutants and the respective wild-type plants with *Alternaria brassicicola* spores and monitored fungal growth and symptom development. Symptoms were scored and infected leaves were subjected to trypan blue staining to visualize cell death and fungal growth. The disease index of *bir2* mutants was significantly higher than in the respective wild-type plants at 7 and 10 days after inoculation (Figure 3-15). This is obvious in the pictures taken at 10 dpi. Trypan blue staining revealed that fungal growth was enhanced in the mutants but cell death occurred and spread also to uninfected areas in *bir2* mutants, which is not the case for wild-type plants. The results show that BIR2 negatively controls cell death formation and has a secondary effect on fungal growth. Taken together, BIR2 differentially affects immunity-related BAK1-regulated processes with a negative regulation of MAMP responses and a positive impact on cell death containment.
**Results**

**Figure 3-15. bir2 mutants show enhanced Alternaria brassicicola-induced cell death**

Col-0, bir2-1 and two independent amiR-BIR2 lines, Ws-0 and bir2-2 were infected with the necrotrophic fungus *Alternaria brassicicola*. The disease index was monitored in Col-0, bak1-4, bir2-1, two independent amiR-BIR2 lines (A), Ws-0 and bir2-2 (B) on days 7 and 10 post inoculation. Results are mean ± SE (n = 18). A representative of at least three independent experiments is shown. Asterisks represent significant differences to wild type (*p<0.5; **p<0.01; ***p<0.001; Student’s t-test). Representative pictures of the symptom development on infected leaves after 10 days were taken in (C) and (D). (E) Trypan blue staining of Col-0, bir2-1 and two independent amiR-BIR2 lines 7 days after inoculation shows necrotic tissue and fungal mycelium. Representative pictures from six biological replicates are represented.
3.2.3.1. *bir2* mutants and amiR-*BIR2* lines exhibit stronger defense responses upon DC3000 inoculation

Besides MAMP responses and *Alternaria*-induced cell death, we wanted to test downstream defense responses in *bir2* lines upon *Pto* DC3000 treatment. The defense marker gene *PR1* and the hormone SA are known to be upregulated upon infection with the virulent bacterial pathogen *Pto* DC3000. In case of mutants showing constitutive cell death like for example *bir1* (Gao et al., 2009), *mpk4* (Petersen et al., 2000) or *mkk1* (Ichimura et al., 2006; Gao et al., 2008), the level of *PR1* and SA are enhanced as compared to wt plants even in basal conditions. These mutants have uncontrolled immune responses coupled with drastic developmental disorders leading to severe dwarfism. To check these defense responses in *bir2* mutants, we performed qRT-PCR analysis of *PR1* expression and quantified SA content in *bir2* mutant lines after *Pto* DC3000 treatment. Compared to Col-0, both amiR-*BIR2* lines and *bir2-1* were showing significantly enhanced *PR1* expression upon *Pto* DC3000 infection (Figure 3-16). SA levels were higher in *bir2-1* mutants as compared to Col-0 (Figure 3-16). In conclusion, it becomes clear that *bir2* mutants exhibit stronger SA-dependent defense responses against *Pto* DC3000 compared to Col-0.
Figure 3-16. bir2 mutant show stronger defense responses against Pto DC3000

(A) GC-MS quantification of SA content in 5-week-old leaves of Col-0 and bir2-1, 24 hours after infiltration with DC3000 or mock control. Results represent mean ± SE (n=6). (B) PR1 expression has been monitored by qRT-PCR in leave material of 5-week-old Col-0, bir2-1 and two independent amiRN-BIR2 lines upon infiltration with Pto DC3000 or mock treatment. Expression values were normalized to EF1α and presented as a ratio to Col-0 mock treated samples. Bars represent mean ratios ± SE (n=3). All experiments were repeated at least three times with similar results. Asterisks represent significant differences from Col-0 (*p<0.05, **p<0.01, ***p<0.001, Student’s t-test).

3.2.3.2. bir2 mutants are more resistant to Pto DC3000

Since the MAMP responses are higher and the basal PR1 expression and SA are higher, we would expect a stronger resistance in BIR2-deficient plants. In order to test this hypothesis, plants were infiltrated with Pto DC3000 and bacterial growth was counted at days 0 and 2 post infiltration. The resistance to the virulent bacteria was increased in bir2 mutants coming along with up to 60-fold less growth compared to the respective WT plants at day 2 after infection (Figure 3-17). This result confirms our hypothesis that stronger MAMP response coupled to higher defense responses in bir2 leads to higher resistance to Pto DC3000 by limiting bacterial propagation.
3.2.3.3. **BIR2-deficient plants have no significant defect in BL responses**

In order to check whether BIR2 modulates the BR pathway, a hypocotyl growth assay has been performed on Col-0, bir2-1, two independent amiR-BIR2 lines, Ws-0 and bir2-2 using bak1-4 and bak1-1 as control. Seedlings were grown on vertical ½ MS plates supplemented or not with 10 nM BL during 6 days under long day conditions and the hypocotyl length was measured using the ImageJ software (Figure 3-18). While bak1-4 and bak1-1 show reduction in sensitivity to BL, no clear differences could be observed in the BIR2-deficient plants compared to Col-0 (Figure 3-18).
To support the BL experiment, we performed a brassinazole (BRZ)-induced hypocotyl growth inhibition assay. BRZ is a triazole compound that inhibits BR biosynthesis by reversibly and specifically blocking *DWARF4* activity (Asami et al., 2000). The decreased BR content in the plant leads to a reduction of hypocotyl growth in the dark. We compared the hypocotyl growth of Col-0, *bir2-1*, two independent amiR-*BIR2* lines, Ws-0, *bak1-1* and *bir2-2* in the presence or not of 10 µM BRZ (Figure 3-18). No clear differences could be observed between Col-0 and the *BIR2*-deficient lines without BRZ treatment showing that loss-of *BIR2* has no effect in BL-dependent processes. Furthermore, the hypocotyl length of *bir2* mutants treated with BRZ had similar lengths as Col-0, confirming that there are no obvious effects of *BIR2* on the brassinolide pathway.
Figure 3-18. Knockdown and knockdown of BIR2 has no significant impact on BL responses
Col-0, bak1-4, bir2-1, two independent amiRNA-BIR2 lines (A), Ws-0, bak1-1 and bir2-1 (B) grown vertically for 6 days in long day conditions on ½MS agar plates supplemented or not with 10 nM brassinolide. Hypocotyl length was measured and presented as a ratio of length of treated (T) and untreated (NT). Bars represent the ratio mean ± SE. Asterisks represent significant differences from Col-0 (*p<0.05, **p<0.01, ***p<0.001, Student’s t-test). (C) Phenotypes of Col-0, bir2-1 and two independent amiR-BIR2 lines are shown to illustrate normal growth of bir2 mutants. Growth phenotypes of Col-0, bir2-1, two independent amiR-BIR2 (D), Ws-0 and bir2-2 lines (E) with or without 10µM BRZ grown in the dark. Representative seedlings from at least 15 replicates are shown.
3.2.4. Complementation of bir2-1 and bir2-2 mutants

To confirm that the phenotypes observed in the T-DNA insertion are indeed due to the insertion in BIR2 and not due to any side effect of second-site insertion in bir2-1 and bir2-2, both mutant lines were stably transformed with a construct containing an 1830-base-pair promoter and the coding region of BIR2. Two independent lines for the complementation of bir2-2 were used for further studies and both lines exhibit elf18-induced oxidative burst similar to Ws-0. For the bir2-1 mutant, one line was showing a complemented phenotype. It exhibits Col-0 WT phenotype in terms of morphology, DC3000 growth, Alternaria-induced cell death formation and elf18-induced ROS production. This all set of experiment confirms that the phenotype is indeed due to the mutation at the BIR2 locus.
Results

Figure 3-19. Expression of the coding region of BIR2 under its own promoter fully complements all observed phenotypes.

(A) Morphological phenotype of Col-0, bir2-1 and complemented bir2-1 ProBIR2-BIR2 plants.

(B) Wild-type Col-0, bir2-1 and complemented bir2-1 ProBIR2-BIR2 plants were infiltrated with 10^4 cfu/ml Pto DC3000 and growth of the bacteria was monitored at the indicated time points. Results represent mean ± SE (n=8).

(C) Disease index of Col-0, bir2-1 mutants and complemented bir2-1 ProBIR2-BIR2 at the indicated times after inoculation with A. brassicicola. Results represent means ± SE (n=10). Asterisks represent significant differences from Col-0 (*p<0.05, **p<0.01, ***p<0.001, Student’s t-test).

(D) Representative pictures of A. brassicicola symptom development in Col-0, bir2-1 mutants and complemented bir2-1 ProBIR2-BIR2 plants. ROS production measured over a period of 30 min represented as RLUs in leaf discs of Col-0, bir2-1 and bir2-1 ProBIR2-BIR2 (E), Ws-0, bir2-2 and two independent bir2-2 ProBIR2-BIR2 lines (F) after elicitation with 100 nM elf18. Results are mean ± SE (n = 9). All experiments were repeated at least three times with similar results.
3.2.5. **BIR2 overexpression negatively affects BL and elf18 signaling**

Col-0 plants have been stably transformed with a 35S-\textit{BIR2-YFP} construct in the pB7YWG2 vector (Karimi et al., 2002) and two independent lines have been selected. They present slightly smaller rosette diameter than wild type and exhibit wrinkled leaves with tendencies to upward curling (Figure 3-20). These lines were subjected to several functional analyses.

BIR2 mutant lines did not exhibit any defect in BL signaling. However, we cannot exclude that the lack of phenotype is due to redundancy in the BIR family. To confirm this hypothesis we tested the effect of BRZ on hypocotyl growth of 35S-\textit{BIR2-YFP} lines. Interestingly, 35S-\textit{BIR2-YFP} lines shows differences upon BRZ treatment that are slightly more drastic than those observed in \textit{bak1-4} mutants (Figure 3-20). In a second step, BL-induced hypocotyl growth experiments have been performed on Col-0, \textit{bak1-4} and 35S-\textit{BIR2-YFP} lines. Interestingly, while \textit{bak1-4} is partially insensitive to BL, 35S-\textit{BIR2-YFP} lines present a significant insensitivity (Figure 3-20). This result shows that 35S-\textit{BIR2-YFP} affects BL signaling although their morphology at adult stage is not typical to BL-deficient plants. In conclusion, \textit{BIR2} overexpression leads to a decrease in BL sensitivity pointing to a role of BIR2 not only in MAMP and cell death but also in BL signaling.

These lines were also subjected to elf18-induced growth inhibition assays together with \textit{bak1-4} and Col-0 in order to test the effect in MAMP signaling. Both overexpressing lines present a decrease in responses to elf18 confirming the negative regulation of MAMP responses by BIR2 (Figure 3-20).
Results

Figure 3-20. BIR2 overexpression negatively affects BL and elf18 signaling

(A) Morphological phenotype of Col-0 and two independent 35S-BIR2-YFP overexpressing plants. (B) Col-0, two independent 35S-BIR2-YFP lines and bak1-4 were subjected to seedling growth inhibition assay after 100nM elf18. The one-way ANOVA method was performed combined with the Tukey’s honest significant difference (HSD) test. Significant differences are indicated with different letters (p < 0.01). (C) Hypocotyl length phenotypes of Col-0, bak1-4 and 35S-BIR2-YFP grown in the dark. Mean ± SE of 15 replicates are shown. (D) Col-0, bak1-4 and two independent 35S-BIR2-YFP lines grown vertically 6 days in long day conditions on ½ MS agar plates supplemented or not with 10 nM Brassinazole. Hypocotyl length was measured, ratio of size of treated on untreated seedlings was calculated and compared to the Col-0 control. Asterisks represent significant differences from Col-0 (*p<0.05, **p<0.01, ***p<0.001, Student’s t-test).
3.2.6. BIR2 influences BAK1-dependent complex formation

3.2.6.1. BAK1 dissociates from BIR2 upon ligand binding

In order to understand the molecular mechanism leading to the negative regulatory role of BIR2 on BAK1-dependent MAMP signaling, we hypothesized that BIR2 may sequester BAK1 at basal state and release it once MAMPs are perceived by the cognate receptor FLS2 or EFR. We checked the amount of BIR2 interacting with BAK1 after flg22 or control treatment by co-immunoprecipitation. Less BIR2 was bound to BAK1 in the presence of flg22 compared to mock-treated controls (Figure 3-21). Relative quantification of immunoprecipitated BIR2 proteins shows that at least 1/3 of BIR2 is released from BAK1 within 5 min after flg22 treatment, thereby increasing the pool of BAK1 available for binding to FLS2. Furthermore, treatment with different ligands such as pep1 or BL also leads to a partial release of BIR2 from BAK1 (Figure 3-21). This experiment suggests that BIR2 participates in the control of BAK1 in an unstimulated state. Once the ligand is perceived, BAK1 is released from BIR2 to enable binding to ligand-binding receptors.
Figure 3-21. BAK1 dissociates from BIR2 upon several ligands treatments
(A) Arabidopsis seedlings of the indicated genotypes were treated for 5 min with 1 μM flg22 or 1 μM pep1 for 5 min, or 10 nM BL for 90 min. Immunoprecipitation (IP) was performed with anti (α)-BAK1 antibody. Co-immunoprecipitated BIR2 was detected with anti-BIR2 antibody (WB: α-BIR2) and precipitation of BAK1 detected with anti-BAK1 antibody (WB: α-BAK1). Western blot analysis with α-BIR2 and α-BAK1 antibodies of protein extracts before IP show protein input. Coomassie brilliant blue (CBB) staining of the membrane shows protein loading. (B) Signal intensity of co-immunoprecipitated BIR2 was quantified using ImageJ software relative to the precipitated BAK1 signal after background subtraction. Mock-treated Col-0 was set to 1.
3.2.6.2. **BIR2 modulates BAK1-FLS2 interaction upon flg22 treatment**

*Bir2* and amiR-*BIR2* lines exhibit enhanced responses to the flg22. We propose the hypothesis that BIR2 is negatively regulating the interaction between BAK1 and FLS2. This negative regulation might control the amount of BAK1 molecules interacting with FLS2. We first checked FLS2 protein level by Western blot and interestingly amiR-*BIR2* and *bir2-1* lines present elevated amounts of FLS2 protein levels. This effect is independent of SA since *NahG* expression doesn’t restore FLS2 levels (Figure 3-22). BAK1 proteins were then immunoprecipitated and the amount of interacting FLS2 proteins was detected and quantified. Interestingly, we do see that the level of FLS2 interacting with BAK1 was enhanced in flg22-treated *bir2-1* line compared to Col-0. This difference, which was observed in all experiments performed, confirms that a bigger amount of BAK1-FLS2 interaction is occurring in *bir2* mutants compared to WT suggesting that BIR2 negatively regulates BAK1-FLS2 complex formation (Figure 3-22). In the reverse experiment, *BIR2*-overexpressing plants treated with flg22 present reduced FLS2-BAK1 interaction (Figure 3-22). Overexpressor lines have similar amount of FLS2 compared to WT confirming that the effect is not due to changes in FLS2 expression levels but that BIR2 has indeed a direct negative regulatory role on receptor complex formation.
Figure 3-22. BIR2 controls BAK1-FLS2 interaction levels independently of SA
(A) Western blot analysis with α-FLS2 and α-BAK1 antibody detects protein levels of FLS2 and BAK1 in Col-0, amiR-BIR2 #1, bir2-1, NahG, NahG bir2-1 and 35S-BIR2-YFP #2 plants treated or not with 1 µM flg22. Coomassie brilliant blue (CBB) staining of total protein reveals equal loading. (B) and (C) Arabidopsis seedlings of the indicated genotypes were treated for 5 min with 1 µM flg22 or a mock control. Immunoprecipitation (IP) was performed with anti (α)-BAK1 antibody. Co-immunoprecipitated FLS2 was detected with anti-FLS2 antibody (WB: α-FLS2) and precipitation of BAK1 detected with anti-BAK1 antibody (WB: α-BAK1). (D) ROS production measured over a period of 30 min represented as RLUs in leaf discs of Col-0, bak1-4, bir2-1, NahG, NahG bir2-1 after elicitation with 100 nM flg22. Results are mean ± SE (n = 9). All experiments were repeated at least three times with similar results.

3.2.7. Does BIR2 influence the activity/phosphorylation level of BAK1?

Earlier studies showed that overexpression of BAK1 leads to spontaneous cell death. This spreading cell death might be correlated with unwanted phosphorylation events due to loss of control of BAK1. We therefore hypothesized that the spreading cell death might be correlating with a loss of BAK1 phosphorylation...
control by BIR2. In order to check the involvement of BIR2 in this regulation, we performed immunoprecipitation on Col-0, bak1-4 and amiR-BIR2 #1 seedlings. Col-0 seedlings treated with BL or a M/DAMP cocktail containing elf18, flg22 and pep1 have been used as controls. As expected, phosphorylated BAK1 was detectable upon BL treatment (Figure 3-23). A clear band was found as well in the M/DAMP-treated sample. In the amiR-BIR2 line, no phosphorylated BAK1 was detected suggesting that either BIR2 might act in another way than control of BAK1 basal phosphorylation state, or BIR2 homologues might act redundantly with BIR2 taking over the role in control of BAK1 basal phosphorylation state. More detailed analyses on the phosphorylation events between BAK1 and BIR2 are needed to understand their role in BAK1-dependent signaling.

![Figure 3-23](image.png)

**Figure 3-23. BIR2 knockdown doesn’t affect BAK1 phosphorylation state**

*Arabidopsis* seedlings of the indicated genotypes were treated for 5 min with a cocktail containing 1 μM flg22, 1 μM elf18 and 1 μM pep1 for 5 min, or 10 nM BL for 90 min. Immunoprecipitation (IP) was performed with α-BAK1 antibody. Precipitation of BAK1 was detected with α-BAK1 antibody and with an α-phosphoSer/Thr (α-phos) antibody. Western blot analysis of protein extracts with α-BAK1 antibodies before IP shows BAK1 proteins input.

**3.2.8. Identification of potential BIR2 interactors by co-immunoprecipitation followed by mass spectrometry analysis**

To identify potential interactors that might be controlled by BIR2 or might modulate the BIR2 function, a co-immunoprecipitation followed by MS analysis has been performed. The high quality of the α-BIR2 antibody allowed us to per-
form this experiment with wild type plants. The experiment has been performed in the Col-0 and the Ws-0 ecotypes with the respective knockdown or knockout plants (amiR-BIR2 #1 and bir2-2) as negative controls for the experiment to be able to detect unspecific interactions. 10 g of seedlings from each genotype were used as starting material. The immunoprecipitate was then submitted to LC-MS/MS analysis. Candidates were sorted among all co-immunoprecipitated proteins through three main criteria: the peptide signal intensity that represent the amount of peptide signal per protein, through the fact that candidates appears in Col-0 and Ws-0 but not in both negative controls and depending on the relevance of the candidate. BIR2 appears to have the strongest peptide intensity in all experiments, BIR2 proteins being absent in bir2-2 and strongly reduced in amiR-BIR2 line, confirming that the antibody has a strong affinity for BIR2 (Figure 3-24). Interestingly, BIR3 is present in all samples tested at a quite strong intensity suggesting that some affinity of the antibody to BIR3 is occurring. BAK1 can be co-immunoprecipitated in Col-0 and Ws-0 but also appears to a lesser extent in the negative controls. This might occur because of its interaction with BIR3, the BIR protein that had the highest affinity to BAK1 in all interaction studies. As proof of concept, calnexins, calreticulins and ten different 14-3-3 were found to interact with BIR2 in our experiment as well. Interestingly, three LRR-RLKs named RLK1, RLK2 and RLK3 were found in our interactors candidate list. Two superoxide dismutases, enzymes involved in reactive oxygen species production were also found, possibly linking BIR2 to ROS production. Four ATPases and a number of phosphatases were identified as well. In the future, further experiments will be done to confirm the interaction of BIR2 with those candidates.
## Results

### Figure 3-24. IP followed by LC-MS/MS analysis led to the identification of BIR2-interacting candidates

Immunoprecipitation on 12-day-old Col-0, amiR-BIR2, Ws-0 and bir2-2 was followed by LC-MS/MS analysis in order to identify BIR2-interacting partners. Potential interacting proteins were sorted by protein family. The colour (from white to black) represents the peptide signal intensity in a logarithmic scale.

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**Note:** The table shows the peptide signal intensity for each protein family, with Col-0, Ws-0, amiR-BIR2 #2, and bir2-2 as conditions. The intensity scale ranges from 0 to 9, with higher values indicating stronger signal.
3.3. The BAK1-Interacting Receptor 3

In a second part of this thesis, we started to characterize the BAK1-interacting receptor 3 (BIR3) that shows a very high affinity to BAK1 and is a close relative of the previously described BIR2. However, the characterization is not as complete as for BIR2, but it brings some new insights about the function of BIR proteins.

3.3.1. Localization of BIR3

As an LRR-RLK, BIR3 is expected to localize to the plasma membrane. In order to verify the potential membrane localization of BIR3, stable transgenic Arabidopsis lines expressing a 35S-BIR3-YFP construct were produced. The localization was visualized using laser scanning confocal microscopy and the presented pictures show an intersection at the equatorial zone of the plant epidermal cells. Without treatment, YFP fluorescence localizes to the periphery of the cell and plasmolysis analysis confirmed the plasma membrane localization of BIR3-YFP fusion protein (Figure 3-25).

Figure 3-25. Subcellular localization of the BIR3-YFP fusion protein
35S-BIR3-YFP was expressed in Arabidopsis plants. The localization was visualized by laser scanning confocal microscopy. The pictures show an intersection at the equatorial zone of plant epidermal cells. Plasmolysis was triggered by addition of 850 mM NaCl (on the lower panel). Arrows indicate YFP-labelled plasma membrane retracted from the cell wall.
3.3.2. Phenotypical analysis of *BIR3*-deficient T-DNA lines

3.3.2.1. Characterization of *bir3* T-DNA insertion lines

To unravel the function of *BIR3* in BAK1-dependent signaling pathways, two T-DNA insertion lines in the *BIR3* locus were used. To confirm a loss of *BIR3* transcripts in those lines, RT-PCR analysis was used with two primer pairs. We cannot detect any transcript for *bir3*-2. In *bir3*-1 residual transcript is strongly reduced compared to Col-0 (Figure 3-26). We cannot exclude that the detected product are only non-functional truncated products and PCR nesting the insertion in *bir3*-1 will be needed to verify this hypothesis. All together, both lines present drastic reduction in transcript levels and were used for functional analysis in the different BAK1-dependent pathways.

![Figure 3-26. *bir3* mutant lines show decreased *BIR3* transcripts](image)

(A) Schematic representation of *bir3*-1 and *bir3*-2 T-DNA insertions on the *BIR3* coding region (B) *BIR3* gene expression in Col-0, *bir3*-1 and *bir3*-2 was measured by semi-quantitative PCR analysis using two different primer pairs A and B compared to *EF1α* expression. Representative gel pictures are presented.
3.3.2.2. **BIR3-deficient plants have no significant defects in BL responses**

BAK1, the co-receptor or enhancer of multiple receptors, is known to be involved in the brassinosteroid pathway with BRI1, the MAMP pathways with FLS2 and EFR, the cell death pathway and most likely the DAMP pathway with PEPR1. We first analyzed the role of BIR3 in BL-induced responses in Col-0, *bir3-1* and *bir3-2* plants. First, no clear differences in etiolation could be observed between Col-0 and the *bir3* mutants showing that mutants present no defect in photomorphogenesis. Considering the BL assay, even if a very slight inhibition of the hypocotyl growth could be observed in *bir3-2* compared to Col-0, no statistical significance could confirm it (Figure 3-27). We conclude here that mutation in BIR3 doesn’t clearly influence the BL pathway. The lack of phenotype could be explained by the potential redundancy of BIR proteins.

![Image](image.png)

**Figure 3-27. BIR3 has no significant impact on BL responses**

(A) Seedling growth phenotypes of Col-0, *bak1-4*, *bir3-1* and *bir3-2* grown in the dark for 6 days. Representative seedlings from at least 15 replicates are shown. (B) Col-0, *bak1-4*, *bir3-1* and *bir3-2* were grown vertically for 6 days in long day conditions on ½ MS agar plates supplemented or not with 10 nM Brassinolide. Hypocotyl length was measured and presented as a ratio of length of treated (T) and untreated (NT). Bars represent the ratio mean ± SE (n=18). Asterisks represent significant differences from Col-0 (*p<0.05, **p<0.01, ***p<0.001, Student’s t-test).
3.3.2.3. *bir3* mutants show enhanced responses to elf18 and flg22

The influence of BIR3 on the MAMP pathway was also tested. *bir3-1* and *bir3-2* show a stronger growth inhibition of seedlings upon both flg22 and elf18 compared to Col-0. ROS production upon elf18 in *bir3* mutants and Col-0 confirm the hypersensitivity of *bir3* lines to MAMP treatments (Figure 3-28). Taken together, *bir3-1* and *bir3-2* show higher responses to MAMPs than Col-0 pointing to a negative regulation of the MAMP pathway by BIR3.

Figure 3-28. *bir3* mutants are hypersensitive to MAMP treatment

(A) ROS production measured over a period of 30 min represented as RLUs in 5-week-old leaf discs of Col-0, *bir3-1*, *bir3-2* and *bak1-4* triggered by 100 nM elf18 (B) Total oxidative burst over 30 min upon elf18. Mean and Standard error out of 9 replicates are represented on the graphs. (C) Fresh-weight ratio of 12-day-old Col-0, *bir3-1* and *bir3-2* seedlings grown for 7 days with or without 100 nM of the indicated peptide. The bar graph represents the average fresh-weight ratio ± SE (n=48). Asterisks represent significant differences from Col-0 (*p<0.05, **p<0.01, ***p<0.001, Student’s t-test).
3.3.2.4. *bir3* mutants show no altered *Alternaria brassicicola* phenotype

To test the level of cell death control in *bir3* mutant lines, infection with the fungus *Alternaria brassicicola* was performed and the disease index was calculated after 7 and 10 days. In this assay, no differences could be observed between *bir3* mutants and Col-0 (Figure 3-29) while *bak1-4* and *bir2-1*, used here as control, were showing stronger disease symptoms than Col-0. This points to no effect of *BIR3* knockout in cell death control.

![Figure 3-29. *bir3* mutation have no effect on *Alternaria*-induced cell death](image)

The disease index was monitored in Col-0, *bak1-4*, *bir2-1*, *bir3-1* and *bir3-2* on days 7 and 10 after inoculation with *Alternaria brassicicola* spores. Bars represent the ratio mean ± SE (n>10). Asterisks represent significant differences from Col-0 (*p<0.05, **p<0.01, ***p<0.001, Student’s t-test). A representative of at least three independent experiments is shown.

3.3.3. Phenotypical analysis of *BIR3* overexpressing lines

To get a full overview on BIR3 function, we looked at the effect of overexpression of *BIR3*. Col-0 plants were transformed with a 35S-*BIR3* construct without any tag in the pB2GW7 vector (Karimi et al., 2002). Again, 35S-*BIR3* lines at the T1 generation showed a mild BL-insensitivity morphology. T2 lines exhibit a 1:2:1 segregating phenotype with 25% of WT-looking plants, 50% of mild dwarfism and 25% of strong dwarfism similar to *bri1* mutants suggesting that *BIR3* overexpression leads to reduction in BL sensitivity, that only one insertion is present and that the phenotype stoichiometrically follows *BIR3* expression (Figure 3-30). In con-
results, we see that BIR3 overexpression induces a morphological change apparently similar to a BL-insensitivity phenotype. Since BIR3 is a strong interactor of BAK1, we can hypothesize that this phenotype is due to a repression of BAK1 activity by BIR3. Thus, BAK1 cannot perform its function in BL signaling. Further experiments will be performed in the next chapters to decipher the role of BIR3 overexpression in BL, MAMP and cell death pathways.

Figure 3-30. Morphological phenotype of 35-BIR3 lines
(A) Morphological phenotype of Col-0, bak1-4, two independent hemizygous 35S-BIR3 lines and two independent homozygous 35S-BIR3 lines. (B) Rosette diameter of at least 10 replicates was measured and presented in the graph. The bar graph represents mean value ± SE.

3.3.3.1. BIR3 represses BL signaling

Overexpression of BIR3 in adult plants leads to a strong dwarfism reminiscent to bri1 mutants. To confirm the involvement of BL in this phenotype we performed a BL-induced hypocotyl growth experiments and tested the hypocotyl growth in
the dark. 35S-BIR3 lines exhibit extremely small hypocotyl lengths when they are grown in the dark compared to Col-0 and bak1-4 confirming drastic defects in etiolation. BL-induced hypocotyl growth experiments confirmed that BIR3 overexpressing lines exhibit strong insensitivity to BL. Without BL addition, 35S-BIR3 lines show shorter hypocotyls than Col-0 and bak1-4. Ratios of BL-treated hypocotyl length on untreated hypocotyl length are presented (Figure 3-31). Col-0 exhibits a ratio of 1.8 confirming the hypocotyl elongation effect of brassinolide. bak1-4 mutants show a decrease in BL sensitivity with a ratio of 1.3. Finally, 35S-BIR3 lines have a ratio of 1 confirming that they are completely insensitive to BL. This phenotype supports the bri1 null mutant-like morphological phenotype observed on adult plants. BIR3 overexpression lines are not responding anymore to brassinolide, pointing to a negative regulation of BAK1 by BIR3 that dramatically decreases BL signaling.

**Figure 3-31. BIR3 overexpression compromises BL signaling**

(A) Seedling growth phenotypes of Col-0, bak1-4 and two homozygous 35S-BIR3 lines grown in the dark for 6 days. Representative seedlings from at least 15 replicates are shown. (B) Col-0, bak1-4 and two homozygous 35S-BIR3 lines were grown vertically 6 days in long day conditions on ½ MS agar plates supplemented or not with 10 nM brassinolide. Hypocotyl length was measured and presented as a ratio of length of treated (T) and untreated (NT). Bars represent the ratio mean ± SE. Asterisks represent significant differences from Col-0 (*p<0.05, **p<0.01, ***p<0.001, Student’s t-test).
3.3.3.2. Responses to elf18 are strongly reduced in BIR3 overexpressing lines

BAK1 is a multifunctional co-receptor that is not only involved in BL signal transduction but is also an adapter of FLS2 and EFR. We decided to test if BIR3 overexpression also downregulates MAMP responses. ROS production was followed in Col-0, bak1-4 and BIR3 overexpressors. While ROS production is strongly impaired in bak1-4 compared to Col-0 upon treatment with 100 nM elf18, both independent 35S-BIR3 lines showed also a strong reduction in elf18 responses similar to bak1-4 mutant (Figure 3-32). Overexpression of BIR3 leads to a strong decrease in elf18 signaling confirming the hypothesis that BIR3 overexpression is not only affecting BL signaling but also the BAK1-dependent MAMP signaling.

![Graphs showing ROS production](image)

**Figure 3-32. BIR3 overexpression leads to elf18 insensitivity**

(A) ROS production measured over a period of 30 min represented as RLUs in 5-week-old leaf discs of Col-0, bak1-4 and two independent hemizygous 35S-BIR3 lines triggered by 100 nM elf18. (B) Total ROS production over a period of 30 min upon elf18. Mean and SE out of 9 replicates are represented on the graphs. Asterisks represent significant differences from Col-0 (*p<0.05, **p<0.01, ***p<0.001, Student’s t-test).

3.3.3.3. BIR3 overexpressors have enhanced *Alternaria brassicicola*-induced cell death

Compared to bak1 mutant lines presenting increased *Alternaria*-induced cell death, bir3 mutants present a WT-like phenotype in this pathway. Col-0, 35S-BIR3 lines and bak1-4 were inoculated with *Alternaria brassicicola* spore solutions. The disease index was calculated 7 and 12 days post inoculation and pic-
Results

tures were taken. We see here that BIR3 overexpressors present stronger cell death production upon Alternaria infection with lesions spreading from the infection site as seen in bak1-4 mutants (Figure 3-33). In concert with the BL-insensitivity phenotype and the strong decrease in flg22 and elf18 responses, this phenotype mimicks clearly bak1 mutants phenotype. Indeed, bak1 mutants exhibit reduced BL responses, reduced MAMP responses and stronger cell death production, so do the lines overexpressing BIR3 with a more dramatic phenotype in the BL pathway.

Figure 3-33. BIR3 overexpression suppresses cell death control
(A) Col-0, bak1-4 and two independent hemizygous 35S-BIR3 lines were infected with the necrotrophic fungus Alternaria brassicicola. The disease index was monitored 7 and 12 days post inoculation. Results of at least 10 biological replicates and representative of at least three independent experiments are shown. Bars represent ratio mean ± SE. Asterisks represent significant differences from Col-0 (*p<0.05, **p<0.01, ***p<0.001, Student’s t-test). (B) Representative pictures of the symptom development on infected leaves after 12 days were taken.
3.3.3.4. *BIR3* overexpression strongly represses BAK1-FLS2 interaction

Decrease in flg22 and elf18 induced responses in *BIR3* overexpressing plants suggests that BIR3 might affect the ability of BAK1 to interact with FLS2 and EFR upon ligand perception. To test whether the strong decrease in flg22 response seen in 35S-*BIR3* lines can be correlated molecularly with a decrease in BAK1-FLS2 interaction, BAK1 was immunoprecipitated in Col-0 and 35S-*BIR3* seedlings treated or not with 1 µM flg22 during 5 min. The amount of FLS2 proteins interacting with BAK1 was visualized by Western blot. Immunoprecipitated BAK1 is equal in Col-0 and 35S-*BIR3* seedlings and co-immunoprecipitated FLS2 were clearly detectable in Col-0 upon flg22 treatment. However, barely any FLS2 proteins were interacting anymore with BAK1 upon flg22 treatment in the overexpressing lines (Figure 3-34). This experiment provides strong evidence that decrease in flg22 signaling is due to a repression of BAK1-FLS2 association upon flg22 perception. Since BIR3 is a strong interactor of BAK1, this experiment suggests that BIR3 strongly competes with ligand-binding receptors for the interaction with BAK1.

![Figure 3-34. Overexpression of BIR3 leads to strong reduction in BAK1-FLS2 interaction after flg22 treatment](image)

*Arabidopsis* seedlings of the indicated genotypes were treated (+) or not (-) for 5 min with 1 µM flg22. Immunoprecipitation (IP) was performed with anti (α)-BAK1 antibody. Co-immunoprecipitated FLS2 was detected with anti-FLS2 antibody (WB: α-FLS2) and precipitation of BAK1 detected with anti-BAK1 antibody (WB: α-BAK1).
3.3.4. **BIR3 heteromerizes with BIR2 and BIR1 in *N. benthamiana***

Both BIR2 and BIR3 interact with BAK1 and control different pathways depending on BAK1. However, we don’t know yet if complexes are simply dimeric or if they involve more components. Furthermore, we wanted to address whether BIR proteins might interact and regulate each other. We tried to co-immunoprecipitate tagged BIR2 and BIR3 together after transient expression in *N. benthamiana*. Interestingly, BIR2 and BIR3 co-immunoprecipitated with each other illustrating in both combinations tested, confirming that these two proteins indeed interact (Figure 3-35). Furthermore, we tested if BIR1, BIR2 and BIR3 can form heterodimers in a BiFC assay. All three proteins can heterodimerize in this assay. Therefore, this observation indicates that BIR proteins might act together to exert their control on BAK1 bringing another layer of complexity in the BIR-mediated BAK1 regulation.
Figure 3.5. BIR1, BIR2 and BIR3 can heteromerize in vivo

(A) Co-immunoprecipitation of BIR3-myc with BIR2-YFP transiently expressed in N. benthamiana leaves. Total proteins were subjected to immunoprecipitation with anti-YFP antibody and followed by immunoblot analysis with anti-myc and anti-GFP antibodies to detect BAK1 and BIR proteins respectively. (B) Co-immunoprecipitation of BIR2-myc and BAK1-myc with BIR3-YFP transiently expressed in N. benthamiana leaves. Total proteins (input) were subjected to immunoprecipitation (IP) with anti-YFP antibodies followed by immunoblot analysis with anti-myc antibodies to detect BAK1 and BIR2 and anti-YFP antibodies to detect BIR3. (C) Bimolecular fluorescence complementation analysis was performed with full-length protein fused to the N- or the C-terminal of the YFP and expressed under an ubiquitin promoter as indicated. Fluorescence was visualized by epifluorescence microscopy.
4. Discussion

BAK1 is a general regulator of several signaling pathways (and more that are likely to be identified in the future), allowing full signaling capacity of ligand-binding receptors and activation of downstream responses (Chinchilla et al., 2009; Liebrand et al., 2013a). BL, flg22, elf18 and pep1 signal through the interaction of BAK1 with the respective ligand-binding LRR-RLKs BRI1, FLS2, EFR and PEPR1/2 (Chinchilla et al., 2009). BIK1 interacts with BAK1 in order to positively regulate flg22, elf18 and chitin signaling but BAK1 is also critical in RLP-mediated MAMP responses like SCFE1 (Zhang et al., 2013b). Additionally, BAK1 interacts with the LRR-RLK BIR1 and BON1 to regulate cell death containment through a yet unknown mechanism (Gao et al., 2009; Wang et al., 2011).

4.1. BIRs interact constitutively with BAK1 at the plasma membrane

We identified two new BAK1-interacting proteins BIR2 (At3g28450) and BIR3 (At1g27190) by IP-MS/MS analysis (Mazzotta, 2012). More recently, a similar approach showed that both proteins also interact with SERK1 (Smaczniak et al., 2012). BIR2 and BIR3 belong to the LRR-subgroup Xa together with BIR1 and the close homologue of BIR3, At1g69990, that we named BIR4 (Halter et al., 2014). The LRR-subgroup X contains also BRI1 and BRI1-like members as well as EMS/EXS that have all been shown to interact with BAK1 or other SERK proteins (Li et al., 2002; Nam and Li, 2002; Albrecht et al., 2005; Chinchilla et al., 2009; Fabregas et al., 2013) suggesting that LRR-subgroup X members present high affinity for BAK1 or more generally the SERK family. Interestingly, BIR proteins have a short 4,5 LRR extracellular domain suggesting that they are not perceiving a ligand and that they might act preferentially as regulatory factors. In contrast to other BAK1-RLK associations requiring ligand-binding, BIR proteins interact with BAK1 at the plasma membrane in a constitutive manner, as shown by in planta experiments including FRET-FLIM, BiFC and CoIP, indicating that BIR proteins act through a different molecular mechanism than ligand-binding receptors.
Interestingly, the previous published BIR1 (Gao, 2009 #615) was not found in our IP-MS experiments and shows the lowest FRET efficiency indicating that BIR1 interacts only weakly with BAK1 compared to the two newly identified BIR2 and BIR3. As shown in a yeast two-hybrid assay, the BIR1 kinase domain failed to interact with BAK1 suggesting that the extracellular LRR domain or/and the transmembrane domain are needed for the interaction. It remains to be elucidated whether BIRs extracellular domains are generally required for the interaction as shown with BAK1 and its ligand-binding receptor partners.

Taken together, we confirmed that all BIR family members can interact with BAK1 and that BIR2 and BIR3 show the higher affinity for BAK1 compared to BIR1.

4.2. BIR2 and BIR3 function as pseudokinases

As described earlier, BIR1 exhibits autophosphorylation activity indicating that it is an active kinase (Gao et al., 2009). A kinase-dead mutant of BIR1 partially complements the bir1 phenotype suggesting that the kinase activity of BIR1 is necessary at least for some aspect of its function (Gao et al., 2009). In general, protein kinases rely on the Lys of the Val-Ala-Ile-Lys (VAIK) motif to position ATP during the phosphotransfer, on the Asp of the His-Arg-Asp (HRD) motif within the catalytic loop acting as a catalytic residue and on the Asp within the Asp-Phe-Gly (DFG) motif in the activation loop to bind Mg$^{2+}$ to coordinate the $\beta$ and $\gamma$ phosphates of ATP (Hanks et al., 1988). In BIR2 as well as in BIR3 and BIR4, both HRD and DFG motifs are degenerated. In Arabidopsis, the pseudokinase STRUBBELIG (SUB) exhibits similar mutations as BIR2 and point mutation studies confirmed that SUB does not require kinase activity to function in organ shape control (Chevalier et al., 2005). Furthermore, following a phylogenetic approach, we could show that BIR1 falls into a separate clade than the three other BIR members. The BIR1 clade includes homologues that, beside the D in the HRD motif that is not present, contain the key residues for kinase activity while homologues in the BIR2-4 clade contain several mutations in those key residues. In contrast to BIR1, additional experiments performed in our lab didn’t reveal autophosphorylation for the three other members (Mazzotta, 2012). Characterization of the crystal structure of the BIR2 kinase domain confirmed its inability to bind
ATP and therefore to exert any kinase activity (Blaum et al., 2014). Therefore, BIR2 belongs to the large group of pseudokinases or atypical kinases (Castells and Casacuberta, 2007). Although we are lacking structural and functional evidences, BIR3 and BIR4 most likely also fall into this group because of the lack of conserved residues usually necessary for kinase activity. Taken together, all these observations suggest that BIR2 as well as BIR3 and BIR4 are unable to phosphorylate their substrate and exert their function independent of this activity. More than 10% of the predicted human kinases are actually atypical kinases. In animals, several pseudokinases have been characterized. They can act as scaffold proteins, helpers in the formation of multiprotein complexes or can control the activity of active kinases. However, only few plant pseudokinases have been characterized until now such as SUB (Chevalier et al., 2005), CORYNE (Nimchuk et al., 2011) and hopZ-ETI-deficient1 ZED1 (Lewis et al., 2013; Lewis et al., 2014). In Arabidopsis, 13% of the kinome are non-active pseudokinases and this statistic rises to ~20% for the RLK family (Boudeau et al., 2006; Castells and Casacuberta, 2007) implicating that evolutionary development lead to inactive variants that have evolved new functions independent of their enzymatic activity. Arise of the BIR pseudokinases, most likely happened after the duplication of a BIR1 ancestor enabling BIR2 and BIR3 to diverge from the BIR1 function.Conserving the ability to interact with BAK1, we propose that during evolution, BIR2, BIR3 and BIR4 might have developed different new modes of BAK1 regulation as compared to BIR1.

4.3. Differential regulation of BIRs gene expression

BIR expression levels were analyzed using available microarray data. Firstly, BIR4 is only very weekly expressed if at all compared to the three other BIR genes that are expressed in all tissues. Importantly, BIR1 and BIR2 are strongly induced upon application of several P. syringae strains. However, this observation is not true when Arabidopsis is treated with the virulent Pto DC3000 strain suggesting that expression of BIR genes is under control of some effectors. Furthermore, BIR2 is strongly upregulated upon flg22 treatment while BIR1 only present a slight increase in expression. This observation supports the absence of phenotypes of bir1 mutants in the flg22 pathway (Gao et al., 2009). We therefore
hypothesized that BIR2 might be a component of MAMP signaling pathways. *BIR3* is antagonistically expressed showing downregulation upon treatment with biotrophic bacteria or several MAMPs, suggesting that BIR3 might have a different function than BIR2 in the defense pathway.

Taken together, microarray analysis revealed an expression pattern that is a first hint for a potential role of BIRs in defense against bacteria especially the MAMP pathway. Interestingly, each member is differentially regulated suggesting that the functions might diverge between the different members. In order to get insights into the role of the BIR family in *Arabidopsis*, we decided to focus on the functional analysis of *BIR2* and *BIR3*.

**4.4. Knocking out BIR2 differentially affects BAK1-related pathways**

Implication of BIR2 in the different BAK1-related pathways was tested. *BIR2*-defective lines did not show any significant difference to WT in etiolation and BL-induced hypocotyl growth-induction assays suggesting that loss of *BIR2* does not affect BL signaling. This observation is supported by the unaffected expression of *BIR2* by BL. But redundancy in the BIR family might also explain this lack of phenotype. Importantly, BIR2 negatively regulates MAMP responses. Indeed, flg22 and elf18 early and late responses are strongly enhanced in *bir2* mutants. In the opposite assay, overexpression of *BIR2* strongly compromises flg22-induced responses confirming that BIR2 negatively regulates BAK1-dependent MAMP responses. Furthermore, these observations correlate with stronger disease resistance, higher SA production as well as higher *PR1* induction in response to *Pto* DC3000 in the mutants. Depletion of *BIR2* also leads to a loss of cell death control. Restriction of necrosis upon *Alternaria brassicicola* application is lost in *bir2* mutants exhibiting bigger lesions coming along with an augmented growth of the fungi. While *bak1* mutants exhibit a loss of MAMP and BL signaling and cell death containment, *bir2* present no changes in BL signaling, stronger MAMP signaling and a loss in cell death containment. In conclusion, we could show that BIR2 differentially affects BAK1-dependant pathways and that the pathways are independently regulated.
4.5. BIR2 negatively controls MAMP signaling directly at the BAK1-FLS2 complex formation

A great emphasis has been given to study positive regulators of signaling. However, negative regulation is necessary to keep signaling pathways under control. Indeed, the rice Xb15 and the Arabidopsis KAPP phosphatases have been shown to negatively regulate innate immunity and cell death via direct interaction with ligand-binding receptors (Gómez-Gómez et al., 2001; Park et al., 2008). Moreover, in Arabidopsis, BR signaling is doubly controlled at the BRI1 receptor level. In a first step, a BRI1 auto inhibitory domain is released upon BL perception and as a second step, dissociation of the negative regulator BKI1 allows BRI1-BAK1 dimerization (Wang et al., 2005; Wang and Chory, 2006; Jaillais et al., 2011b). Thus, multiple mechanisms are used to avoid signaling in the absence of ligand. The possibility that an active kinase is controlled by an inactive pseudokinase is reminiscent to the Janus tyrosine kinases although the kinase and the pseudokinase domains are here situated in the same protein (Boudeau et al., 2006). Indeed, in JAK isoforms, the pseudokinase domain directly interacts with the kinase domain in the absence of ligand to control its activity in the absence of cytokines (Boudeau et al., 2006).

BIR2 has a negative regulatory effect on flg22 and elf18 signaling. MAMP signaling depends partially on SA and therefore upregulation of MAMP responses in bir2 mutants could be possibly due to side effect of the cell death. However, bir2 mutants show increased MAMP response through an SA-dependent and independent mechanisms since flg22 signaling is only partially reduced when SA is downregulated by expression of the bacterial NahG gene (Halter et al., 2014). Thus, a second hypothesis emerges with BIR2 directly affecting MAMP responses at the receptor level for example by the control of BAK1 complex formation. Co-IP experiments showed that BAK1 binding to FLS2 is increased in bir2 mutants confirming that BIR2 has a direct negative regulatory effect on BAK1-FLS2 complex formation. Interestingly, as observed with BIR1 expression levels, FLS2 protein content is augmented in bir2 mutants. Furthermore, crossing of bir2 mutants with NahG lines didn’t affect FLS2 protein levels indicating that this phenomenon is independent of the SA pathway. Unwanted MAMP signaling might
occur in the mutants inducing $FLS2$ expression in a feedback regulatory loop (Zipfel et al., 2006). Lack of BIR2 could lead to transient $FLS2$-$BAK1$ dimerization in the absence of ligand. Although the interaction rate is much lower, this hypothesis implies that negative regulation of $BAK1$ by BIR2 is necessary even in the absence of ligand. We propose here that BIR2 mediates this negative regulatory effect through direct interaction with BAK1 and most possibly the whole SERK protein family.

However, this increase of $FLS2$ expression might be the explanation for the augmentation of $BAK1$-$FLS2$ dimers upon flg22 treatment. To confirm a direct negative role of BIR2 on complex formation, the IP experiment has been performed in the $BIR2$ overexpressors since $FLS2$ levels are not affected in this background. In this complementary assay, $BAK1$-$FLS2$ dimerization level is clearly reduced showing a direct negative effect of BIR2 on $BAK1$-$FLS2$ interaction. Interestingly, $bir1$ mutants did not exhibit any increase in flg22 responses (Gao et al., 2009) suggesting that BIR2 evolved a new function in this pathway compared to BIR1.

It will be interesting to address whether this mode of action is also true for EFR and PEPR1. Moreover, BAK1 has been shown recently to play a positive role in SOBIR1-dependent RLP-mediated MAMP-triggered immunity. Although a direct interaction of BAK1 with SOBIR1-RLP complexes has not been reported yet (Liebrand et al., 2013a), it would be interesting to address whether responses to SCFE1 (Zhang et al., 2013b) are also augmented in $bir2$. Furthermore, we could show that BIR2 also interacts with SERK1 and further experiments will possibly show an interaction with the other SERK members. We can expect that BIR2 also function in a similar way in pathways requiring the other SERK members (Chinchilla et al., 2009; Liebrand et al., 2013a).

Taken together, we deciphered here a new regulatory mechanism of receptor complexes signaling where an LRR-RLK negatively controls another LRR-RLK via direct interaction in order to limit downstream responses.

### 4.6. BIR2 negatively regulates BAK1 at basal state

BIR2 negatively controls flg22 signaling through regulation of $BAK1$-$FLS2$ association. We hypothesized that BIR2 functions at a basal state by competing
with FLS2 for the interaction with BAK1. Dissociation of BAK1 from BIR2 is therefore expected upon treatment allowing the free BAK1 to be recruited by a ligand-binding receptor. Stimulation with either flg22, BL or pep1 provokes partial dissociation of BIR2-BAK1 complexes while simultaneous stimulation with BL, flg22, elf18 and pep1 leads to the dissociation of the majority of BIR2-BAK1 duplexes, suggesting that in the absence of ligand, BAK1 is kept in complex with BIR2 in subpools with specific receptors in preformed complexes (Halter et al., 2014). Further evidence supporting the existence of preformed complexes was provided by visualization of the BRI1-BAK1 heteromers by extended microscopic imaging (Bücherl et al., 2013).

Binding of the ligand to its cognate receptor might on one hand lead to conformational changes and on the other hand create an additional binding site for BAK1 as shown for the FLS2-flg22-BAK1 crystal structure, increasing the affinity of BAK1 to FLS2. Upon ligand perception, BAK1 shuttles from BIR2 to the ligand-binding receptors. The bak1-5 mutant nicely exemplifies this hypothesis. In bak1-5 plants, BAK1 constitutively interacts with FLS2, BRI1 or EFR (Schwessinger et al., 2011). Correlating with a stronger affinity with the ligand-binding receptors, interaction level of BAK1 for BIR2 interaction drastically decreases in bak1-5 (Halter et al., 2014).

This hypothesis implies that dissociation of BIR2-BAK1 duplexes precedes FLS2-BAK1 association. Since BAK1-FLS2 oligomerization occurs within less than a 1s (Schulze et al., 2010), we would expect a fast dissociation and association implicating a close vicinity of the three proteins inside the membrane. Alternatively to an affinity shift after ligand binds to the ligand-binding receptor, an active mechanism through BAK1 modification leading to dissociation might occur. The activated receptor would then directly modify BAK1 possibly through phosphorylation provoking the dissociation. In a first step, the activated receptor would phosphorylate BAK1 provoking its release from BIR2. In a second step, BAK1 would then be recruited.
Figure 4-1. Schematic representation of BIR2 regulation.
At unstimulated state, BIR2 constitutively interacts with BAK1. This interaction keeps BAK1 under control in order to avoid unwanted BAK1-PRR interaction. Upon ligand perception, BAK1 dissociated from BIR2 and is incorporated in a complex with the PRR enabling signal transduction and MAMP-triggered immune response.

4.7. Cell death controlled by BIR2 and BAK1

Both bir2 and bak1 mutants are unable to contain cell death. bir1 mutants are also compromised in this pathway. Following genetic investigations, the authors propose that two independent R gene-mediated pathways, one PAD4-dependent and the other SOBIR1-dependent are constitutively active in bir1 mutants, leading to SA-dependent autoimmune response (Gao et al., 2009). In addition, BIR1 and BAK1 interact with BON1 (Wang et al., 2011) that negatively regulates another R gene, the TIR-NB-LRR SNC1 suggesting that the bir1 cell death phenotype includes SNC1-dependent autoimmune response. A guarding model might be true for BIR2 as well where loss of BIR2 is sensed and activates a yet unknown guard system. It would be interesting to check if SOBIR1 also acts in BIR2-mediated cell death control. Furthermore, overexpression of SOBIR1 leads to similar autoimmune responses suggesting that SOBIR1 acts as the defense activator (Gao et al., 2009). However, the signaling pathway possibly depending on an R gene remains to be elucidated. Recently, a study using a suppressor screen identified the components leading to autoimmune cell death phenotype in mekk1,
mkk1mkk2 and mpk4 mutants (Gao et al., 2008; Kong et al., 2012). This MAPK pathway negatively regulates SUMM1 (SUPPRESSOR OF mkk1mkk2 1), also known as MEKK2 (Kong et al., 2012; Su et al., 2013). MEKK2 activates SUMM2, a CC-NB-LRR when MPK4 downregulation is lost (Kong et al., 2012; Zhang et al., 2012; Su et al., 2013). Furthermore, inactivation of MPK4 by the HopAI effector leads to SUMM2-mediated immunity (Zhang et al., 2012). Similar to SOBIR1, MEKK2 overexpression leads to strong defense activation, in this case through SUMM2. In a similar way to MPK4, loss of BAK1, BIR1 or BIR2 might activate a component like MEKK2 or SOBIR1.

Interestingly, overexpression of BAK1 also leads to runaway cell death (Belkhadir et al., 2012). Simultaneous overexpression of BRI1 suppresses the runaway cell death suggesting that BAK1-interacting partners such as BIR1 or BIR2 are necessary to avoid autoimmunity (Belkhadir et al., 2012). BIR1 and BIR2 proteins might act as docking platforms for BAK1 for its negative control. Loss of function of one of these two proteins activates autoimmunity possibly through a mimic of BAK1 overexpression. It will be of first interest in the future to determine through double mutant analysis whether the cell death phenotype in bir1 and bir2 mutants is genetically dependent on BAK1 or other SERK proteins.

It remains to be elucidated whether BAK1 proteins uncontrolled by the BIR family possibly phosphorylate a yet unknown component at the plasma membrane leading to cell death formation. Unfortunately, we couldn’t detect an increase in autophosphorylation activity of BAK1 in bir2 mutants suggesting either that other BIRs take over BIR2 function or that it works through a different mechanism. The same experiment in bir1, bir1bir2 or even bir1bir2bir3 triple mutants might confirm the hypothesis of a phosphorylation-dependent mechanism.

Another hypothesis would explain why mutation and overexpression of BAK1, BIR1 and BIR2 leads to cell death formation. Some components might sense integrity of BAK1-BIR dimers suggesting that a tight regulation of protein levels needs to take place and that a system similar to a guarding system would detect an imbalance in BIR-BAK stoichiometry. In the future, new components need to be discovered in order to decipher RLK-mediated cell death control. Genetic analysis by using suppression screen as well as Co-IP-MS analysis will give
chances to identify new components involved in the RLK-mediated cell death containment.

4.8. Are there more components in the BAK1-BIR2-FLS2 regulation?

Mechanistic dissection generally implicates to first work on simple systems consisting of few proteins as we did with BAK1, BIR2 and FLS2. However, more components likely participate in this regulation. BIK1 plays a positive role in flg22 signaling as well as in cell death containment (Veronese et al., 2006; Lu et al., 2010b; Zhang et al., 2010; Laluk et al., 2011). Upon flagellin perception, BIK1 and BAK1 transphosphorylate each other and subsequently phosphorylate FLS2 (Lu et al., 2010b). Although BIK1 appears to be a downstream component of BIR2-BAK1-FLS2, we cannot exclude a direct interaction of BIK1 with BIR2 as well. BSK1, another RLCK, resembles BIK1 in terms of function and might act similarly as BIK1 (Shi et al., 2013b; Shi et al., 2013a). Furthermore, BON1 interacts directly with BAK1 and BIR1 (Wang et al., 2011). bon1 mutants present increased cell death in a SNC1-dependant manner (Yang and Hua, 2004). It would be therefore interesting to investigate on one hand whether BON1 interacts with BIR2 or other BIR members and on the other hand if BON1 itself might influence BAK1-ligand-binding receptor complex formation.

Members of the LRR X protein family interact preferentially with SERK members. However, whether BIR2 regulates other receptors than BAK1 and the SERKs remains an open question. Furthermore, we cannot exclude a regulation of BAK1 by BIR2 together with a third protein in a multimeric complex. To answer those questions, we aimed to identify new BIR2 interactors by IP with α-BIR2 antibodies followed by MS analysis. Several potential new interactors were identified. While it remains to be confirmed whether the interaction really occurs in planta, some candidate might provide new insight into BAK1 and BIR2 regulation.

A huge bunch of 14-3-3 proteins were identified. They are known to play a role in BL signaling and to interact with BAK1 (Gampala et al., 2007; Oecking and Jaspert, 2009; Jaspert et al., 2011). However, redundancy in this huge family render genetic analysis complicated. In the future, the use of multiple mutants will
provide evidences for a role of 14-3-3 in defense signaling and especially in BAK1-related mechanisms. Furthermore, three LRR-RLKs were identified suggesting that BIR2 might play a role in the regulation of these receptors or inversely that these receptors control BIR2-mediated regulation. Recently, a direct regulation of ROS production by BIK1 via direct interaction with RbohD has been described (Kadota et al. 2014). We identified superoxide dismutases as potent interactors of BIR2 that might provide another direct link between receptor and ROS production. Furthermore, the calcium-sensing CAS receptor that regulate defense signaling possibly at the plasma membrane or at the chloroplast was also found (Han et al., 2003; Nomura et al., 2012).

Investigation of the interaction together with in depth functional analysis will validate a role of these promising candidates together with BIR2 in BAK1-dependent mechanisms or more generally in defense signaling.

4.9. **BIR3, another negative regulator of BAK1**

In addition to BIR2, we started analyzing the function and mechanism of BIR3. This topic is of course not yet complete but exerts additional insights into the function of BIR family proteins. BIR3, another member of the BIR family was co-immunoprecipitated with BAK1. Through the all subfamily, BIR3 exhibits the strongest interaction with BAK1. While BIR1 and BIR2 expression are upregulated upon biotrophic bacteria or MAMP treatment, BIR3 expression is diminished suggesting that its regulation of defense could be opposite to what is observed for BIR1 and BIR2. Kinase domain of BAK1 is not able to phosphorylate BIR3 kinase domain in in vitro experiments suggesting that BIR3 acts differentially to BIR1 and BIR2. To shed light on BIR3 function, we tested the role of BIR3 in the different BAK1-related pathways. Both bir3 mutant lines showed no significant differences in BL and cell death pathways compared to WT suggesting that it does not function in these pathways or that other BIRs are redundant to BIR3. Interestingly, flg22 and elf18 early and late responses were increased in both knockout lines pointing to a specific role of BIR3 in MAMP signaling. BIR3 does not affect cell death control and BL signaling but negatively regulates MAMP responses showing that it shares the same function with BIR2 in MAMP signaling and that BIR2 and BIR3 differentially regulate BAK1-dependent pathways.
The lack of phenotype or mild phenotype in single mutant lines can often be explained by redundancy. In order to overcome this problem, we generated BIR3 overexpressing lines. These lines exhibit strong dwarfism reminiscent of bri1 mutant phenotypes. BL assays confirmed the strong BL insensitivity in those lines. Further characterization of BIR3 overexpressors showed a strong decrease in flg22 and elf18 insensitivity and a loss of cell death control. Furthermore, response to chitin is not disturbed by BIR3 overexpression supporting that BIR3 only regulates MAMP responses mediated by BAK1. Although we need to confirm that BIR3 is not acting by directly repressing ligand-binding receptors, we can assume that BIR3 as a strong BAK1-interacting receptor directly inhibits BAK1 and likely other members of the SERK family. Interestingly, BIR2 overexpression also leads to downregulation of BL and flg22 responses together with a higher necrosis formation upon Alternaria brassicicola application. The BL insensitivity is clear in BL hypocotyl growth assays, but adult 35S-BIR2-YFP plants are only slightly smaller and do not exhibit the typical BL-insensitive morphology like bak1 or bri1 mutants suggesting that BIR3 is more prominent in the BL pathway than BIR2.

Hemizygous BIR3 overexpressing lines have bigger rosettes than the homozygous lines suggesting that BL insensitivity in BIR3 overexpressors is stoichiometrically correlating with its expression level. BIR3 has the highest affinity for BAK1 within the BIR family. It might directly repress and therefore titrate out BAK1. BIR3 would bind BAK1 rendering it unavailable for ligand-binding receptors resulting in downregulation of BAK1-dependent pathways implicating that BIR3 would outcompete BAK1 from ligand-binding receptor complexes.

Taken together, overexpression of BIR3 leads to a complete block of MAMP signaling and uncover two additional roles of BIR3 as a repressor of BL signaling and as a positive regulator of cell death.

4.10. Do BIR2 and BIR3 act together?

We tested the interaction of BIR proteins with each other and could show that BIR1, BIR2 and BIR3 can form heteromers when transiently expressed in N. benthamiana. This suggests that BIR proteins might act in concert to perform their regulation on BAK1. This finding brings a new layer of complexity in BAK1
regulation by BIR proteins. We can imagine that, for example, BIR2 might perform its regulation not only as dimer with BAK1 but by interacting with BAK1 and BIR3 or BIR1 at the same time. Furthermore, we cannot exclude that BIRs can regulate each other. One could imagine that one BIR protein regulates another through scaffolding or transphosphorylation, and this BIR would then act on BAK1. For example, BIR2 might regulate BIR1 in the cell death pathway and the lack of BIR1 regulation in bir2 mutant would explain the cell death phenotype. In the future, in depth study of BIRs trans-regulation will shed light on this layer of control.

4.11. Ancestral function of BAK1 and BIRs

Lower plants like Physcomitrella or Selaginella are known to respond to several MAMPs and to BL. However, phylogenetic analysis could not identify any close homologue of BRI1 or FLS2 in these species suggesting that the perception of these signals relies on different mechanisms than in higher plants (Boller and Felix, 2009; Cheon et al., 2013). Homologues of BAK1 are present with an homology suggesting that its function could be conserved (Boller and Felix, 2009). Moreover, three homologues close to BIR1 are present in P. patens and S. moellendorfii. Presence of only BIR1 homologues suggests that the three other BIR proteins appeared later and that mutation rendered them pseudokinases with new functions. The main function of BIR1 is to control cell death via R gene-dependent pathways. It will be interesting to find out if these ancestral BAK1 and BIR1 have identical functions as described for higher plants; if they can interact with each other and/or transphosphorylate. However BAK1 might have been a transducer of signal for ancestral receptor or a ligand-binding receptor for ancestral ligand, a role of BAK1 in cell death control in moss and fern would point to an ancestral role of BAK1 as a cell death control component together with BIR1. Furthermore, if BAK1-BIR1 module exists at an early stage of evolution, it would suggest that BIR2-4 evolved to regulate younger functions of BAK1. In the future, study of BAK1 and BIR1 homologues in lower plants will bring insight into their ancestral function.
4.12. Conclusion

In the last decade, BAK1 has emerged as a co-receptor in a variety of pathways as well as an important positive regulator of cell death control. Study of BIR2 and BIR3 led to the understanding of a new BAK1 regulatory mechanism by negatively affecting BAK1 complex formation with ligand-binding receptors. Further molecular studies are needed to dissect the specific mechanistic differences between BIR1, BIR2 and BIR3, to understand how cell death control and interaction and suppression of BAK1-dependent signaling pathways are mechanistically achieved.
5. **Summary**

Transmembrane leucine-rich repeat (LRR) receptors are commonly used innate immune receptors in plants and animals but can also sense endogenous signals to regulate development. BAK1, a plant LRR-receptor-like kinase (RLK) with a small extracellular domain, interacts with several ligand-binding LRR-RLKs to positively regulate their functions. BAK1 is involved in brassinosteroid-dependent growth and development, innate immunity, and cell-death control by interacting with the brassinosteroid receptor BRI1, immune receptors, such as FLS2 and EFR, and the small receptor kinase BIR1, respectively. In vivo study of BAK1 complex partners by LC/ESI-MS/MS led to the identification of two novel BAK1-interacting RLKs, BIR2 and BIR3.

Functional analyses of bir2 mutants show differential impact on BAK1-regulated processes, such as hyperresponsiveness to microbe-associated molecular patterns (MAMP), enhanced cell death, and resistance to bacterial pathogens, but have no effect on brassinosteroid-regulated growth. BIR2 interacts constitutively with BAK1, thereby preventing interaction with the ligand-binding LRR-RLK FLS2. MAMP perception leads to BIR2 release from the BAK1 complex and enables the recruitment of BAK1 into the FLS2 complex. These results provide evidence for a new regulatory mechanism for innate immune receptors with BIR2 acting as a negative regulator of MAMP-triggered immunity by limiting BAK1-receptor complex formation in the absence of ligands.

In a second part, functional analysis of BIR3 overexpressing lines has been performed. These lines present a strong decrease in BL signaling with a morphology resembling bri1 mutants. MAMP signaling and cell death control are also downregulated pointing to a negative regulatory role of BIR3 on BAK1.
6. Zusammenfassung

Transmembrane Rezeptoren mit Leuzin-reichen Wiederholungen (LRRs) sind häufig verwendete Rezeptoren in der angeborenen Immunität von Pflanzen und Tieren. Aber sie können auch endogene Signale erkennen um Entwicklungsprozesse zu regulieren. BAK1, eine pflanzliche Rezeptor ähnliche Kinase (RLK) mit einer kleinen extrazellulären Domäne interagiert mit mehreren Liganden-bindenden LRR-RLKs um ihre Funktion positiv zu regulieren. BAK1 ist in Brassinosteroid-abhängiger Entwicklung, pflanzlicher Immunität und Zelltod Kontrolle involviert indem es mit dem Brassinosteroid Rezeptor BRI1, den Immunrezeptoren FLS2 und EFR und der kleinen Rezeptorkinase BIR1 interagiert. In vivo Experimente von BAK1 Komplexpartnern mittels LC/ESI-MS/MS führte zur Identifikation von zwei neuen BAK1 interagierenden RLKs, BIR2 und BIR3.


Im zweiten Teil wurden funktionelle Analysen von BIR3 überexprimierenden Pflanzen durchgeführt. Diese Linien zeigen eine starke Einschränkung im BL Signalweg mit einer Morphologie die bri1 Mutanten ähnelt. Außerdem sind MAMP Signalisierung und Zelltod Kontrolle vermindert, was auf eine negativ regulatorische Rolle von BIR3 auf BAK1 hinweist.
7. References


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reveals amino acid residues critical for receptor formation and activation of BR signaling. Mol Cells 27, 183-190.


### 8. Appendix

#### 8.1. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
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<tr>
<td>BAK1</td>
<td>BRI1-associated kinase</td>
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<tr>
<td>BES1</td>
<td>BRI1 EMS suppressor 1</td>
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<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
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<td>BiFC</td>
<td>Bimolecular fluorescence complementation</td>
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<td>BIK1</td>
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<td>BAK1-like 1</td>
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<td>eMAX</td>
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<td>ETI</td>
<td>Effector-triggered immunity</td>
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<td>NB-LRR</td>
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<td>s</td>
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<td>SA</td>
<td>Salicylic acid</td>
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<td>SERK</td>
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<td>SNC1</td>
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<td>SOBIR1</td>
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8.2. Primers list

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