# Identification and Functional Analysis of *in vitro*/*in vivo* Phosphorylation Sites of the *Arabidopsis* BAK1 Interacting Receptor Kinase BIR2

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# List of abbreviations

| | |
|---|---|
| °C | Degree Celsius |
| µ | Micro ($10^{-6}$) |
| $ | Dollar sign |
| % | Percent sign |
| 35S | Promoter of cauliflower mosaic virus |
| A | Alanine |
| aa | Amino acid |
| *A. brassicicola* | *Alternaria brassicicola* |
| Ade | Adenine |
| ADP | Adenosine diphosphate |
| Ala | Alanine |
| *Alternaria* | *Alternaria brassicicola* |
| amiRNA | Artificial microRNA |
| AMP-PNP | Adenylyl-imidodiphosphate |
| APAF-1 | Apoptotic protease activating factor 1 |
| *Arabidopsis* | *Arabidopsis thaliana* |
| ARF-GEF | ADP ribosylation factor - guanine nucleotide exchange factor |
| Asp | Aspartate |
| *A. thaliana* | *Arabidopsis thaliana* |
| ATP | Adenosine triphosphate |
| *At*Pep1 | *Arabidopsis thaliana* peptide 1 |
| *A. tumefaciens* | *Agrobacterium tumefaciens* |
| avr | Avirulence |
| avrB | Avirulence protein of *P. syringae pv. glycinea* |
| ave1 | Avirulence on Ve1 |
| avrPto | Avirulence protein of *Pseudomonas syringae pv. tomato* |
| avrRpm1 | Avirulence protein 1 from *P. syringae* pv. *maculicola* |
| avrRps4 | Avirulence protein of *Pseudomonas syringae* |
| avrRpt2 | Avirulence protein 2 from *P. syringae* pv. *tomato* |
| BAK1 | BRI1-associated kinase |
| BES1 | BRI1–EMS-suppressor 1 |
| BIK1 | Botrytis-induced kinase 1 |
| BIN2 | Brassinosteroid insensitive 2 |
| BIR | BAK1-interacting RLK |
| BIR | Baculovirus inhibitor-of-apoptosis repeat |
| BKI1 | BRI1 kinase inhibitor 1 |
| BKK1 | BAK1-like 1 |
| BL | Brassinolide |
| BLK | BIK1-like kinase |
| BON1 | Bonzai 1 |
| bp | Base pair |
| BR | Brassinosteroid |
| BRI1 | Brassinosteroid-insensitive 1 |
| BSK1 | BR signaling kinase 1 |
| BSA | Bovine serum albumin |
| BSU1 | *bri1* suppressor 1 |
| BZR1 | Brassinazole resistant 1 |
| C | Cysteine |
| $Ca^{2+}$ | Calcium ion |
| CARD | Caspase-activation and recruitment domain |
| CASK | $Ca^{2+}$/calmodulin-activated serine kinase |
| CBB | Coomassie brilliant blue |
| CBEL | Cellulose-binding elicitor lectin |
| CC | Coiled-coil |
| cDNA | Complementary DNA |

| | |
|---|---|
| CDG1 | Constitutive differential growth 1 |
| CDPK | Calcium dependent protein kinase |
| CEBiP | Chitin elicitor binding protein |
| CED-4 | *Caenorhabditis elegans* caspase 4 |
| CERK1 | Chitin elicitor receptor kinase 1 |
| Cf | *Cladosporium fulvum* |
| cfu | Colony forming units |
| $Cl^-$ | Chloride ion |
| CLV | Clavata |
| CNL | CC-NB-LRR |
| Co-IP | Co-immunoprecipitation |
| Col-0 | Columbia-0 |
| CPK28 | Calcium dependent protein kinase 28 |
| CRE1 | Cytokinin response 1 |
| CRN | Coryne |
| CSK | Dual-specificity kinases |
| csp | Cold shock protein |
| CSM | Complete synthetic medium |
| CT | C-terminus |
| CuRe1 | Cuscuta receptor 1 |
| Catalytic spine | C-spine |
| D | Aspartate |
| DAMP | Danger-associated molecular pattern |
| DD | Death domain |
| DMSO | Dimethyl sulfoxide |
| DNA | Desoxyribonucleic acid |
| dNTPs | Deoxyribonucleotide triphosphate |
| DORN1 | Does not respond to nucleotides 1 |
| dpi | Days post inoculation |
| dsRNA | Double-stranded RNA |
| E | Glutamic acid |
| ECD | Extracellular domain |
| *E. coli* | *Escherichia coli* |
| EDS1 | Enhanced disease susceptibility 1 |
| EFR | EF-TU receptor |
| EGF | Epidermal growth factor |
| e.g. | for example |
| EIX | Ethylene inducing xylanase |
| eMAX | Enigmatic MAMP activity from *Xanthomonas* |
| ERECTA | Erecta (er) |
| ESI | Electrospray ionization |
| ET | Ethylene |
| ETI | Effector-triggered immunity |
| EtOH | Ethanol |
| ETR1 | Ethylene response 1 |
| ETS | Effector-triggered susceptibility |
| EVR | Evershed |
| F | Phenylalanine |
| FDR | False discovery rate |
| FGFR1 | Fibroblast growth factor receptor 1 |
| FLS | Flagellin-sensing |
| fwd | Forward |
| g | Gram |
| *g* | Gravitational acceleration |
| G | Glycine |
| GBP | β-glucan binding protein |
| GFP | Green fluorescent *protein* |
| h | Hour |

| | |
|---|---|
| $H^+$ | *Hydrogen ion* |
| HCD | Higher-energy collisional dissociation/higher-energy C-trap dissociation |
| HcrVf2 | Homologue of *Cladosporium fulvum* resistance of the Vf region 2 |
| His | Histidine |
| HopAO1 | Effector of *Pseudomonas syringae* |
| HopM1 | HRP (hypersensitive response and pathogenicity) outer protein M1 |
| *Hpa* | *Hyaloperonospora arabidopsidis* |
| HPLC | High-performance liquid chromatography |
| HR | Hypersensitive response |
| HRP | Horseradish peroxidase |
| HG | Heptaglucoside |
| IP | Immunoprecipitation |
| IRAK | Interleukin-1 receptor-associated kinase |
| JA | Jasmonic acid |
| JM | Juxtamembrane |
| K | Lysine |
| $K^+$ | Potassium ion |
| KAPP | Kinase-associated protein phosphatase |
| kb | Kilo base pair |
| kDa | Kilo Dalton |
| KD | Kinase domain |
| KO | Knock-out |
| L | Liter |
| L | Leucine |
| LB | Lysogeny broth |
| LC-MS/MS | Liquid chromatography-mass spectrometry/mass spectrometry |
| Leu | Leucine |
| LORE | Lipooligosaccharide-specific reduced elicitation |
| LPS | Lipopolysaccharide |
| LRR | Leucine-rich repeat |
| LYK5 | LysM-containing receptor-like kinase 5 |
| LYM | Lysine-motif |
| LysM | Lysine motif |
| M | Molar |
| m | Milli ($10^{-3}$) |
| $Mg^{2+}$ | Magnesium ion |
| MAMP | Microbe-associated molecular pattern |
| MAPK | Mitogen-activated protein kinase |
| MEKK | MAPK / ERK (extracellular signal-regulated kinase) kinase kinase |
| min | Minute |
| MKK | Mitogen activated protein (MAP) kinase kinase |
| MPK | MAP kinase |
| MS | Murashige and Skoog |
| MS | Mass spectrometry |
| MyD88 | Myeloid differentiation factor 88 |
| m/z | Mass to charge |
| n | Nano ($10^{-9}$) |
| n | Sample size |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| *N. benthamiana* | *Nicotiana benthamiana* |
| NB-LRR | Nucleotide-binding leucine-rich repeat |
| NDR1 | Non-race specific disease resistance 1 |
| NF | Nodulation factors |
| NLP | Ethylene-inducing peptide 1 (Nep1)-like protein |
| NO | nitric oxide |
| NOD-LRR | Nucleotide-binding oligomerization domain leucine-rich-repeat |
| OD | Optical density |
| OE | Overexpression |

| | |
|---|---|
| OG | Oligogalacturonide |
| P | Proline |
| P-site | Phosphorylation site |
| PAD | Phytoalexin deficient |
| PAMP | Pathogen-associated molecular pattern |
| PBL | AvrPphB Susceptible1 (PBS1)-like proteins |
| PBS1 | AvrPphB susceptible 1 |
| PCD | Programmed cell death |
| PCR | Polymerase chain reaction |
| PEP | Posterior error probability |
| PEPR | Pep1 receptor |
| PG | Polygalacturonase |
| PGIP | PG inhibitor protein |
| PGN | Peptidoglycan |
| PKA | Protein kinase A |
| PME | Pectin methyl esterase |
| PopP2 | Pseudomonas outer protein P2 (effector of *Ralstonia solanacearum)* |
| PP2A | Protein phosphatase 2A |
| PP2C | Protein phosphatase 2C |
| PR | Pathogenesis related |
| PRR | Pattern recognition receptor |
| PSKR | PSK receptor |
| PSK | Phytosulfokine |
| PTI | PAMP or pattern triggered immunity |
| PTM | Post-translational modification |
| Pto | AvrPto/AvrPtoB interacting kinase |
| *Pto* | *Pseudomonas syringae pv. tomato* |
| PUB12/13 | Plant U-Box 12/13 |
| PYD | Pyrin domain |
| R | Arginine |
| RaxX | Required for activation of Xa21 X |
| RBOHD | Respiratory burst oxidase homolog D |
| RBPG1 | Responsiveness to botrytis polygalacturonases 1 |
| RCR3 | Extracellular cysteine protease 3 |
| ReMAX | Receptor of eMAX |
| Rev | Reverse |
| R-gene | Resistance gene |
| RIN4 | RPM1-interacting protein 4 |
| RLCK | Receptor-like cytoplasmic kinase |
| RLK | Receptor-like kinase |
| RLP | Receptor-like protein |
| RLU | Relative light units |
| RME | Receptor-mediated endocytosis |
| RNA | Ribonucleic acid |
| ROS | Reactive-oxygen species |
| rpm | Ration per minute |
| RPM1 | Resistance to *Pseudomonas syringae pv. maculicola* protein 1 |
| RPP1 | Recognition of *Peronospora parasitica* 1 |
| R-protei n | Resistance protein |
| RPS | Resistance to *Pseudomonas syringae* protein |
| RRS1 | Resistance to *Ralstonia solanacearum* 1 |
| R-spine | Regulatory spine |
| RT | Room temperature |
| s | Second |
| S | Serine |
| SA | Salicylic acid |
| SAG10 | Senescence associated gene 101 |
| SAR | Systemic acquired resistance |

| | |
|---|---|
| ScFE1 | *Sclerotinia* culture filtrate elicitor1 |
| Sec | Second |
| Ser | Serine |
| SERK | Somatic embryogenesis receptor kinase |
| SILAC | Stable isotope labeling by amino acids in cell culture |
| SNC1 | Suppressor of NPR1-1 constitutive 1 |
| SOBIR1 | Suppressor of *bir1* |
| SOC | Super optimal broth (SOB) with catabolite repression |
| Sod | Superoxide dismutase |
| SR160 | 160-kDa systemin cell-surface receptor |
| STAND | Signal transduction ATPases with numerous domains |
| STD NMR | Saturation transfer difference- nuclear magnetic resonance |
| SUB | Strubbelig |
| T | Threonine |
| T3SS | Type III secretion system |
| $T_a$ | Annealing temperature |
| TAL | Transcription activator-like |
| TF | Transcription factor |
| TGF-β | Transforming growth factor-beta receptor |
| Thr | Threonine |
| TIR | Toll/interleukin-1 (IL-1) receptor |
| TLR | Toll-like receptor |
| $T_m$ | Melting temperature |
| TM | Transmembrane |
| TMM | Too many mouths |
| TMV | Tobacco mosaic virus |
| TNL | TIR-NB-LRR |
| Trp | Tryptophan |
| Tyr | Tyrosine |
| Ug99 | Uganda in 1999 (date of first characterization) |
| untr. | Untreated |
| Ura | Uracil |
| V | Volt |
| V | Valine |
| Ve | Verticilium |
| VRK3 | Vaccinia-related kinase 3 |
| v/v | Volume per volume |
| w/v | Weight per volume |
| WAK1 | Wall-associated kinase 1 |
| WB | Western blot |
| WNK | With no lysine (K) |
| WRKY | Tryptophane, arginine, lysine, tyrosine containing transcription factor |
| Ws-0 | Wassilewskija-0 |
| wt | Wild type |
| XA21 | *Xanthomonas* resistance 21 |
| XB15 | XA21-binding proteins 15 |
| Y | Tyrosine |
| Y2H | Yeast-two-hybrid |
| YFP | Yellow fluorescent *protein* |
| YPD | Yeast extract peptone dextrose |

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# 1. Introduction

## 1.1 Plant-microbe interaction

Environmental signals influence our daily life, and lead to adaptive responses of our immune systems to deal best with the surrounding situation, or we can simply move away from the source of danger. In contrast to us, plants are sessile organism and have to overcome challenging conditions at their fixed location. Various abiotic stresses (temperature, soil moisture, salinity, soil fertility, emission of greenhouse gases, light condition, etc.) (Ahmad & Prasad, 2012) have an impact on the fitness of plants, and force them to quickly adapt to those conditions by reprogramming and modification of their response system (Cramer et al., 2011). Nevertheless, plants have to manage attacks by biotic stresses such as microorganisms (viruses, bacteria, fungi and oomycetes), insects, grazing animals, nematodes, or other plants/weeds. Due to the importance of plants as crops for the human diet, Pennisi (2010) has listed the most critical biological threats for food security. Most of the serious diseases are caused by fungi (*Puccinia graminis* Ug99, *Mycosphaerella fijiensis, Phakopsora pachyrhizi, Magnaporthe oryzae).* The fungus *M. oryzae* mainly attacks rice species and leads to a disease called rice blast. Rice blast is a worldwide problem, and experts have calculated that each year this fungus destroys harvests that could feed up to 60 million people (Pennisi, 2010). Other top pathogens are the oomycete *Phytophthora infestans* and the cassava brown streak virus. Last but not least a parasitic plant, *Striga hermonthica* (witchweed), leads to yield losses of about $1 billion per year, affecting 100 million people, especially in Africa (Pennisi, 2010). These are only some examples, underlining the strong impact of pathogens on plants, and consequentially on our human life. However, plants are not defenseless. They have established a fine-tuned detection system, which relies on a two-layered innate immune system, including the action of different types of receptors. This innate immune system, where each cell contains its own set of immune receptors and can act autonomously, is related to the innate immune system in animals (Ausubel, 2005; Nürnberger et al., 2004). An additional immune strategy occurs in animals, the adaptive immunity, but this lacks in plants. The adaptive immunity is based on specialized immune cells and organs. By somatic mechanisms antigen receptors are generated, which are clonally distributed on lymphocytes (T cells and B cells) (Thompson, 1995). In plants and animals, the first layer of the innate immune system is based on the recognition of pathogen-associated molecular patterns (PAMPs) (Medzhitov & Janeway, 1997) or danger/damage-associated molecular patterns (DAMPs) (Matzinger, 1994), mediated by pattern recognition receptors (PRRs), which are stuck in the plasma membrane. In general, PAMPs are highly conserved structures in pathogens, but they do not occur in the host. This detection system is an essential mechanism of the host to distinguish between self or non-self (Medzhitov & Janeway, 2002)**.** DAMPs are endogenously delivered molecules of the host, but can reach the PRRs through cellular damage, such

as injuries caused by the pathogen, or DAMPs can be secreted under biotic stress condition (Boller & Felix, 2009). The cell surface-based recognition system is called PAMP-triggered immunity (PTI), whereas the second layer of innate immunity takes place within the cell, and is known as effector-triggered immunity (ETI). Certain immune receptors called resistance (R)-proteins can interfere with effectors or virulence factors, which are released by the pathogen (Dangl & Jones, 2001; Jones & Dangl, 2006).

It does not matter whether plant or animal, both have developed strategies to overcome pathogen attacks, and evolutionary improvement of their machineries is still going on.

## 1.2 The plant innate immune system

Exposition to microbes, or other organisms is not necessarily a sign for plants to be at risk. Some organisms might even support the functionality of plants by building up a symbiotic relationship. Mycorrhizal fungi are associated with the roots of over 90 % of all plant species and improve the nutrient status of their host plant. On the other hand, the fungus needs the host plant for its growth and reproduction (Bonfante & Genre, 2010; Smith & Read, 2008). Obviously, plants have to carefully distinguish between suitable and pathogenic microbes (De Souza, Granada, & Sperotto, 2016). A further definition, which has to be done, is the correct classification of the pathogenic life style to activate specific defense responses in the plant cell. Biotrophic and hemibiotrophic microorganisms rely on keeping their target organism alive, and feed on their metabolites. In contrast, necrotrophic pathogens live and feed on dead cells (Davidsson et al., 2013). In the case of the hemibiotrophic life style, the pathogens can switch to a necrotrophic behavior at the end of their life cycle (Glazebrook, 2005). Harmful microorganisms have to find a way into the plant tissue. This movement is secured on several levels in the plant. Most pathogens penetrate the leaf or root surface through wounds or natural openings, such as gas pores (stomata) or water pores (hydathodes), and enter the plant interior (Chisholm et al., 2006; Jones & Dangl, 2006). On this way, microbes have to deal with waxes and hairs on the surface of the plant tissue. The next barrier is represented by the cell wall, a rigid and cellulose-based surrounded structure of each plant cell. Some pathogens release cell wall degrading enzymes, such as pectin methyl esterases (PMEs) (Valette-Collet et al., 2003), endo-polygalacturonases (PGs) (Ten Have et al., 1998), and endo-β-1,4-xylanases (Choquer et al., 2007), all produced by the fungus *Botrytis cinerea*; or cellulases, pectinases, and hemicellulases by the bacterial genera *Pectobacterium* and *Dickeya* (Davidsson et al., 2013). To stop this attacking process, plants have proteinaceous inhibitors in their cell wall, such as xylanase inhibitors and PG inhibitor proteins (PGIPs), which are released to block the activity of pathogenic enzymes (Beliën et al., 2006). If the pathogens overcome these first obstacles, they will end up in the apoplast, known as extracellular space. The last layer, before entering the plant cell, is formed by the plasma membrane, a phospholipid bilayer with

embedded proteins. At this site, the plant innate immune system is active (Figure 1-1) (Jones & Dangl, 2006). The classification into PTI and ETI, and their corresponding features (PAMPs, DAMPs – PRRs and effectors – R-proteins, respectively) are under discussion due to inaccuracy in the definition of the terms (Thomma, Nürnberger, & Joosten, 2011). Despite all that, these designations will be used to have a simplified orientation in the plant innate immune system.

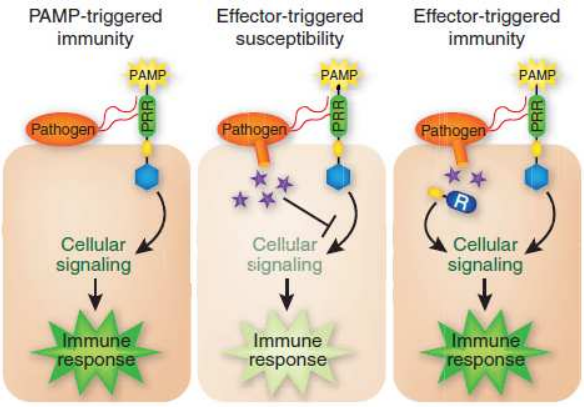



**Figure 1-1: Simplified model of plant innate immunity, illustrating PTI, ETS and ETI (Pieterse et al., 2009).**
PTI is based on pattern recognition receptors (PRR), sensing pathogen-associated molecular pattern (PAMP). The kinase domain (blue) is involved in cellular signaling, and activation of immune responses. In ETS, pathogens release effectors (purple stars) into the host cell, leading to an inhibition of PTI. Cellular plant resistance (R)-proteins (dark blue and yellow) can detect effectors, resulting in signaling of ETI, and activation of immune responses.

### 1.2.1 PAMP-triggered immunity (PTI)

The basal first layer of plant innate immunity occurs on the plasma membrane level, involving PRRs as major players. The diverse extracellular domains of the receptors can fish for PAMPs and DAMPs. PAMPs, also known as elicitor or ligand (related to PRRs), classically defined as highly conserved molecules with functional importance for the fitness of various microorganisms (Medzhitov & Janeway, 1997; Nürnberger & Brunner, 2002). These molecules might be also present in nonpathogenic microbes, wherefore the alternative term microbe-associated molecular pattern (MAMP) is abundantly used in literature, too (Boller & Felix, 2009). Apoplastically released effectors can be detected by membrane-localized R-proteins, but originally belong to ETI. Newman et al. (2013) have created an overview of the, so far known, PAMPs and DAMPs, and their connected plant receptors, which is modified and added by several publications in the last years, highlighting the enormous output in this rather young research field.

#### 1.2.1.1 PAMPs of bacteria, and their plant receptors

The two major bacterial pathogens for crops are *Pseudomonas syringae* and *Ralstonia solanacearum* (Mansfield et al., 2012), causing high yield losses, and having a strong impact on the agricultural system. An improved understanding of the detection machinery is a relevant aim, for a better defense against these microbes.

The best known PAMP is located in the flagellum, the moving apparatus of bacteria, thus a wide-spread feature in the kingdom of bacteria. The PAMP-active part is further classified as a N-terminal sequence

of 22 amino acids (flg22) (Felix et al., 1999), which is located within the internal conserved part of the flagellum filament (Kunze et al., 2004). This PAMP is detected by a PRR called flagellin sensing 2 (FLS2) (Gómez-Gómez & Boller, 2000). Sensing of flg22 is a common feature in higher plants, whereas basal plants, such as the moss *Physcomitrella patens*, does not carry an FLS2 ortholog (Boller & Felix, 2009). Interestingly, the *Arabidopsis* ecotype Wassilewskija-0 (Ws-0) shows a flagellin insensitivity, due to a stop-codon mutation in the kinase domain (KD) of FLS2 (Gómez-Gómez & Boller, 2002). FLS2 belongs to one of the largest kinase families, the receptor-like kinases (RLKs), represented by more than 610 members in *Arabidopsis* (Shiu & Bleecker, 2001b). Plant RLKs are closely related to the *Drosophila melanogaster Pelle* kinases (Belvin & Anderson, 1996) and the mammalian interleukin-1 receptor-associated kinases (IRAKs) (Cao, Henzel, & Gao, 1996). The kinases of the RLK/Pelle family can have two main configurations; (1) membrane-spanning RLKs, and (2) receptor-like cytoplasmic kinases (RLCKs). The first group of proteins contain an extracellular domain (ECD), a transmembrane domain (TM), and an intracellular KD. The second group of kinases miss an ECD, as well as a TM (Shiu & Bleecker, 2001a, b). Some membrane-spanning RLKs are PRRs, such as FLS2. The ECD of FLS2 contains 28 leucine-rich repeats (LRRs), the most common ECD-motif of RLKs (Shiu & Bleecker, 2001b), with a conserved amino acid sequence of LxxLxxLxLxxNxLt/sGxIPxxLG, where x represents any amino acid (Jones & Jones, 1996). The discovery of the FLS2 co-receptor, BRI1-associated kinase 1 (BAK1) (Li et al., 2002; Nam & Li, 2002), has been elementary (Chinchilla et al., 2007a; Heese et al., 2007) for further understanding of PAMP-signaling, and induced downstream responses. Chinchilla et al. (2007a) have shown that BAK1, a further LRR-RLK, acts as a positive regulator in flg22-signaling, and forms ligand-dependent *in vivo* complexes with FLS2. In flg22-untreated material BAK1-FLS2 complexes are barely detectable. In 2013, Sun et al. have published the crystal structure of FLS2 and BAK1 ECDs, showing flg22 as a molecular glue to connect the receptor with the co-receptor. *Botrytis*-induced kinase 1 (BIK1), a RLCK involved in *Arabidopsis* resistance to necrotrophic fungi (Veronese et al., 2006), can interact with FLS2 and BAK1. The RLCK is rapidly phosphorylated upon flg22 perception in a FLS2- and BAK1-dependent manner. BIK1, on the other hand, also phosphorylates BAK1 and FLS2, likely to enhance those kinase activities. BIK1 phosphorylation leads to a dissociation of BIK1 from the FLS2-BAK1 complex (Zhang et al., 2010b). Events further downstream of BIK1 are not well investigated, yet. So far it is shown that soluble BIK1 phosphorylates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase respiratory burst oxidase homolog D (RBOHD), which is essential for its activation, and ROS production is induced as a defense mechanism (Kadota et al., 2014; Li et al., 2014). BIK1 turnover is regulated by calcium-dependent protein kinase 28 (CPK28) via phosphorylation, too (Monaghan et al., 2014). Beside the mentioned PTI responses, flg22 mediates the activation of a phosphorylation cascade in *Arabidopsis* (Nühse et al., 2000), by activating a downstream mitogen-activated protein kinase pathway, composed of mitogen-activated protein kinases (MAPK)/ extracellular signal-regulated kinase (ERK) kinase kinase 1 (MEKK1), mitogen activated protein (MAP)

kinase kinase 4 and 5 (MKK4 and MKK5), and MAP kinase 3 and 6 (MPK3 and MPK6) for the activation of transcription factors (TF) (Asai et al., 2002). However, Ichimura et al. (2006) have reported, that MEKK1 is not required for flg22-induced activation of MPK3/MPK6, but instead MPK4. Mészáros et al. (2006) have demonstrated that in addition to MPK3/MPK6, flg22 stimulates activation of MPK4, and the involvements of further MPKs. These findings open the discussion about two parallel MAPK cascades, involved in flg22-signaling. Flg22-sensed FLS2 receptors are endocytosed from the plasma membrane, and this internalization depends on the cytoskeleton, proteasome function, as well as receptor activity (Robatzek, Chinchilla, & Boller, 2006). The receptor-mediated endocytosis (RME) of FLS2 seems to rely on two distinct endocytic trafficking routes, depending on the FLS2 activation status (Beck et al., 2012). Further, FLS2 endocytosis needs a functional Rab5 GTPase pathway (Pfeffer, 2005; Ueda & Nakano, 2002; Ueda et al., 2001). The cytoplasmic amino acid sequence of FLS2 contains a conserved PEST-like motif, known for ubiquitin-triggered receptor endocytosis (Haglund et al., 2003; Zipfel et al., 2004). Indeed, upon flg22 treatment FLS2 can interact with the E3 ubiquitin ligases plant U-box 12 and 13 (PUB12 and PUB13) in a BAK1-dependet manner (Lu et al., 2011). Another negative controller of this signaling pathway is a small LRR-RLK, called BAK1-interacting RLK 2 (BIR2), which inhibits BAK1-FLS2 interaction in the absence of bacterial flagellin (Halter et al., 2014a, b). Other regulatory steps can be performed by phosphatases, such as kinase-associated protein phosphatase (KAPP), which is able to interact with the KD of FLS2 to trigger dephosphorylation (Gómez-Gómez, Bauer, & Boller, 2001). Likewise, the phosphatase type 2A (PP2A) interacts with BAK1 to control its activity (Segonzac et al., 2014).

Another well-known PAMP is part of the elongation factor Tu (Ef-Tu), the most abundant (5 to 9 % of total cell protein) bacterial protein (Kunze et al., 2004). It functions in the ribosomal translation machinery (Böhm, 2016), and might be released, and accessible through lysis of dying bacterial cells during plant colonization (Furukawa et al., 2014; Zipfel et al., 2006). Zipfel et al. (2006) have identified the corresponding PRR, Ef-Tu receptor (EFR), which directly recognizes a conserved N-acetylated 18 amino acid-long epitope. EFR is a 21 LRRs-containing RLK, and creates a ligand-dependent heterodimerized complex with BAK1 (Roux et al., 2011). Phosphorylation is also an important modification for elf18-induced signaling, including BIK1 activity (Lu et al., 2010; Schulze et al., 2010).

The cell walls of bacteria contain peptidoglycan (PGN), which consist of glycan chains that are cross-linked by oligo-peptides (Newman et al., 2013). In *Arabidopsis*, PGN-binding is mediated by two plant proteins, lysine-motif domain protein 1 and 3 (LYM1 and LYM3) (Willmann et al., 2011), named after their specific extracellular motifs. LYM1 and LYM3 are receptor-like proteins (RLPs), a class of membrane-spanning proteins lacking the intracellular KD. Due to this lack of an intracellular signaling domain, RLPs need interaction partners for signal transduction. In 1999, Jeong, Trotochaud, and Clark have published clavata 2 (CLV2), a LRR-RLP involved in maintaining the balanced meristematic stem cell population. Additionally, this group could show a stabilizing effect on clavata 1 (CLV1), a LRR-RLK,

which dependents on CLV2. That was the first evidence that RLPs might be linked to RLKs to act as a signaling receptor complex. So far, the most common interaction partners of RLPs are BAK1 and suppressor of *bir1-1* (SOBIR1) (Gao et al., 2009), two small LRR-RLKs (Albert et al., 2015; Fradin et al., 2009; Liebrand et al., 2013; Zhang et al., 2014; Zhang et al., 2013). In the case of PGN-sensing, LYM1 and LYM3 establish a complex with a LysM-RLK, named chitin elicitor receptor kinase 1 (CERK1) (Iizasa, Mitsutomi, & Nagano, 2010; Petutschnig et al., 2010; Willmann et al., 2011). Recently the PRR of another PAMP was identified, lipooligosaccharide specific reduced elicitation (LORE) (Ranf et al., 2015). The *Arabidopsis* receptor LORE, a RLK with an extracellular lectin S-domain, recognizes lipopolysaccharides (LPSs), the major component of the outer membrane of Gram-negative bacteria (Newman et al., 2013).

A novel PAMP (old name RsE2) is purified from the bacterial pathogen *R. solanacearum* (Melzer, 2013, Dr. Eric Melzer and Katja Fröhlich personal communication). This PAMP is recognized by LRR-RLP32 (Fan, 2016). Bacterial cold shock proteins (csp), which could be implicated in antiterminate transcription of genes (Bae et al., 2000) act as PAMPs, too (Felix & Boller, 2003). Recently the receptor of csp22 (Felix & Boller, 2003) was identified, cold shock protein receptor (CORE) (Wang et al., 2016, Saur et al., 2016).

The sulfated protein required for activation of Xa21 X (RaxX), or its derived epitope RaxX21-sY is a conserved molecule in *Xanthomonas oryzae* pv. *oryzae* and many other *Xanthomonas* species, and is required for Xa21-mediated response in rice (Pruitt et al., 2015). In the same bacterial genus, a PAMP called enigmatic MAMP of *Xanthomonas* (eMax) was identified together with its recognition receptor, receptor of eMax (ReMax), contributing to host defense (Jehle et al., 2013). There are further not fully identified bacterial PAMPs, with partly unknown PRRs. Bacterial DNA (Yakushiji et al., 2009), and bacterial superoxide dismutase (Sod) (Watt et al., 2006) are just two examples (Zipfel, 2014).

#### 1.2.1.2 PAMPs of fungi/oomycetes, and their plant receptors

The best known PAMP produced by fungi is chitin, the major component of the fungal cell wall. Chitin is a polysaccharide, consisting of β(1-4)-linked N-acetylglucosamine (GlcNAc) residues. In oomycetes, the cell walls are composed of β-glucans and cellulose. The β-glucan binding protein (GBP) from soybean might play an active part in binding *Phytophtora sojae*-derived 1,6-β-linked and 1,3-β-branched heptaglucoside (HG) (Fliegmann et al., 2004).

Moreover, the cellulose-binding elicitor lectin (CBEL) of *Phytophthora* can function as a PAMP, whereas the receptor remains unknown (Gaulin et al., 2006; Mateos, Rickauer, & Esquerré-Tugayé, 1997; Séjalon-Delmas et al., 1997). The *Arabidopsis* major chitin receptor belongs to the class of LysM-RLKs, and named LysM-containing receptor-like kinase 5 (LYK5), which is able to build up a chitin-induced complex with CERK1 (Cao et al., 2014). Recently Yamada et al. (2016) have published that the RLCK,

avrPphB susceptible 1 (PBS1)-like 27 (PBL27) can interact with the chitin receptor complex CERK1-LYK5, and additionally with the MAPK cascade (Shinya et al., 2014). This interesting finding underlines the importance of RLCKs as linker between ligand perception at the plasma membrane, and signal transduction to the nucleus, or other cellular compartments.

The necrotrophic fungus *Sclerotinia sclerotiorum* triggers typical immune responses in *Arabidopsis*. The inducible elicitor, named *Sclerotinia* culture filtrate elicitor 1 (SCFE1) is perceived by LRR-RLP30 (Zhang et al., 2013). Another LRR-RLP, RLP42/responsiveness to *Botrytis* polygalacturonases 1 (RBPG1) can detect endo-PGs, released by the necrotrophic fungus *B. cinerea* (Zhang et al., 2014).

In wild potato *Solanum microdontum*, a LRR-RLP, called elicitin response (ELR) is identified (Du et al., 2015), which shows response to elicitin, a conserved molecule of the oomycete genus *Phytophthora*. Another highly conserved oomycete PAMP is detected in parsley by Nürnberger et al. (1994). The 13 amino acid-long epitope of a secreted transglutaminase, known as Pep-13, is a surface exposed fragment. RLKs and RLPs in wheat (*Triticum aestivum*) mediate resistance to diverse *Puccinia* strains (Feuillet, Schachermayr, & Keller, 1997; Jiang et al., 2013; Zhou et al., 2007). Orphan PAMPs are ergosterol (Klemptner et al., 2014), arachidonic acid (Bostock, Laine, & Kuc, 1982; Dedyukhina, Kamzolova, & Vainshtein, 2014), and siderophores (also delivered by bacteria, Meziane et al., 2005).

#### 1.2.1.3 PAMPs of other plants, and their plant receptors

*Cuscuta reflexa*, also known as dodder, is a stem holoparasite, infesting most dicotyledonous plants. The group of Dr. Markus Albert have identified a tomato cell surface RLP, cuscuta receptor 1 (CuRe1), mediating improved resistance to *Cuscuta* species (Hegenauer et al., 2016). The corresponding PAMP, derived from the parasitic plant, is still under investigation.

#### 1.2.1.4 Atypical pathogenic factors, and their plant receptors

Some PAMPs are not specific for a single class of pathogens, such as necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) (Bailey, 1995). These 24-26 kDa proteins are highly conserved and secreted by various pathogens; oomycetes like *Phytophthora* species (Fellbrich et al., 2002; Feng et al., 2014; Qutob, Kamoun, & Gijzen, 2002), *Pythium aphanidermatum* (Veit et al., 2001), and *Hyaloperonospora arabidopsidis* (Cabral et al., 2012), in fungi such as *B. cinerea* (Schouten, van Baarlen, & van Kan, 2008), *Moniliophthera perniciosa* (Garcia et al., 2007), *Mycosphaerella graminicola* (Motteram et al., 2009), *S. sclerotiorum* (Dallal Bashi et al., 2010) and *Verticillium dahliae* (Zhou et al., 2012), and in bacteria *Pectobacterium carotovorum* (Mattinen et al., 2004). Cytotoxic NLPs carry a 20 amino acid-long sequence (nlp20) that induces plant defense responses in *Arabidopsis*, and in related plant species (Böhm et al., 2014). Furthermore, the corresponding receptor, LRR-RLP23 is identified and described in Albert et al. (2015).

As described earlier, the definition of PTI and ETI, as well as PAMP or effector is not that clear (Thomma, Nürnberger, & Joosten, 2011). The following samples of pathogen detections take place on plasma membrane level by membrane-fused LRR-RLPs, but the detected structures have close similarities to effectors and mainly cause typical ETI responses in plants.

Tomato ethylene-inducing xylanase receptor 1 and 2 (EIX1 and EIX2) are identified as receptors for a virulence factor delivered by *Trichoderma viride*, a fungus. This factor is part of an endoxylanase protein, and termed ethylene-inducing xylanase (EIX) (Dean & Anderson, 1991; Ron & Avni, 2004). Functionality studies show that five amino acids, exposed to the surface of EIX, are important for the recognition, but not essential for protein activity, and therefore classifying EIX as a PAMP (Thomma, Nürnberger, & Joosten, 2011). However, EIX perception leads to a hypersensitive response (HR), typical for ETI (Ron & Avni, 2004). Another example of an identified LRR-containing receptor in tomato is *Verticillium* receptor 1 (Ve1) recognizing avirulence on Ve1 (ave1) (De Jonge et al., 2012; Kawchuk et al., 2001; Thomma, Nürnberger, & Joosten, 2011), and mediating resistance to fungal *Verticillium* race 1 strains, and bacteria *Xanthomonas axonopodis* pv. *citri* (Newman et al., 2013). Ave1 might also be classified as an apoplastic effector; likewise the avirulence (avr) effectors, released by *Cladosporium fulvum*, a biotrophic fungus. In tomato, several *C. fulvum* (Cf) genes, encoding potential R-proteins are identified, and can interfere with their corresponding avr effectors, but there are membrane-anchored, too (Balint-Kurti, Jones, & Jones, 1995; Dickinson, Jones, & Jones, 1993; Jones et al., 1993, 1994; Rivas & Thomas, 2005; Stevens & Rick, 1988). Nevertheless, some described PAMPs are able to induce HR, an immune response atypical for PTI (Naito et al., 2008). Further investigations and identifications of pathogenic peptides/proteins might help to clarify the classification in the plant innate immune system.

#### 1.2.1.5 DAMPs, and their plant receptors

DAMPs are endogenous signals of the host plant during colonization by pathogens. The hormone systemin is classified as a primary signal, released upon cell injury (Pearce et al., 1991). Recently a likely involved tomato LRR-RLK is identified (Lei Wang, personal communication). Another well-known DAMP is peptide 1 (pep1), a 23 amino acid-long fragment of a cytosolic protein of *Arabidopsis*. The LRR-RLKs pep1 receptor 1 and 2 (PEPR1 and PEPR2) act as binding receptors for pep1 (Huffaker, Pearce, & Ryan, 2006; Yamaguchi et al., 2010; Yamaguchi, Pearce, & Ryan, 2006). Plant cell wall fragments are typical DAMPs, such as oligogalacturonides (OGs) and cutin (Denoux et al., 2008; Schweizer et al., 1996). OGs, for instance, can be recognized by an epidermal growth factor (EGF)-RLK, wall-associated kinase 1 (WAK1) (Brutus et al., 2010). Choi et al. (2014) have observed that released ATP, due to cell damage, can act as a DAMP in *Arabidopsis*, too. Immune responses are triggered by recognition of extracellular ATP through a lectin-RLK, does not respond to nucleotides 1 (DORN1). Discovery of

further DAMPs might be a task for future work, whereas some hints for potential DAMPs already exist (Bianchi, 2007; Boller & Felix, 2009; Tanaka et al., 2014).

#### 1.2.1.6 Output of PAMP/DAMP perception

Related to PTI, and thus to the perception of PAMPs/DAMPs via PRRs, are certain activation steps, mediating immune response within the plant cell. These defense responses are temporally scheduled (Boller & Felix, 2009). The earliest measurable responses are detectable after a few seconds up to five minutes. Ion fluxes across the membrane are changed (Boller, 1995; Garcia-Brugger et al., 2006; Wendehenne et al., 2002; Zimmermann et al., 1997), including an increased influx of $H^+$ and $Ca^{2+}$, and an efflux of $K^+$, $Cl^-$ and $NO^{3-}$. Output is a membrane depolarization, which is visible in electrophysiological studies (Mithöfer, Ebel, & Felle, 2005). Especially $Ca^{2+}$, moved into the cytoplasmic space, might serve as a second messenger to promote further signaling steps, like opening of other channels, or the activation of calcium-dependent protein kinases (CDPKs) (Blume et al., 2000; Hrabak et al., 2003; Lecourieux et al., 2002; Ludwig, Romeis, & Jones, 2004; Ranf et al., 2008). After a few minutes a new response is detectable, the oxidative burst, which can be recorded by $H_2O_2$-dependent luminescence of luminol (Chinchilla, Boller, & Robatzek, 2007b). The oxidative burst is caused by reactive oxygen species (ROS), mainly $H_2O_2$, which react with DNA, proteins, and membrane lipids to reduce photosynthesis, increase electrolyte leakage, and expedite senescence, and cell death (Lamb & Dixon, 1997; Sharma & Davis, 1997). Major source of ROS production is a flavoprotein called NADPH oxidases, and in *Arabidopsis* the homolog RBOHD (Torres, Dangl, & Jones, 2002). Moreover, the production of nitric oxide (NO), a well-known second messenger in animals, is reported (Asai, Ohta, & Yoshioka, 2008). Next response steps lead to the activation of phosphorylation-dependent MAPK cascades, and finally the activation of tryptophane, arginine, lysine, tyrosine (WRKY)-type TFs (Asai et al., 2002). All these immune responses are activated within the first five minutes after detection of a PAMP/DAMP.

Responses, which take place within the next 30 minutes are listed here; elicitor-activated PRRs, such as FLS2-flg22, undergo endocytosis after 10-20 minutes (Robatzek et al., 2006), and transcriptional reprograming, caused by the MAPK cascade-activated TFs occurs in this timeframe, too (Colcombet & Hirt, 2008; Jonak et al., 2002). This leads not only to the production of the stress hormone ethylene (Spanu et al., 1994), also the cross-talk of the phytohormones jasmonic acid (JA) and salicylic acid (SA) is regulated; two hormones, which often behave antagonistically (Feys & Parker, 2000; Kunkel & Brooks, 2002; Pieterse et al., 2012). SA is a typical hormone produced during attacks by biotrophic pathogens (Segonzac & Zipfel, 2011). In contrast, JA is generated after infection by necrotrophic pathogens (Glazebrook, 2005; Kazan & Manners, 2008). SA, along with other factors, plays a major role in stomatal closure to avoid further penetration of pathogens (Melotto et al., 2006; Zhang, He, & Assmann 2008). Furthermore, it is demonstrated that PAMP perception, and resulting SA production

can trigger systemic acquired resistance (SAR) in *Arabidopsis* (Loake & Grant, 2007; Mishina & Zeier, 2007).

Antimicrobial substances like phytoalexins are released, too (Mao et al., 2011; Tsuji et al., 1992). Another output is callose deposition, which belongs to the latest responses of PTI, and can take hours to days. Callose, a (1,3)-β-glucan cell wall polymer, is accumulated between the plasma membrane and the cell wall, for a reinforcement of the damaged cell barrier, due to invading pathogens (Ellinger & Voigt, 2014).

### 1.2.2 Effector-triggered susceptibility (ETS)

During an evolutionary arms-race, persistent pathogens have learned to overcome this first layer of innate immune response. Pathogenic bacteria deliver effectors or virulence factors into host cells, using the type III secretion system (T3SS) (Abramovitch, Anderson, & Martin, 2006; Block et al., 2008; McCann & Guttman, 2008; Zhou & Chai, 2008). Whereas in fungi and oomycetes such a secretion system is not identified, yet. However, biotrophic organisms can produce haustoria, specialized infection organs, which might be responsible for the delivery of effects into the cytoplasmic space. Parasitic nematodes use a specialized feeding organ, known as the stylet, to inject their effector proteins into plant vascular cells (Davis et al., 2008). Some effectors are released into the extracellular space, and thus known as apoplastic effectors. The term effector itself is defined as secreted molecule, specific to single, or a few related species**.** Moreover, an effector contributes to virulence, by targeting host physiology, and thus promotes its own lifestyle (Bent & Mackey, 2007; Chisholm et al., 2006; Jones & Dangl, 2006; Thomma, Nürnberger, & Joosten, 2011). Several studies have showed that effectors can interfere with PTI, and inhibit the immune response (Abramovitch, Anderson, & Martin, 2006; Boller & Felix, 2009; Chisholm et al., 2006; Hogenhout et al., 2009; Jones & Dangl, 2006). The effector HRP outer protein M1 (HopM1), released by the bacterium *P. syringae*, targets an ADP ribosylation factor-guanine nucleotide exchange factor (ARF-GEF) protein, which is involved in vesicle transport in the host cell (Nomura et al., 2006), indicating that a manipulation of these transport systems supports the performance of the pathogen in the host. Further effectors are identified, and able to block the MAPK cascades (He et al., 2006; Mukherjee et al., 2006). Some bacterial strains make coronatine, a JA mimic, suppressing SA-mediated defense to biotrophic pathogens (Brooks, Bender, & Kunkel, 2005; Zhao et al., 2003), and thus induce stomatal opening (Melotto et al., 2006). Transcription activator-like (TAL) effectors from *Xanthomonas* and *Ralstonia* species are transported to the plant nuclei to bind to the promotors of host disease susceptibility genes, and thus activate their expression, and enhance infection (Schornack et al., 2013). These examples show the strong influence of effectors on host plant immunity, leading to effector-triggered susceptibility (ETS).

### 1.2.3 Effector-triggered immunity (PTI), and output

Anyway, plants have adapted to this situation by establishing intracellular immune receptors, named R-proteins, which belong to a subclass of the superfamily signal transduction ATPases with numerous domains (STAND) (Danot et al., 2009). In 1971, Flor has described the R-protein-effector immunity system as a gene-for-gene resistance strategy. R-proteins are located in the cytoplasmic space, and are divided into two major structural groups, nucleotide-binding leucine-rich repeat (NB-LRR) proteins having an additional N-terminal (1) coiled-coil (CC)-domain, or (2) Toll/interleukin-1 receptor (TIR)-domain (Collier & Moffett, 2009; Meyers et al., 2003). CC-NB-LRRs (CNL) occur in monocots and dicots, whereas TIR-NB-LRRs (TNL) are restricted to dicots (Jacob, Vernaldi, & Maekawa, 2013; Yue et al., 2012). The LRR-domain of R-proteins might serve as a sensor for pathogen-derived effectors; in a direct or indirect manner (Krasileva, Dahlbeck, & Staskawicz, 2010). There are only a few examples, confirming a direct interaction of effector and R-protein (Cui, Tsuda, & Parker, 2015), whereas the indirect recognition model, also known as the guard hypothesis, is quite established. Prominent R-proteins, underlining the indirect detection, are the CNL receptors resistance to *P. syringae pv. maculicola* 1 (RPM1), and resistance to *P. syringae* 2 (RPS2). Both proteins constitutively guard their co-factor RPM1-interacting protein 4 (RIN4). Effectors secreted by *P. syringae* (avirulence protein of *P. syringae pv. glycinea* (avrB), avirulence protein 1 from P. *syringae pv. maculicola* (avrRpm1), and avirulence protein 2 from *P. syringae pv. tomato* (avrRpt2)) interfere and modify RIN4, sensed by the R-proteins, and then activate ETI response (Axtell & Staskawicz, 2003; Kim et al., 2005a; Mackey et al., 2003).

In the last years, numerous effectors of bacteria, fungi, oomycetes, and nematodes are described (Boller & Felix, 2009; Chisholm et al., 2006; Davis et al., 2008). Almost all known oomycete- distributed effectors carry a conserved RxLR-motif (Bozkurt et al., 2012; Rehmany et al., 2005), that seems to be important as a translocation signal (Bhattacharjee et al., 2006). Infection of plant by pathogenic viruses can cause ETI, too. The potato CNL receptor Rx mediates a strong resistance response to a potato virus X coat protein, results in local cell death in transient expression experiments in tobacco (Bendahmane, Kanyuka, & Baulcombe, 1999). The plant detection system for viral infections bases on the recognition of double-stranded RNA (dsRNA) by ribonuclease dicer-like proteins (Ding, 2010).

The general immune response of plants, after detection of an effector, is HR (Greenberg & Yao, 2004). Classical symptoms are locally restricted cell death that should block the propagation of biotrophic pathogens (Morel & Dangl, 1997). Thus, HR is not the best immune response regarding attacks by necrotrophic enemies, which depend on PTI. Beside HR, SAR is often connected to ETI, too (Dangl & Jones, 2001). SAR requires the hormone SA, and is associated with expression of pathogenesis-related proteins, to contribute to durable systemic resistance (Durrant & Dong, 2004).

### 1.2.4 Arms-race between pathogen and plant host

These observations are not the end of the story. There are already many evidences for underlining the arms-race between host and pathogen. Co-evolution causes creation and modification of virulence factors, as well as immune receptors, to gain the best survival strategy.

A genomic study of field-isolated *P. syringae* strains have revealed that the pathogens are under strong selective pressure, causing the creation of a changed flagellin epitope, flgII-28. This 28 amino acid-long PAMP induces immune responses in many *Solanaceae* species, but seems to be not perceived by FLS2 (Cai et al., 2011; Clarke et al., 2013). Only a few months ago, Hind et al. (2016) have published the matching tomato receptor of flgII-28, flagellin-sensing 3 (FLS3).

In ETI, new studies have revealed that plant host proteins are not that specific for a certain effector. For instance, RIN4 is targeted by multiple *P. syringae* effectors (avr*Rpm*1, avr*Rpt*2, and avrB) (Kim et al., 2005b; Mukhtar et al., 2011). Interestingly some effectors target several proteins such as *P. syringae* effector avrRpt2 that has proteolytic activity against at least five *Arabidopsis* proteins (Chisholm et al., 2005; Takemoto and Jones, 2005). Likewise, the avirulence protein of *P. syringae pv. tomato* (avr*Pto*), another effector of *P. syringae*, which can interfere with FLS2, EFR and BAK1, but can be also detected by the R-protein Pto (Scofield et al., 1996; Shan et al., 2008; Xiang et al., 2008; Xing et al., 2007). These findings have induced rethinking of current models. Van Der Hoorn and Kamoun (2008) have described a third model for R-protein-effector interaction, the decoy model, a modification of the guard hypothesis. Four major players are involved in this model; the operative target (1) of the effector (2) and a decoy (3) for the effector, sensed by a R-protein (4), to trigger immune response. The decoy protein should mimic a potential target of the effector, likely it has only a function in inhibiting the virulence effect of the effector on the operative protein, but can indirectly mediate HR responses by interfering with a R-protein. A further modification of this model is mentioned in Sarris et al. (2015). The observation of two *Arabidopsis* TNLs, carrying a WRKY-domain (Narusaka et al., 2009), has raised the question, whether this domain, normally related to TFs, can act as a decoy-domain, due to the fact that many effectors targeting WRKY proteins (Cui et al., 2015). In fact, the group of Dr. Jonathan D.G. Jones could confirm that the C-terminal WRKY-domain of resistance to *R. solanacearum* 1 (RPS1) can interact with two bacterial effectors, avirulence protein of *P. syringae* 4 (avr*Rps*4) and *Pseudomonas* outer protein P2 (PopP2). Moreover, R-proteins prefer to act as a complex (Eitas & Dangl, 2010), same case for RPS1 which works in a heterodimeric complex with resistance to *P. syringae* 4 (RPS4) (Williams et al., 2014). Sarris et al. (2015) hypothesize that one of the R-proteins (here RPS1) act as a decoy whereas the second R-protein (here RPS4, lacking a WRKY-domain) is essential for triggering the immune response, but nevertheless both R-proteins, connected through their TIR-domains have to act as a pair to induce full resistance (Deslandes et al., 2003; Gassmann, Hinsch, & Staskawicz, 1999; Narusaka et al., 2009; Williams et al., 2014).

Arms-race between pathogen and host is still going on. Anyway, it is important to keep in mind that plant defense mechanism not rely on a certain PRR or R-protein. Plants can detect harmful microbes such as bacteria via several detection systems including; perception of flagellin, EF-Tu, lipopolysaccharide, peptidoglycan, and probably other PAMPs/DAMPs, and effectors. Thus, overcoming one of these recognition systems is not necessarily associated with a total break-down of the host plant defense. Same is true for the pathogens, which can inject several different effectors per strain (Baltrus et al., 2011) to avoid detection and suppress defense. The correct balance of virulence factors against immune receptors seems to be a major point to influence the outcome of this battle.

## 1.3 SERKs, LRR-RLKs with multiple functions

### 1.3.1 The SERK family

A central key player in plant innate immunity is a member of the LRR-RLK II group, BAK1, named after its first identified interaction partner brassinosteroid insensitive 1 (BRI1) (Clouse, Langford, & McMorris, 1996; Li et al., 2002; Nam & Li, 2002). In *Arabidopsis*, BAK1 belongs to a small kinase family containing five members, the somatic embryogenesis receptor-like kinases 1 to 5 (SERK1 to 5) (Hecht et al., 2001). Due to this family name, BAK1 is also known as SERK3. The next homolog of BAK1, SERK4, has an additional name too, BAK1-like 1 (BKK1) (He et al., 2007). All SERKs have a short extracellular LRR-domain, consisting of 4.5-5 repeats, followed by a serine-proline rich (SPP)-domain, which might provide a flexible hinge to the ECD (Hecht et al., 2001; Schmidt et al., 1997). The proteins are fixed to the plasma membrane through a single pass TM, and contain a cytoplasmic KD to provide catalytic activity. Homologs of *Arabidopsis* SERKs are identified in basal plants, such as green algae and liverworts (Sasaki et al., 2007), and are widely expanded in higher plant of monocots and dicots (Aan Den Toorn, Albrecht, & De Vries, 2015). Schmidt et al. (1997) have identified SERKs in cell cultures of *Daucus carota*, where the proteins mark embryogenic competence, giving this family its name. Signaling pathways in which SERKs are involved (Fan, Wang, & Bai, 2016; Ma et al., 2016) include: Male sporogenesis (SERK1, SERK2) (Albrecht et al., 2005), separation of floral organs (SERK1, SERK2, BAK1, BKK) (Lewis et al., 2010, Ma et al., 2016), plant vascular development (SERK1, SERK2, BAK1) (Zhang et al., 2016), light signaling (BAK1) (Whippo & Hangarter, 2005), brassinosteroid (BR)-signaling (SERK1, BAK1, BKK1) (Albrecht et al., 2008; He et al., 2007; Karlova et al., 2006; Li et al., 2002; Nam & Li, 2002), PAMP/DAMP-signaling (BAK1, BKK1) (Chinchilla et al., 2007a; Heese et al., 2007), and cell death responses (BAK1, BKK1) (He et al., 2007; Kemmerling et al., 2007). Interestingly, SERK1 to 4 play essential roles in stomatal patterning by interfering with ERECTA family LRR-RLKs (Godiard et al., 2003; Torii et al., 1996), and association with the LRR-RLP too many mouths (TMM) (Nadeau & Sack, 2002; Yang & Sack, 1995), a signal modulator of stomata development (Jordá et al., 2016; Meng et al., 2015). Further SERKs (SERK1, SERK2, BAK1) are required for the plant peptide hormone phytosulfokine (PSK)

signaling detected by the PRR-RLK, peptide hormone phytosulfokine receptor (PSKR1) (Wang et al., 2015). *Arabidopsis* (Col-0) SERK5 seems to have no functional importance, due to amino acid substitution in the catalytically important arginine aspartate (RD)-motif (He et al., 2007). However, SERK5 in *Arabidopsis* ecotype Landsberg erecta (Ler) shows an intact RD-motif, and functional assays reveal an action of SERK5 in BR-signaling and cell death control (Wu et al., 2015). Analyses of SERK genes and proteins have demonstrated that the differences in SERK functions are mostly mediated by distinct protein sequences in the ECD and KD, rather than different gene expression patterns or levels (Aan Den Toorn et al., 2015). Moreover, SERK proteins can be clustered into two distinct groups, one group containing SERK1 and SERK2, and the other cluster contains BAK1, BKK1 and SERK5. The central importance of SERKs is supported by the fact that pathogens have created several effectors targeting and inhibiting BAK1 (Shan et al., 2008).

However, BAK1 is identified as a major co-receptor in many different signaling pathways (Chinchilla et al., 2009; Heese et al., 2007, Ma et al., 2016, Fan et al., 2016), and two important plant signaling pathways involving SERK action, and especially BAK1, will be described in more detail in the following sections.

### 1.3.2 SERKs-dependent BR-signaling

BRs are a special class of plant polyhydroxysteroids that play crucial roles for plant growth and development (Clouse & Sasse, 1998). Genetic screening for BR-signaling mutants in *Arabidopsis* has resulted in the identification of brassinosteroid-insensitive 1 (BRI1) (Clouse, Langford, & McMorris, 1996), a LRR-RLKs with 25 tandem LRRs (Li & Chory, 1997). BAK1 is an important co-receptor of BRI1 in a BR-dependent manner, whereas in yeast-two-hybrids an interaction is detectable, too (Bücherl et al., 2013; Hink et al., 2008; Li et al., 2002, Nam & Li, 2002; Russinova et al., 2004). Three other members of the SERK family are able to interfere with the BR-signaling pathway, SERK1 (Karlova et al., 2006), BKK1 (He et al., 2007), and SERK5 (Wu et al., 2015), too. In the last years, many missing parts of this pathway were discovered (Kim & Wang, 2010; Lozano-Durán & Zipfel, 2015). Upon BR perception, BRI1 heterodimerizes with BAK1 at the cell surface, and the BRI1 kinase inhibitor (BKI1) is released (Jaillais et al., 2011; Wang & Chory, 2006). The BRI1-BAK1 complex is activated by transphosphorylation (Wang et al., 2005b, 2008). Meanwhile, phosphorylated BKI1 interacts with 14-3-3 proteins, and supports BR-signaling (Wang et al., 2011). Further phosphorylation events happen, and might lead to the dissociation of BIK1 (Lin et al., 2013), followed by activation of two RLCKs, brassinosteroid-signaling kinases 1 (BSK1) and constitutive differential growth 1 (CDG1) (Kim et al., 2011; Tang et al., 2008). These kinases interfere with the phosphatase BRI1-suppressor 1 (BSU1) (Kim et al., 2009, 2011; Mora-García et al., 2004) that blocks the GSK3-like kinase brassinosteroid insensitive 2 (BIN2), which is then degraded by the proteasome (Peng et al., 2008). Furthermore, brassinazole resistant 1 (BZR1) and *bri1*-ems-suppressor 1 (BES1) are dephosphorylated by phosphatase PP2A (Tang et al., 2011), and can move

to the nucleus to promote transcriptional reprogramming (He et al., 2005; Sun et al., 2010; Vert & Chory, 2006; Yu et al., 2011). There is evidence for a crosstalk between growth and immune signaling (Albrecht et al., 2012; Belkhadir et al., 2012; Lozano-Durán & Zipfel, 2015). A potential crosstalk is also supported by sharing important kinases such as BSK1, that also interacts with FLS2 (Shi et al., 2013), or the transcriptional activator BZR1 (Lozano-Durán et al., 2013).

### 1.3.3 SERKs in cell death control

For all living organisms cell death is a common feature to regulate cellular processes and/or defense mechanisms. In animals, there are three distinct cell death pathways; apoptosis (Adrain & Martin, 2001), autophagy, and necrosis (Lockshin & Zakeri, 2004). In pathogen-plant interaction, programmed cell death (PCD) is a common response (Jones & Dangl, 2006; Lam, 2004; Raff, 1998), that can be associated with disease resistance or susceptibility, depending on the pathogenic lifestyle (Greenberg & Yao, 2004). Necrotrophic pathogens such as the fungi *Alternaria* and *Cochliobolus* can induce cell death formation in the host plant by releasing toxins such as AAL toxin and victorin, respectively (Akamatsu et al., 1997; Curtis & Wolpert, 2002; Wolpert, Dunkle, & Ciuffetti, 2002). A prominent plant cell death output is as previously mentioned HR, which is related to pathogenic effectors detected by R-proteins (Jones & Dangl, 2006). This knowledge indicates that plant cell death must be strongly controlled, and specific regarding the attacking pathogen. In Kemmerling et al. (2007) and He et al. (2007) such a possible regulator (BAK1) is identified, and can be linked to plant cell death. Knock-out mutants of BAK1 show in necrotrophic-fungal-infection assays an enhanced spreading of the fungi *Alternaria brassicicola* and *B. cinerea*, correlating to an increased cell death formation, and ROS production (Kemmerling et al., 2007; Lam, 2004). He et al. (2007) have reported a further SERK protein, BKK1, involved in cell death control, whereas *bkk1* mutant plants have no obvious growth phenotype. However, *bak1 bkk1* double-null mutants exhibit a seedling-lethality phenotype. This strong phenotype is partially rescued by the *pepr1 pepr2* double mutant, suggesting that the cell death phenotype of the two SERKs might be directly ot indirectly connected to a PEPR-mediated signaling pathway (Yamada et al., 2015). Recently BAK1 overexpression lines in *Arabidopsis* are investigated, and revealed a novel action of BAK1 in cell death control (Belkhadir et al., 2012; Domínguez-Ferreras et al., 2015; Liu et al., 2016). Surprisingly, extensive BAK1 expression leads to increased cell death formation, such as described for *bak1* mutant plants. Thus, BAK1 is not only a negative regulator of cell death control, furthermore the right amount of expressed BAK1 is essential for a regulated cell death output. Similar observations are reported for the BAK1 interactor BIR2 (Halter et al., 2014a). BIR2 overexpression leads to increased spreading of necrotrophic pathogens, as published for *bir2* mutant plants (Halter et al., 2014a; Imkampe, 2015). Further team players in the cell death control are BAK1 interacting RLK 1 (BIR1) and SOBIR1, identified by Gao et al. (2009), and will be described in the next

section. These findings indicate that BAK1 and SERKs in general are important for cell death control, and can interfere with many other cell death signaling-associated proteins. How further signaling transduction works is not clarified, yet. One possibility is the occurrence of one or several guardes, such as R-proteins, which sense the performance of the above-mentioned membrane-localized proteins. The guarding hypothesis might also explain the cell death phenotypes of BAK1 and BIR2. R-proteins could sense the integrity of BAK1 and BIR2, and trigger cell death when disturbance is noticed. The additional involvement of unknown PRRs, and cell death signals cannot be excluded.

## 1.4 The BIR family

The essential importance of BAK1 in diverse signaling pathways raises the question how the protein is regulated and tightly controlled. A *in vivo* BAK1 pull-down assay, followed by mass spectrometry analysis, revealed two potential BAK1 interactors called BAK1 interacting RLK 2 and 3 (BIR2 and BIR3). These two proteins belong to a small LRR-RLKs family in LRR X group, LRR Xa subgroup (Shiu & Bleecker, 2001b). The BIR family contains four members, BIR1 to 4, whereas BIR3 and BIR4 have the highest homology, based on KD amino acid sequence alignments. In an evolutionary context, BIR1 seems to be the oldest member of the family. All BIR proteins are very similar in their structural architecture. The N-terminus is extracellularly localized, and build up by the signal peptide and five LRRs, followed by a TM, and three cytoplasmic sections; juxtamembrane domain (JM), KD, and C-terminus (CT) (Halter et al., 2014a).

BIR1 is described by Gao et al. (2009) with functional impact in the regulation of cell death, as well as in R-protein-mediated immunity. The knock out mutants in *Arabidopsis* show extensive cell death, which can be partly rescued by growing temperatures of 27-28 °C. Nevertheless, constitutively activated defense responses, such as high accumulation of SA, and increased levels of pathogenesis-related gene 1 and 2 (PR1 and PR2) without induction are observed, leading to enhanced resistance against pathogens (e.g. virulent oomycete *Hyaloperonospora parasitica* Noco2). BIR1 can interact with BAK1 and other SERKs, but not with the PRR-RLKs, BRI1 and FLS2 (Gao et al., 2009). Further investigations of *bir1* crossed with positive regulators of ETI have indicated an influence of BIR1 in preventing the activation of R-protein mediated resistance. In the same study SOBIR1, also published as evershed (EVR), modulating floral organ shedding (Leslie et al., 2010), is identified. The cell death phenotype of *bir1* is partly blocked by additional knock out of SOBIR1, and summing up SOBIR1 as a critical constructive regulator of cell death control. New data indicate that BAK1 and SOBIR1 can better interact in the absence of BIR1 (Liu et al., 2016), that might act as a negative regulator of BAK1 complex formation.

BIR2 is one of the novel identified interactors of BAK1. It can directly interact with the intracellular domains of BAK1. Functional screens of *bir2* mutants and BIR2 overexpression (OE) lines, in BAK1-

dependent pathways, show a central role of the protein in PTI and cell death control. In a ligand-free environment BIR2 is constitutively bound to BAK1. Ligand perception such as FLS2-flg22 leads to a dissociation of BAK1 from BIR2. The available BAK1 can interact with FLS2 to support downstream PTI-signaling. Interaction assays and measurements of ligand-induced ROS production of *bir2* and 35S-BIR2 lines support this model, and pointing to a role of BIR2 as a negative regulator in innate immunity (Halter et al., 2014a). Knocking out BIR2 in *Arabidopsis* results in a cell death phenotype. The adult plants are slightly smaller than wild type, and produce spontaneous necrosis in leaves.

BIR3, a further member of the BIR family, is detected by mass spectrometry of *in vivo* BAK1 complexes. *In vitro* and *in vivo* interaction assays have underlined this finding, and confirm BIR3 as an interactor of BAK1 (Halter et al., 2014a). Functional analyses of loss-of-function mutants and OE lines have shown that BIR3 can act as a negative regulator in PTI, as described for BIR2 (Imkampe et al., unpublished). Interestingly BIR3 can directly interact with PRRs (FLS2, EFR, PEPR1), unlike BIR2. Remarkable is the 35S-BIR3 phenotype of *Arabidopsis* plants. The plants show a strong dwarfism, indicating a function of BIR3 in BR-signaling. The confirmation of a BIR3-BRI1 complex, in a ligand-independent manner supports this hypothesis, and treatment of BIR3 OE seedlings with exogenous-applied BR reveals insensitivity. In conclusion, BIR3 has a strong negative impact on BR-signaling. An additional function of BIR3 might be the stabilization of SERKs. *Bir3* plants show reduced BAK1 protein levels compared to wild type (Imkampe, 2015), whereas the protein levels of BAK1 are enhanced by co-expression with BIR3. BKK1 interacts with BIR3, too, and supports a general stabilization effect. Knocking out both, BIR3 and BAK1, result in a spontaneous cell death phenotype (Imkampe, 2015, Imkampe et al., unpublished), comparable to *bak1 bkk1* double mutants (Albrecht et al., 2012). In absence of BIR3 the SERKs, namely BAK1 and BKK1, are not stabilized on the membrane, and this might explain the strong necrotic phenotype due to the unavailability of these proteins for the cell death control. This observation links BIR3 to a SERK-dependent cell death control pathway, as mentioned for BIR1 and BIR2. *Bir3* lines have no noticeable growth phenotype, unlike BIR1 and BIR2, and there is no detection of an altered *A. brassicicola* phenotype (Halter, 2014; Imkampe, 2015). The role of BIR3 in cell death control might be concentrated on stabilization properties to SERKs, and not a direct strong impact on cell death regulation.

BIR4 is not strongly investigated so far. It is only weakly expressed in plant tissues (Halter, 2014), and therefore seems to be not a main actor in BIR protein functions. However, it can interact with BAK1 (Halter et al., 2014a), hinting to be an interesting candidate for plant innate immunity, which should be investigated in more detail in future.

Interestingly BIR proteins seem to play central roles in at least three important plant signaling pathways, PTI-signaling, BR-signaling, and cell death control.

## 1.5 Kinases and their structural and biochemical properties

### 1.5.1 The kinase domain

In 1981, Shoji et al. have published for the first time the complete amino acid sequence of a KD. The protein of interest is a cyclic AMP-dependent protein kinase, known as protein kinase A (PKA). Ten years later the crystal structure of PKA is obtained (Knighton et al., 1991), and allows a closer look on kinase functionality on a structural and biochemical level. In the following description, the catalytically active kinase PKA is used as a prototype for the general explanation of KD construction (Figure 1-2). Hanks, Quinn, and Hunter (1988) have classified the KD into 11 subdomains, which are distributed among two lobes, the N-terminal lobe (N-lobe) and C-terminal lobe (C-lobe). The N-lobe is much more flexible, but also smaller than the C-lobe. The lobes form a cleft for the fixation of ATP.

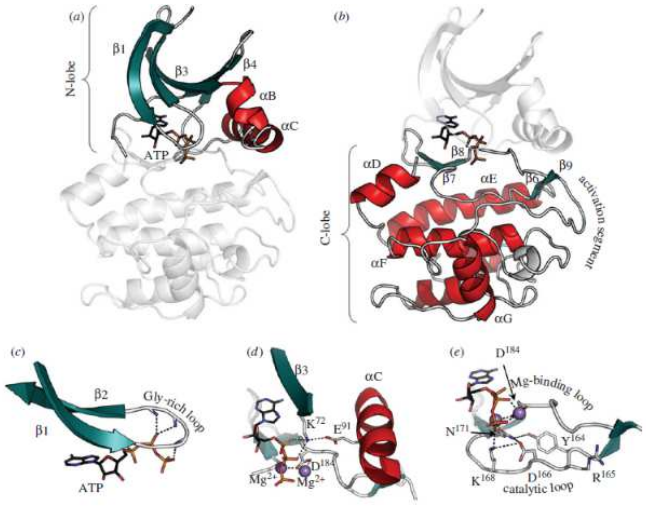



**Figure 1-2: Crystal structure of KD of eukaryotic PKA (Taylor et al., 2012).** In (**a**) and (**b**) functionally important subdomains of N-lobe and C-lobe, respectively, are highlighted (helixes are in red and b-strands are in blue). The bottom panels (**c**-**e**) show modulated zoom in structures. In (**c**) the glycine-rich loop is linked to the phosphates of the ATP, in (**d**) the conserved lysine and glutamic acid fix the confirmation of the kinase, and in (**e**) the catalytic loop with bound ATP, and in purple the $Mg^{2+}$ ions fused to important residues.

The N-lobe starts with subdomain I, also called glycine-rich loop (GXGX$\varphi$G), where $\varphi$ is usually a tyrosine or phenylalanine (Huse & Kuriyan, 2002). This part is highly mobile, and essential for the fixation of the ATP. In subdomain II, a very conserved lysine (K) is present. The lysine is essential for kinase activity, due to its fixation function of the α- and β-phosphate of ATP. The only conserved helix in the N-lobe is located in subdomain III. This C-helix contains a glutamic acid (E) residue, which builds salt bridges to the mentioned lysine, and stabilizes the shape of the protein. A further interesting subdomain is subdomain V, representing the link between N- and C-lobe. Subdomain VIa in the C-lobe is build up by a helix, called E-helix, which is strongly hydrophobic. The catalytic loop is located in subdomain VIb. This loop starts with a highly-conserved motif $\varphi$RDXKXXN, where $\varphi$ can be a tyrosine or histidine. The important residue in that motif is the catalytic aspartate, which is involved in the transfer of the γ-phosphate of ATP to a peptide substrate. A supporting role might have the conserved residues, lysine and asparagine (Hanks & Hunter, 1995; Taylor et al., 2012). In PKA, a single phosphorylation site (T197) in the activation loop (subdomain VII) promotes kinase activity (Knighton et al., 1991; Shoji et al., 1979). Kinases which are regulated on this way typically have this invariant arginine, preceding the aspartate, and are termed RD-kinases (Johnson, Noble, & Owen, 1996). The

arginine residue is clustered with positively charged amino acids, which can inhibit the catalytic functionality (Dardick & Ronald, 2006). The neutralization of this arginine by contact with a negatively charged phosphorylated residue (Hubbard, 1997), such as phosphorylated T197 in PKA, seems to be important for a correct orientation of the aspartate within the DFG-motif (subdomain VII) (Krupa, Preethi, & Srinivasan, 2004), and results in stabilization of the activation loop (Johnson et al., 1996; Nolen, Taylor, & Ghosh, 2004).

In *Arabidopsis* around 10 % of the kinome encodes non-RD kinases, which lack the arginine (Dardick & Ronald, 2006). Some of the non-RD kinases do not show activation through autophosphorylation in the activation loop. Constitutive activity or the use of alternative mechanisms might explain the regulatory features of non-RD kinases (Johnson et al., 1996; Nolen et al., 2004). Interestingly, many plant PRRs belong to this group, such as FLS2 and XA21. Similar observations are made for related animal kinases, namely interleukin-1 receptor-associated kinase 1 (IRAK1) and Pelle (Dardick & Ronald, 2006). These findings could hint to a central function of non-RD kinases in innate immunity and pathogen recognition (Dardick, Schwessinger, & Ronald, 2012). The already mentioned activation loop is classified as subdomain VII. It contains the DFG-motif and ends with the motif APE, another conserved motif**.** The conserved aspartate binds to catalytic magnesium ions necessary for the transfer of the γ-phosphate. The phenylalanine in that motif can build a bridge to the N-lobe by establishing hydrophobic interaction to residues in the C-helix, leading to an altered conformation of the KD (Nolen et al., 2004). Subdomain VIII is connected to substrate binding, too. Also known as P+1 loop, it contains hydrophobic residues (e.g. leucine), supporting the interaction to the peptide substrate (Hanks & Hunter, 1995; Taylor et al., 2012). Nevertheless, the features of the activation loop, and the P+1 loop are linked to the discrimination whether the kinase recognizes a serine/threonine and/or tyrosine as potential phosphorylation site (Chen et al., 2014; Johnson, Noble, & Owen, 1996; Taylor, Radzio-Andzelm, & Hunter, 1995). Subdomain IX and X are helixes (F-helix and G-helix), and both are important as docking sites for the peptide substrate, due to their hydrophobic features (Hanks & Hunter, 1995; Taylor et al., 2012).

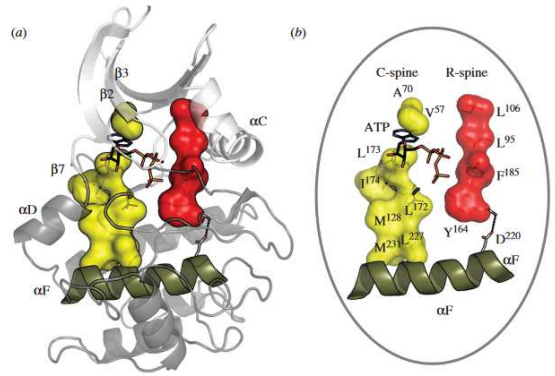



**Figure 1-3: Kinase domain of PKA with highlighted hydrophobic spines (Taylor et al., 2012).**
In (**a**) the two spines; the regulatory (R) spine in red, the catalytic (C) spine in yellow. In (**b**) simplified presentation of the spines and their crucial residue. C-spine is completed by ATP, and linked to the F-helix in the C-lobe.

By comparison of crystal structures of kinases in their active or non-active form, have led to a novel spine concept (Figure 1-3) (Kornev et al., 2006; Kornev, Taylor, & Ten Eyck, 2008; Taylor et al., 2012),

where hydrophobic interactions play a significant role. In active kinases, the group of Dr. Susan Taylor has identified two hydrophobic spines, anchored to the hydrophobic F-helix in the C-lobe (Kornev et al., 2008). The regulatory spine (R-spine) (Kornev et al., 2006) is comprised of four hydrophobic residues (e.g. in PKA, the phenylalanine of the DFG-motif), and typically assembled as a consequence of phosphorylation in the activation loop. The second spine is termed catalytic spine (C-spine) and must be completed by the adenine ring of the ATP (Kornev et al., 2008; Taylor et al., 2012). Disorder of these spines is associated with catalytically inactive kinases (Shaw et al., 2014; Taylor et al., 2013).

### 1.5.2 Phosphorylation, the major PTM

The genomes of living organisms encode for many different proteins, which are functional in numerous cell processes. Their molecular functionality is even increased by certain modifications, called post-translational modifications (PTM) (Eichler & Adams, 2005; Jensen, 2004, 2006; Krishna & Wold, 1993; Mann & Jensen, 2003; Yang, 2005). There are more than 300 types of PTMs, including; sumoylation, sulfation, glycosylation, ubiquitination, and phosphorylation (Ghelis, 2011; Stulemeijer & Joosten, 2008). One-third of all eukaryotic proteins are assumed to be phosphorylated (Olsen et al., 2006), and indicate phosphorylation as one of the most dominant PTMs (Lemeer & Heck, 2009). The cellular importance of phosphate is already described by Westheimer (1987). Phosphorylation plays essential roles in diverse biological signal transductions by altering protein activities, interfering protein-protein interaction, or influencing subcellular localization (Cohen, 2000; Jensen, 2004; Park, Caddell, & Ronald, 2012; Pawson & Scott, 1997; Seet et al., 2006). Kinases and phosphatases are proteins which regulate this reversible protein modification by adding and removing a phosphoryl group from ATP to and off a target peptide substrate, respectively (Champion et al., 2004; Hanks & Hunter, 1995; Hunter, 1995). For instance, the *Arabidopsis* genome encodes 1052 kinases and 162 phosphatases (Wang et al., 2014), underlining the central function of phosphorylation within living cells. The method of phosphorylation can be seen as a molecular switch for turning on or off further processing steps in cells (Martinez-Quiles et al., 2004).

Kinases act as serine/threonine or tyrosine kinases, catalyze the phosphorylation of these specific phosphoacceptors. Most of the animal receptor kinases are tyrosine kinases, with the prominent exception of the transforming growth factor-beta receptor (TGF-β), involved in proliferation and differentiation in many cell types (Roberts et al., 1983; Shiu & Bleecker, 2001b; Sporn et al., 1986). Recent large-scale phosphoproteomis have revealed that tyrosine phosphorylation is extensive in *Arabidopsis*, too (Ghelis, 2011; Mithoe & Menke, 2011; Nakagami et al., 2010, 2012; Sugiyama et al. 2008). Nevertheless, bioinformatic studies of the *Arabidopsis* genome have identified 57 candidates of dual-specificity kinases (DSKs) (Rudrabhatla, Reddy, & Rajasekharan, 2006). DSKs are kinases that are able to phosphorylate serine/threonine and tyrosine residues. Beside these classical phosphorylated

residues, basic amino acids such as arginine, lysine and histidine can be phosphorylated, too (Cieśla, Fraczyk, & Rode, 2011). The detection of these phosphoramidates is rather complicated, and the understanding of their role in signal transduction is a task for future research. However, the *Arabidopsis* genome encodes 17 two-component histidine kinases (Schaller, Kieber, & Shiu, 2008; Stock, Robinson, & Goudreau, 2000) with different function as ethylene receptors (Bleecker & Kende, 2000) or cytokinin receptors (Inoue et al., 2001; Lohrmann & Harter, 2002; Urao, Yamaguchi-Shinozaki, & Shinozaki, 2001).

### 1.5.3 Mass spectrometry for the identification of peptides and P-sites

Reversible phosphorylation occurs on specific sites within the protein. The detection of these phosphorylation sites (P-sites) is a problematic task, for example due to the instability of the amino acid-bound phosphate, especially for phosphorylated serine and threonine. Moreover, phosphorylation is an active process, happening within seconds (Schulze et al., 2010), and can be stable up to several minutes/hours. The occupancy of a P-site of a protein population can differ; for instance, 5 % of a phosphorylated protein can be sufficient to activate signaling pathways, even so 95 % of the protein population remains unmodified (Jensen, 2006).

The most common method to detect P-sites in plant proteins is by mass spectrometry (MS), due to the fact that a phosphorylated amino acid has an increased mass of adding 80 Da to the unmodified mass. In general, MS is a method based on the determination of the mass-to-charge ratio (m/z) of peptide ions. In the 1990s this more sensitive and precise method has replaced the Edman degradation, developed and published by Edman (1949)**.** The MS analysis can be separated into three parts; the ion source, the mass acceptor and the detector. The most common MS technique for the detection of P-sites is called liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), and will be explained in more detail in this section (Figure 1-4) (Jensen, 2006; Steen & Mann, 2004; Zhang et al., 2010a).

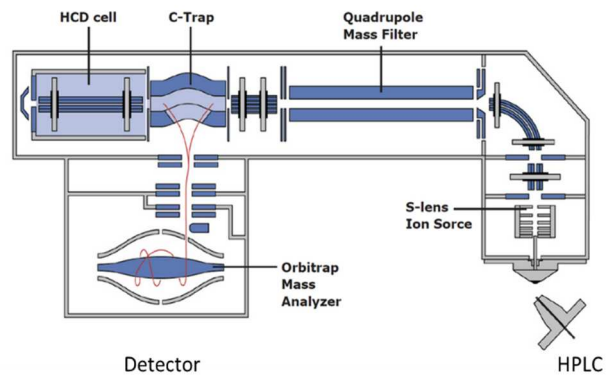



**Figure 1-4: Schematic overview of a LC-MS/MS apparatus (https://www.wur.nl/en/product/Q-ExactivePlus-Orbitrap-LC-MSMS.htm).**
Peptides separated by HPLC are ionized at the ion source (e.g. ESI), and non-charged peptides are removed by the S-lens. Ionized peptides fly into first MS (here Quadrupole), and are further fragmented in the HCD cell. Fragmented precursor ions are transduced into the second mass analyzer (here Orbitrap), which is linked to a detector for estimating m/z values of peptide of interest.

The generation of the samples is the first important step. Even so that the MS is a rather sensitive instrument by analyzing ng levels of certain proteins (Jensen, 2006), an enrichment of the protein of interest is suitable. Affinity methods are used for the enrichment, such as immunoprecipitation (IP). Pull-down of an individual protein can be achieved by using specific antibodies against the protein, or directly against the modification. Protein-specific antibodies can be used with the attention on the binding site, which should be not masked by a potential P-site (serine, threonine or tyrosine) (Erba et al., 2005). Homogeneity of the protein is a further useful step to improve quality of the MS analysis. A SDS-PAGE can achieve a separation of the protein of interest. Processing of the samples on a western blot can also lead to loss of material, thus, in some cases it is beneficial to exclude this further purification step. Isolated gel pieces, containing the sample, can be processed further by protein digestion into peptides. Peptides are short amino acid sequences, and much easier to handle then the whole protein, because of the physico-chemical properties of proteins (Steen & Mann, 2004). Sequence specific proteases are used to digest the proteins, which can be performed in-solution, in-gel or on-beads, depending on the condition of the samples. Trypsin is the most commonly used protease, and cleaves on the carboxy-terminal position of arginine and lysine residues (Zhang et al., 2010a). Those created peptides have an optimal size and a suitable charge for MS analysis. Other used enzymes are e.g. Asp-N (cleaves peptide bonds N-terminal to aspartic acid residues) and Glu-C (cleaves peptide bonds C-terminal to glutamic acid residues). Phosphopeptides can be further enriched, but this step depends on the accessible amount of protein. Enrichment can be achieved in two classical ways; by immobilized metal-affinity chromatography (IMAC) (Posewitz & Tempst, 1999; Stensballe, Andersen, & Jensen, 2001) or $TiO_2$ columns/beads (Larsen et al., 2005; Pinkse et al., 2004), both aim an increased quantity of phosphorylated peptides. Next, phosphopeptides are injected onto a nano-flow high-performance liquid chromatography (nanoHPLC). In the HPLC, the liquid samples are forced at high pressure to move through a column, packed with reversed-phase material (C18). The peptides are eluted from these columns using a solvent gradient, obtaining a segregation of the peptides according to their hydrophobicity, where hydrophilic peptides have the fastest elution (Steen & Mann, 2004).

The end of the column is a needle-like structure, where the phosphopeptides flow through, and reach the ion source. There are two main ion sources, called electrospray ionization (ESI) (Fenn et al., 1989) and matrix-assisted laser desorption/ionization (MALDI) (Hillenkamp et al., 1991). In ESI, which takes place at atmospheric pressure, the analytes derive from the fine needle, and droplets containing the peptides are released by creating fine mist (Fenn et al., 1989). The establishment of high voltage between the needle tip and the counter electrode causes an electric field, created by the droplets. The droplets evaporate, and the peptide molecules get charged by picking up one or more protons from the solvent. The aerially formed ions are sampled into the high-vacuum region of the mass acceptor. A tandem MS apparatus contains two individual mass analyzers, and the electrosprayed peptides are

firstly separated. An isolated precursor ion is further fragmented by using a method called higher-energy collisional dissociation/higher-energy C-trap dissociation (HCD) (Olsen et al., 2007). The advantages of this method are based on a high-resolution ion detection, an increased ion fragmentation (Jedrychowski et al., 2011), and low mass cutoff (seen in linear ion traps) is avoided (enabling the detection of the reporter fragment ion m/z 216 for tyrosine phosphorylation). Cleavage at the amide bond of the peptides is received by collision of the activated ions with nitrogen molecules. Electron transfer dissociation or collision-induced dissociation are other methods of fragmentation. The resulting ions are called b-ions, if the charge is linked to the amino-terminal, and y-ions, if the charge is retained by the carboxy-terminal part of the peptide. In quadrupole instruments (Figure 1-4) y-ions are more common. These first steps can be carried out by MS analyzers, such as ion trap or quadrupole. For instance, a quadrupole consists of energized rods. The voltage can be adjusted for preselection of certain m/z of ions. A continuous voltage permits the detection, and scanning of a m/z range. To improve the output, especially to determinate P-sites, and to increase the sensitivity for certain ions of interest, the products of the fragmentation can be transmitted to a second mass analyzer, such as time of flight (TOF), Fourier transform ion cyclotron resonance (FTICR or FT), and Orbitrap. Orbitrap is a rather new MS analyzer (Hardman & Makarov, 2003; Hu et al., 2005; Makarov, 2000; Makarov et al., 2006; Olsen et al., 2009; Perry, Cooks, & Noll, 2008), and based on electrostatic fields, where the trapped ions orbit around a central electrode, and the axial oscillations of the formed ion rings are detected by their image current, induced on the outer electrode. The frequency of the axial oscillations only dependent on the m/z of the ions. In all mass analyzers, a detector is needed to record either the charge induced, or the current produced, when an ion passes by or hits its surface. Classical detectors are electron multipliers, Faraday cups, microchannel plates, and photomultiplier tube detectors (Church et al., 1999; Prior & Wang, 1977; Yang et al., 1993; Yang & Church, 1991). The formed signal in the detector can be converted into a mass spectrum, illustrating the intensity versus the m/z of a peptide. Moreover, chromatograms can be recorded when the MS is connected to a HPLC. For instance, a base peak chromatogram can give information regarding quality, and a guess about the range of the amount loaded when compared to a standard run of similar complexity. Such a chromatogram shows the detected ions in relation to abundance versus time, whereby the most intensive peak is set to 100 %. Mass spectra contain information about the mass and relative intensity of the peptide. Identification of individual peptides is achieved by matching the spectra against sequence databases (Steen & Mann, 2004). Computational analysis methods use a set of algorithms that efficiently extracts information from raw MS data, and allow very high peptide identification rates, as well as detection of PTM sites (Cox & Mann, 2008; Schulze, 2015). A regularly used program is called MaxQuant (Cox & Mann, 2008), but there are several others, such as Mascot (Perkins et al., 1999), Sequest (Yate et al., 1995), or Open MS (Sturm et al., 2008). By using search engines, like Andromeda, the MS spectra can be searched against an organism specific database, and additionally against the

protein sequence of interest, if this is known. To confirm a predicted P-site, statistic parameters are investigated. For the processing, the false discovery rate (FDR) value usually is set to a low value, such as 1 %, to reduce the risk of wrong-positive results. For each peptide, the posterior error probability (PEP) value, a sum of different statistical analyses based on the selected FDR, is estimated, and should be lower than 0.01, indicating that the observed phosphopeptide is identified with a likelihood of more than 99 % (Käll et al., 2007). Another important value is related to the exact localization of the phosphate in the peptide sequence. To address this issue, the spectrum of an identified peptide is compared with a theoretical spectrum, where phosphates are linked to potential amino acids as phosphate acceptors. The probability score for each position is normalized, and P-sites are valued according to this localization score (Macek, Mann, & Olsen, 2009). Thus, these values should be rather high; for instance, a serine with a localization value higher than 0.75 can be considered as a true P-site. Quantification of MS data needs additional efforts, such as label-free, stable isotope labeling by amino acids in cell culture (SILAC), or dimethyl-labeling (Steen & Mann, 2004; Zhang et al., 2010a). However, the properties of phosphopeptides normally lead to low intensities and qualities, which makes the analysis more complicated. The used MS method in this thesis for the determination of P-sites is not quantitative, and the intensities can only give a rough estimate with regard to quantitative aspects. Given a similar input of material, regarding total protein amount and quality, and provided that MS chromatograms confirm a similar quality of the chromatography as well as comparable overall intensities, total peptide intensities and the total number of peptides identified can be employed to achieve an estimation when aiming for a rough comparison of the abundance of phosphopeptides in different samples within the same data set.

Despite the mentioned limitations, MS analysis is a fundamental method to detect P-sites in proteins of interest. Databases predict potential P-sites such as Musite (Gao et al., 2010), PhosPhAt (Zulawski, Braginets, & Schulze, 2013), and PlantPhos (Lee, Bretana, & Lu, 2011), and can allow a first and/or supporting screen to detect possible P-sites.

## 1.6 Aims of the thesis

The recognition of plant pathogens in the host plant is primarily mediated by PRRs, acting as watchdogs at the plant surface. BAK1, and other SERKs play crucial roles as co-receptors of these PRRs in diverse signaling pathways (Fan, Wang, & Bai, 2016; Ma et al., 2016). Signaling has to be tightly controlled, thus the discovery of BIR2 as a negative regulator of BAK1 in a ligand-dependent manner, has added novel aspects to the understanding of regulatory processes in plant immunity (Halter et al., 2014a, b). BIR2 constitutively binds BAK1 and prevents interaction of BAK1 with ligand-binding receptors. Once ligands bind to the PRRs BIR2 is released from BAK1 and allows BAK1 to act as a co-receptor and positive regulator of the PRRs. BIR2 can be transphosphorylated by BAK1 *in vitro*, and dissociation of a kinase complexes is often mediated by phosphorylation-dependent mechanisms. Thus, the aim of this work is the investigation of phosphorylation events within the BIR2-BAK1 complex to elucidate the mechanism used to achieve dissociation of BIR2 from BAK1 upon ligand stimulation of the plant by identifying and analyzing BIR2 phosphorylation sites.

# 2. Materials and Methods

## 2.1 Materials

### 2.1.1 Organisms

#### 2.1.1.1 Plant lines

Transient expression assays are performed in *Nicotiana benthamiana* plants. Furthermore *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) and Wassilewskija-0 (Ws-0) are used in this study. Based on these ecotypes different T-DNA insertion lines, overexpression lines are available, and listed in following table.

| Genotype | Classification of genotype | Reference/Source |
|---|---|---|
| *bak1-4* (in Col-0) | SALK_116202, T-DNA insertion in BAK1 (At4g33430) | Kemmerling et al., 2007 |
| *bir2-2* (in Ws-0) | Wiscβ-P25D5 (tilling line from Wiscβ-Pool), T-DNA insertion in BIR2 (At3g28450) | Halter et al., 2014a |
| 35S-BIR2-YFP (in Col-0) | pB7YWG2_BIR2 | Halter et al., 2014a |
| 35S-BIR2-YFP (in *bak1-4*) | pB7YWG2_BIR2 in SALK_116202 | Thierry Halter |

*Bir2-2* is the major used BIR2 knock out line for the functional and interaction assays in this thesis. This 185 base pairs (bp) 5'-upstream of start codon T-DNA insertion line is described as a null mutant, concerning transcript and protein level (Halter, 2014).

#### 2.1.1.2 Fungal strains

The yeast strain PJ69-4A (*Saccharomyces cerevisiae*) is used for yeast-two-hybrid assays. Cell death phenotypes of plants are studied by treatment with the necrotrophic fungal pathogen *Alternaria brassicicola* (MUCL20297).

#### 2.1.1.3 Bacterial strains

For cloning and transformation purposes *Escherichia coli* strains DH5α (F-(Φ80lacZΔM15) Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1)), TOP10 (F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G ), Mach1 (F– Φ80*lac*ZΔM15 Δ*lac*X74 *hsd*R(rK–, mK+) Δ*rec*A1398 *end*A1 *ton*A) as well as the *Agrobacterium tumefaciens* strain GV3101 (T-DNA- vir+ rifr, pMP90 genr ) are used.

### 2.1.2 Media and antibiotics

For the cultivation of the plants and microbes different media are needed and listed here.

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

In some cases, the autoclaved media are supplemented with antibiotics which are listed in the following table.

| Antibiotic | Final concentration | Solvent |
|---|---|---|
| Kanamycin | 50 µg/ml | water |
| Rifampicin | 50 µg/ml | methanol |
| Spectinomycin | 100 µg/ml | water |
| Gentamycin | 25 µg/ml | water |
| Carbenicillin | 50 µg/ml | water |
| Hygromycin | 40-50 µg/ml | water |

### 2.1.3 Vectors

Following vectors are used in this study, and are listed in the table.

| Vector | Characteristics | Reference/Source |
|---|---|---|
| pCR8/GW/TOPO | Ori Puc, rrnB, T2, rrnB, T1, attP1, attP2, ccdB, Sm/Spr | Thermo Fisher Scientific |
| pGWB1 | attR1, attR2, ccdB, Kanr, Hygr | Nakagawa et al., 2007 |
| pGWB16 | attR1, attR2, ccdB, Kanr, Hygr, 4x-Myc | Nakagawa et al., 2007 |
| pB7YWG2/GW | 35S promoter, YFP-fusion at the C-terminus | VIB, Ghent |
| pGBKT7/GW | 2a ori f1ori pUC ori attR1 and attR2 ccdB Cmr pADH1 TT7 & ADH1 GAL4 BD c-Myc Kanr TRP1 | RfB/Invitrogen (Postel et al., 2010) |
| pGADT7/GW | 2a ori pUC ori attR1 and attR2 ccdB Cmr pADH1 tADH1 GAL4 AD HA LEU2 Ampr | RfB/Invitrogen (Postel et al., 2010) |

### 2.1.4 Primers

Designed primers are ordered by Eurofins MWG Operon, and diluted to a final concentration of 100 pmol/µl in nuclease-free water.

| Name | Sequence 5'-> 3' | Purpose |
|---|---|---|
| Myc_rev | GCTTTTGTTCACCGTTAATTAACCC | Sequencing |
| pgl3_fwd | GTGTGTTTGGTGCTGCTGCA | Sequencing |
| BIR2_within_fwd | GGTTATGTAGCTCCTG | Sequencing |
| BIR2_KD_fwd | AAGTGGACAAGGAGACGAAGA | Cloning |
| BIR2_ms_rev | TCACACTTTCTCGTTCTCTTGCG | Cloning |

| | | |
|---|---|---|
| BIR2_os_rev | CACTTTCTCGTTCTCTTGCG | Cloning |
| BIR2_cds_fwd (PGL3For1) | ATGAAAGAGATCGGCTCAACCAA | Cloning |
| M13_fwd | TGTAAAACGACGGCCAGT | Sequencing |
| M13_rev | CAGGAAACAGCTATGACC | Sequencing |
| BIP89promF (BIR2) | CTTGGAACAGATGGAGTATATC | Sequencing (within promotor) |
| BIR2_prom_fwd | GATATTTCACCTTCGTGTCCA | Cloning (start of promotor) |
| BIR2S263A-fwd | ACAAGGAGACGAAGAGCCGGTTTAACCGAAGT | Site-directed mutagenesis |
| BIR2S263A-rev | ACTTCGGTTAAACCGGCTCTTCGTCTCCTTGT | Site-directed mutagenesis |
| BIR2S263D-fwd | TGGACAAGGAGACGAAGAGACGGTTTAACCGAAGTAGGAG | Site-directed mutagenesis |
| BIR2S263D-rev | CTCCTACTTCGGTTAAACCGTCTCTTCGTCTCCTTGTCCA | Site-directed mutagenesis |
| BIR2T283A-fwd | CGTAGTCATAAGCTTGCTCAAGTGTCTTTGT | Site-directed mutagenesis |
| BIR2T283A-rev | ACAAAGACACTTGAGCAAGCTTATGACTACG | Site-directed mutagenesis |
| BIR2T283D-fwd | CGTAGTCATAAGCTTGATCAAGTGTCTTTGTT | Site-directed mutagenesis |
| BIR2T283D-rev | AACAAAGACACTTGATCAAGCTTATGACTACG | Site-directed mutagenesis |
| BIR2S286A-rev | GGCTTCTGAAACAAAGCCACTTGAGTAAGCTT | Site-directed mutagenesis |
| BIR2S286A-fwd | AAGCTTACTCAAGTGGCTTTGTTTCAGAAGCC | Site-directed mutagenesis |
| BIR2S286D-rev | GGCTTCTGAAACAAATCCACTTGAGTAAGCTT | Site-directed mutagenesis |
| BIR2S286D-fwd | AAGCTTACTCAAGTGGATTTGTTTCAGAAGCC | Site-directed mutagenesis |
| BIR2S389A-fwd | AATGGGACACTTCACGCGTTGTTGGATTCAA | Site-directed mutagenesis |
| BIR2S389A-rev | TTGAATCCAACAACGCGTGAAGTGTCCCATT | Site-directed mutagenesis |
| BIR2S389D-fwd | AATGGGACACTTCACGACTTGTTGGATTCAAAT | Site-directed mutagenesis |
| BIR2S389D-rev | ATTTGAATCCAACAAGTCGTGAAGTGTCCCATT | Site-directed mutagenesis |
| BIR2T466A-fwd | GAGAGTAGTTTCATGGCTGGTGACTTAGGAG | Site-directed mutagenesis |
| BIR2T466A-rev | CTCCTAAGTCACCAGCCATGAAACTACTCTC | Site-directed mutagenesis |
| BIR2T466D-fwd | GAGAGTAGTTTCATGGATGGTGACTTAGGAGA | Site-directed mutagenesis |
| BIR2T466D-rev | TCTCCTAAGTCACCATCCATGAAACTACTCTC | Site-directed mutagenesis |
| BIR2T533A-fwd | GGTAGAATCGCTGAGGCATTCGATGAAAACA | Site-directed mutagenesis |
| BIR2T533A-rev | TGTTTTCATCGAATGCCTCAGCGATTCTACC | Site-directed mutagenesis |
| BIR2T533D-fwd | GGTAGAATCGCTGAGGACTTCGATGAAAACATT | Site-directed mutagenesis |
| BIR2T533D-rev | AATGTTTTCATCGAAGTCCTCAGCGATTCTACC | Site-directed mutagenesis |

| | | |
|---|---|---|
| BIR2S585A-fwd: | GAGAAACAAGGCTATGCGTTCTCCGAACAAG | Site-directed mutagenesis |
| BIR2S585A-rev | CTTGTTCGGAGAACGCATAGCCTTGTTTCTC | Site-directed mutagenesis |
| BIR2S585D-fwd | GAGAAACAAGGCTATGACTTCTCCGAACAAGAC | Site-directed mutagenesis |
| BIR2S585D-rev | GTCTTGTTCGGAGAAGTCATAGCCTTGTTTCTC | Site-directed mutagenesis |
| BIR2S587A-fwd | CAAGGCTATTCGTTCGCCGAACAAGACGACG | Site-directed mutagenesis |
| BIR2S587A-rev | CGTCGTCTTGTTCGGCGAACGAATAGCCTTG | Site-directed mutagenesis |
| BIR2S587D-fwd | CAAGGCTATTCGTTCGACGAACAAGACGACGA | Site-directed mutagenesis |
| BIR2S587D-rev | TCGTCGTCTTGTTCGTCGAACGAATAGCCTTG | Site-directed mutagenesis |

### 2.1.5 Antibodies

The following tables show the 1. and 2. antibody used for expression checks or pulldown assays.

| **1. Antibody** | **Source** | **Use** | **Reference/Provider** |
|---|---|---|---|
| α-GFP | goat | 1:10000 | Acris |
| α-Myc | rabbit | 1:5000 | Sigma-Aldrich |
| α-BAK1 | rabbit | 1:5000 | Agrisera |
| α-FLS2 | rabbit | 1:2500 | Agrisera |
| α-BIR2 | guinea-pig | 1:2000 | Custom made |
| α-BIR2 | rabbit | 1:1000 | Custom made |

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

### 2.1.6 Chemicals

Chemicals and reagents are purchased in standard purity from Carl Roth (Karlsruhe), Sigma-Aldrich (Taufkirchen), Merck (Darmstadt), Duchefa (Haarlem), Qiagen (Hilden), Invitrogen (Karlsruhe), Fluka (Buchs) or Applichem. Restriction enzymes are used for DNA studies and the kinase inhibitor K252a are obtained from Thermo Fisher Scientific (St. Leon-Rot).

## 2.2 Methods

### 2.2.1 DNA analysis

#### 2.2.1.1 Transformation of *Escherichia coli*

Carefully defrosted competent *E. coli* cells are mixed with 2-4 µl plasmid DNA and kept on ice for 15 min. The heat shock is performed by a pre-heated water bath at 42 °C for 90 sec. After the supplement

of SOC medium, bacterial cells are incubated 1 h at 37 °C and then plated on LB plates with appropriate antibiotic. Colonies are grown overnight (overnight corresponds to approximately 12-16 h) at 37 °C.

#### 2.2.1.2 Transformation of *Agrobacterium tumefaciens*

Depending on the characteristic of the competent *A. tumefaciens* GV3101 cells, either a transformation is done by electroporation or temperature change. Regarding the electroporation, melted bacterial cells are inoculated with 1 µl plasmid DNA and transformed into a cuvette. Electroshock are performed by 1500 V. The other type of competent cells is incubated with 1-5 µg of plasmid DNA on ice for 5 min, then 5 min in liquid nitrogen and further 5 min in 37 °C (water bath). The next steps are common for both kind of competent cells. 300-500 µl of LB medium is added to the cells and after shaking duration of 1.5-2 h at room temperature, samples are poured on selective LB plates (strain has rifampicin and gentamycin resistance). *A. tumefaciens* GV3101 cells are propagated at 28 °C.

#### 2.2.1.3 Bacterial plasmid extraction

A 5 ml overnight-culture of *E. coli* cells, carrying the plasmid of interest are harvested by centrifugation at 13000 rpm at room temperature. The lysis and purification steps are performed according to the user manual of the GeneJET Plasmid Miniprep Kit (Thermo Scientific - #K0503). Purified plasmid DNA is stored at -20 °C, and concentrations are measured by Nanodrop.

#### 2.2.1.4 Plant genomic DNA extraction

The isolation of plant genomic DNA is done according to Edwards, Johnstone, and Thompson (1991). A middle-size leaf is grinded in a 1.5 ml Eppendorf-tube, and then lysed in 200 µl Edwards-buffer (200 mM Tris/HCl, pH 7.5; 250 mM NaCl; 25 mM EDTA pH 8; 0.5 % SDS (w/v)). Clear extraction is treated with 200 µl isopropanol. The precipitation of the genomic DNA took about 10 min by room temperature. A centrifugation step separated DNA pellet from supernatant. Pellet is washed in 70 % ethanol and centrifuged again. Finally, the DNA pellet had to dry and then is resolved in 50 µl 10 mM Tris/HCl pH 8.5. The solution is stored by 4 °C or directly used for running a PCR.

#### 2.2.1.5 Polymerase Chain Reaction (PCR)

Depending on the involved DNA polymerase different PCR setups and programs are used. For cloning purposes, the proofreading polymerase Pfu (Thermo Scientific) is used, and general PCRs (genotyping, etc.) are done with the homemade Taq polymerase (see following tables). Further information can be read in the user manual of Thermo Scientific.

PCR set up for different DNA polymerases:

| **Components in a 20 µl reaction** | **Taq** | **Pfu** |
|---|---|---|
| Template DNA | variable | 50 pg-1 µg |
| Water, nuclease-free | variable | variable |
| Polymerase buffer | 1 x (+$MgCl_2$) | 1 x (+$MgSO_4$) |
| dNTPs | 125 µM | 125 µM |
| Forward primer | 0.5 µM | 0.5 µM |
| Reverse primer | 0.5 µM | 0.5 µM |
| DNA polymerase | 0,5 µL | 0,5 µL |

PCR program for Taq PCR:

| **Time** | **Temperature (°C)** | **Cycle (x)** |
|---|---|---|
| 2 min | 94 | 1 |
| 15 sec | 94 | 30-35 |
| 30 sec | variable ($T_m$ -3) | |
| variable (1 kb in 1 min) | 72 | |
| 10 min | 72 | 1 |
| ∞ | 4 | 1 |

PCR program for Pfu PCR:

| **Time** | **Temperature (°C)** | **Cycle (x)** |
|---|---|---|
| 2 min | 95 | 1 |
| 30 sec | 95 | 25-35 |
| 30 sec | variable ($T_m$ -5) | |
| variable (1 kb in 2 min) | 72 | |
| 10 min | 72 | 1 |
| ∞ | 4 | 1 |

### 2.2.1.6 Colony PCR

To have a quick check of successfully cloned and transformed plasmids a colony PCR is performed. This PCR based on the description of the Taq PCR, whereas there are slight changes in the PCR setup. A tip with a bit bacterial material is incubated for 10 min in a PCR tube containing 10 µl of $ddH_20$. Then, further components of a PCR such as 1 µl dNTPs, 2 µl 10 x Taq buffer + $MgCl_2$, 1 µl forward primer, 1 µl reverse primer, 0.5 µl Taq and 4.5 µl of $ddH_20$ are included.

### 2.2.1.7 Site-directed mutagenesis PCR

Site-directed mutagenesis is proceeded to create mutations leading to another amino acid. In this study potentially phosphorylated serine (S) and threonine (T) are mutated to alanine (A) or aspartic acid (D). Used primers are listed in section 'Primers'. A pCR8 vector carrying the BIR2_ms (with stop) or BIR2_os (without stop) sequence, under the control of the endogenous promotor of BIR2 (1830 bp

upstream of start codon), are used as templates for the PCR, which has following setup (A) and program (B):

A:

| **Components in a 50 µl reaction** | **Pfu DNA polymerase** |
|---|---|
| Template DNA | 0.4 ng/µl |
| Water, nuclease-free | variable |
| Polymerase buffer + $MgSO_4$ | 1 x |
| dNTPs | 150 µM |
| Forward primer | 0.6 µM |
| Reverse primer | 0.6 µM |
| DNA polymerase | 1.5 µl |

B:

| **Time** | **Temperature (°C)** | **Cycle (x)** |
|---|---|---|
| 10 min | 95 | 1 |
| 50 sec | 95 | 3 |
| 15 min | 68 | |
| 50 sec | 95 | 20 |
| 50 sec | variable ($T_m$ -5) | |
| variable (1 kb in 2 min) | 68 | |
| 20 min | 68 | 1 |
| ∞ | 12 | 1 |

For the digestion of the template plasmid 1 µl of DpnI and 5 µl 10 x Tango buffer are added to each PCR amplicon. Incubation time is 2 h at 37 °C; then 25 µl of each reaction are transformed into competent *E. coli* cells.

#### 2.2.1.8 Restriction enzyme digestion of DNA

To confirm the successful cloning of a plasmid or to cut out a certain DNA fragment, a digest is performed. The digestion is performed as given in the user manual (Thermo Scientific). Digested fragments are analyzed by agarose gel electrophoresis.

#### 2.2.1.9 DNA agarose gel electrophoresis

For DNA fragment separation as well as confirmation of successful cloning or transformation, the PCR amplicons are mixed with 6 x Orange DNA loading dye (Thermo Scientific) and loaded on a 1 % agarose gel in 1 x TAE buffer (40 mM Tris, 1 mM EDTA pH 8.5) with 1:5 diluted DNA stain peqGreen (Peqlab). Agarose gels are run at 90-100 V in 1 x TAE buffer. As a marker the GeneRuler 1 kb DNA ladder (Thermo Scientific) is used. Visualization of the DNA are obtained by using UV-Transilluminator (Infinity-3026 WL/26 MX, Peqlab).

#### 2.2.1.10 Purification of DNA fragments from agarose samples

DNA fragments of interest are cut out of the agarose gel with a clean knife, and transformed into a 1.5 ml Eppendorf tube. DNA is then extracted by using the GeneJet Gel Extraction Kit (Thermo Scientific) and according its user manual.

#### 2.2.1.11 Gateway TOPO cloning

To insert DNA fragments of interest into a cloning vector such as the pCR™8/GW/TOPO®TA Cloning®Kit (Life Technologies), some pre-steps are done. The purified PCR product, which might be the full length coding sequence (cds) or the kinase domain (KD) of a gene with (ms) or without (os) stop codon is treated with dATP to create A overhangs. 7.9 µl PCR amplicon is mixed with 1 µl dATPs, 1 µl 10 x Taq-buffer and 0.1 µl Taq polymerase, and incubated for 10 min at 72 °C. 4 µl of these created fragments are directly mixed with 1 µl salt solution and 1 µl pCR™8/GW/TOPO®TA vector, and then incubated 5 min at room temperature. The TOPO reaction is finally transformed into competent *E. coli* cells. Entry vectors are checked via digestion or PCR and is then sent for sequencing.

#### 2.2.1.12 DNA sequencing

Especially entry vectors are sent for sequencing to confirm successful TOPO cloning and avoid inaccuracies of the nucleotide sequence. Therefore, the light run service of GATC Biotech is used. 5 µl of template DNA with following concentrations; plasmid DNA 80-100 ng/µl or PCR product 20-80 ng/µl, are mixed with 5 µl of a primer (5 pmol/µl). Incoming results are analyzed by using CLC Main Workbench.

#### 2.2.1.13 Gateway LR reaction

After confirmation of sequence the entry vector is mixed with the destination vector for recombination of the insert by using the Gateway® LR Clonase® II Enzyme Mix kit (Life Technologies). 50-150 ng (0.4 µl) entry and destination vector are pipetted into 0.8 µl 1 x TE-buffer (pH 8). 0.4 µl LR clonase II enzyme mix are added, and the reaction is started at 25 °C for 1 h or overnight. The reaction is stopped by including 0.2 µl proteinase K into the solution and a 10 min incubation step at 37 °C. The reaction is inserted into competent *E. coli* cells. The success of the LR reaction is analyzed by restriction digestion or PCR.

### 2.2.2 Protein analysis

#### 2.2.2.1 Total protein extraction from plant material

Certain amounts (100-200 µg) of grinded and frozen plant material are transferred into a 1.5 ml Eppendorf tube. Then in a ratio of 1:1 or 1:2 cold extraction buffer (50 mM Tris/HCl pH 8, 150 mM

NaCl, 1 % Nonidet p40 and 1 tablet of proteinase inhibitor cocktail (Roche) per 10 ml solution) are added and mixed with the help of a drill, till the solution is totally homogenized. Protein extraction is performed at 4 °C or on ice for 30-60 min with frequency mixing. To gain clean extract, the solution is centrifuge 2 x by 14000 rpm at 4 °C. Transferring the extracted solution into a fresh 1.5 ml Eppendorf tube reduces the risk of unspecific binding to the plastic wall of the tube. Finally, 5 x SDS loading dye (312.5 mM Tris/HCl pH 6.8, 10 % (w/v) SDS, 25 % (v/v) β-mercaptoethanol, 50 % (v/v) glycerol, 0.05 % (w/v) bromphenol blue) in a ratio 1:5 is supplemented, and the whole mixture is boiled at 95 °C for 5 min, till it could be directly used on a SDS-PAGE or stored at -20 °C.

#### 2.2.2.2 Protein concentration measurements

Protein concentration is measured with the Biorad DC protein assay kit (Biorad, Hercules, USA). The kit protocol is slightly modified to reduce the amount of needed sample. Into a cuvette following working reagents are mixed; 100 µl of solution A' and 800 µl of solution B. After adding 10 µl clean extract, and waiting 15 min the absorbance at 750 nm can be read on a photometer.

#### 2.2.2.3 SDS-PAGE

Frozen protein solution is re-boiled (95 °C for 5 min), and after a short cooling time loaded on homemade 8 % or 10 % SDS polyacrylamide gels (with a 5 % stacking gel part), depending on the molecular weight of the proteins. The gel chamber system of BioRad is used for the whole analysis. Gels are fixed into a running tank, and covered with 1 x SDS-running buffer (25 mM Tris base, 192 mM glycine, 0.1 % (w/v) SDS). Additional to the protein samples, 5 µl of the PageRuler prestained protein ladder (Thermo Scientific) are pipetted into a pocket. The gels are run at approximately 150 V (or fixed at 40 mA per two gels) and stopped after the required separation of the ladder. After removing the gels out of the apparatus and the glass plates, the gels are further proceeded to immunoblot analysis or directly stained in coomassie blue staining (0.025 % (w/v) Coomassie Brilliant Blue R-250, 45 % (v/v) methanol, 10 % (v/v) acetic acid) for visibility of the proteins.

#### 2.2.2.4 Immunoblot analysis

Separated proteins on a SDS-PAGE are electroblotted from the gel on a PVDF membrane (Roche) using Biorad Tetra Blotting Module. Hereby the membrane had to be pre-activated for 1 min in 100 % methanol, and then washed in 1 x transfer buffer (25 mM Tris base, 192 mM glycine). The blotting is performed in 1 x transfer buffer by 4-10 °C at 110 V for 1 h. The blotted membranes are further incubated in 5 % milk (low fat milk powder) in PBS-T (137 mM NaCl, 27 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.4, 0.1 % Tween 20) for 1 h at room temperature to block unspecific antibody-binding sites. Afterwards, the membranes are treated with the primary antibody in 5 % milk in PBS-T overnight

at 4-10 °C. 3 x washing for 5 min in PBS-T is efficient to remove disturbing remains of primary antibody, and membranes are incubated with the secondary antibody in 5 % milk in PBS-T for 1 h at room temperature. Again 3 x washing with PBS-T, then signal detection by using ECL reagent or ECLprime reagent (GE Healthcare). ECL reagent is based on reactions with the horseradish peroxidase coupled to secondary antibody, and treatment is done according the manufacturer's instructions. The detection of the signal is obtained with the CCD camera Amersham Imager 600 (GE Healthcare). Finally, the membrane is stained in Coomassie staining solution to visualize equal protein loading. In this thesis BIR2 mutant lines are cloned into a myc-tag included vector (pGWB16), leading to a molecular weight shift of BIR2-myc fusion protein of about 10-15 kDa (calculated about 5 kDa) on western blot.

#### 2.2.2.5 Co-immunoprecipitation

For co-immunoprecipitation (Co-IP) of certain proteins of interest, a protein extraction is performed like described above. In the meantime, protein A agarose beads (Roche) are incubated with antibodies. For that purpose, 20 µl of beads per sample (resulted of 150-200 mg material) are washed 2 x in washing solution A (50 mM Tris/HCl pH 8, 150 mM NaCl, 1 tablet of proteinase inhibitor cocktail (Roche) per 10ml solution), and then mixed with a variable amount of antibodies. Binding are performed by gently shaking at 4 °C for 1-1.5 h. A certain amount (~15 µl) of clear protein extract with adjust concentration are removed, boiled in 5 x SDS loading dye and used as an input/expression control. Remaining solution is mixed with antibodies bound beads in a 1:1 ratio to reduce the amount of Nonidet p40 in reaction, which can otherwise interrupt binding of protein to antibody. Reaction is obtained at 4 °C within 1 h. Separation of the supernatant from beads are done by 1500 rpm, 4 °C, and 1 min. Beads are washed 2 x with washing solution A (50 mM Tris/HCl pH 8, 150 mM NaCl) and 1 x with washing solution B (50 mM Tris/HCl pH 8). Finally, 15 µl of 5 x SDS loading dye are added and samples are boiled at 95 °C for 5 min before further used on SDS-PAGE.

#### 2.2.2.6 Mass spectrometry for the detection of *in vivo* phosphorylation sites of BIR2

The pull down of BIR2-YFP protein for mass spectrometry (MS) is performed in a similar way then the above described co-immunoprecipitation. Approximately 5 g grinded leaf or seedling material (of stably transformed *Arabidopsis* plants with 35S-BIR2-YFP construct in Col-0 or *bak1-4* background) are used and extracted in 10 ml extraction buffer which is supplemented with additional phosphatase inhibitor tablets (Roche). Duration of extraction is 1-1.5 h at 4 °C. Protein extract is spun down 2 x at 4 °C, 14000 rpm, 10 min. 333 µl GFP-trap beads (Chromotec) are added to protein extract and binding took place at 4 °C for 1-1.5 h. Washing steps are performed like descripted above (see 'Co-immunoprecipitation`). Then 50 µl of 5 x SDS loading dye are included, and mixture is boiled at 95 °C for 5 min. Afterwards solution is directly loaded on a 8 % SDS-PAGE because of the instability of the

phosphate-group on the amino acid. Gel is then stained with Coomassie Brilliant Blue R-250 and destained with 25 % (v/v) isopropanol and 10 % (v/v) acetic acid. Whole gel with colored protein band is sent to the proteomics Core Facility of the University of Tübingen, Proteome Center Tübingen, headed by Prof. Dr. Boris Maček. Dr. Mirita Franz, and further proceeded by Johannes Madlung and sample preparation is done by Silke Wahl or Irina Droste-Borel. There, in gel digestion is performed followed by nanoLC-ESI-MS/MS analysis on a UHPLC Easy-nLC 1200 (column: inner diameter 75 µm, length 20 cm and reversed phase material: C18, pore size 1.9 µm) coupled to a QExactive HF Hybrid Quadrupol-Orbitrap mass spectrometer (Thermo Scientific both); method: 90 min, Top7 ('sensitive'). For the processing of the MS raw data MaxQuant software suite v. 1.5.2.8 (Cox & Mann, 2008, 2011) is applied. Using Andromeda-search engine the spectra are searched against an *Arabidopsis* database plus the available protein sequence of BIR2-YFP. The analysis of the raw data is done with a setting of 1 % for the FDR (False Discovery Rate). Obtained results include the table Phospho(STY)Site. For statistical evidence of a phosphorylation site, the localization probability of the potential site has to be larger than 0.75 (75%) and the PEP value (Post Error Probability) smaller than 0.01. The PEP (Posterior Error Probability, Käll et al., 2007) is calculated for each peptide. A peptide PEP value lower than 0.01 indicates that the peptide is identified with a probability of more than 99 %.

## 2.2.3 Plant analysis

### 2.2.3.1 Cultivation of plants

*A. thaliana* plants are grown on soil for 5-6 weeks under controlled short day conditions (8 hours light, 16 hours dark, 22 °C, 110 $mEm^{-2}s^{-1}$, 60 % relative humidity). These parameters are also set for any kind of pathogen assays on plants. For seed production the plants are treated under long day conditions (16 hours light, 8 hours dark). For transient expression assays *N. benthamiana* plants are grown in the greenhouse under long day conditions for 3 weeks. For MS experiments sterilized seeds are sown into 50 ml ½ MS medium with 1 % (v/w) sucrose, and are incubated for 14 days on a shaker under long day condition. For Co-IP experiments sterile seeds are transferred into a six-well-plate with 5 ml ½ MS medium with 1 % (v/w) sucrose in each well, and incubated as described above. The seedlings are removed from medium, and depending on coming assays treated with elicitors. Then seedlings are carefully dried and directly frozen in liquid $N_2$ and stored at -80 °C.

### 2.2.3.2 Transient transformation of *N. benthamiana* by *Agrobacterium tumefaciens*

For transient protein expression in *N. benthamiana* the *A. tumefaciens* strain GV3101 are performed. 5 ml of LB medium supplemented with appropriate antibiotics are inoculated with A*. tumefaciens* cells containing the construct of interest and grown at 28 °C overnight. Cells are harvested by centrifugation at 4000 x *g* for 5 min. The cell pellets are re-suspended in 10 mM $MgCl_2$ and washing is repeated. The

cultures are diluted to $OD_{600nm}$=1 with 10 mM $MgCl_2$ and the strains are mixed to the same rate, also with the silencing inhibitor P19 (Voinnet et al., 2003). 150 µM acetosyringone are added (from a 150 mM stock in DMSO) and the bacteria are incubated for 1-2 h at room temperature. *N. benthamiana* leaves are pierced with a cannula at the infiltration side, and leaves are infiltrated with a needleless syringe. Infiltrated plants are cultivated under long day condition, and leaves are harvested 2-4 days after infiltration. Signal of yellow fluorescent protein (YFP) fusion proteins expressed in the leaves are used for localization analysis.

#### 2.2.3.3 Subcellular localization studies

To study potential side effect of the mutants in the amino acid sequence on the subcellular localization behavior of the mutated protein, translocation assays are performed. BIR2::BIR2-P-site mutant constructs are cloned into pB7YWG2, and are transiently expressed in *N. benthamiana* up to 3 days. On a Leica SP2 confocal microscope the YFP signal is excited using 514 nm laser collecting emission. Expression of proteins are confirmed via western blot.

#### 2.2.3.4 Stable transformation of *Arabidopsis thaliana* by *Agrobacterium tumefaciens*

To obtain stably transformed *Arabidopsis* lines the *A. tumefaciens* strain GV3101 are used, too. Floral dipping is performed by pre-inoculation of 5 ml LB + antibiotics overnight, and then the transfer of this o/n culture into 250 ml fresh LB + antibiotics; incubation time 18-24 h at 28 °C and 250 rpm shaking. Bacterial solution is centrifuged at 5500 x *g* for 20 min at RT. Cell pellets are re-suspended in 5 % sucrose solution, and adjust to an $OD_{600nm}$ of 0.8. Silwet L-77 are added to a final concentration of 0.02 %. Flowering plants which had still a lot of buds are dipped 2x into the bacterial solution. For the next 24 h the dipped plants are covered by a plastic bag to increase the humidity and thereby increase the success of transformation. For ripening plants are grown under long day conditions.

#### 2.2.3.5 Seeds sterilization

Seeds are sterilized with chlorine gas. A certain amount of seeds are sown into a 2 ml Eppendorf tube (open lid), and are placed into a glass desiccator. Additionally, a beaker with 50 ml 12 % sodium-hypochlorite solution and 2 ml 37 % HCl is included. The desiccator lid is closed, and the seeds are sterilized for 4-18 h. Open Eppendorf tubes are placed under sterile bench for 30 min to force a vaporization of the gas which can otherwise intercalate with the germination.

#### 2.2.3.6 Seed selection

Transformed seeds are selected with the help of selection markers. In the case of pGWB vectors hygromycin is added to the ½ MS medium. Plates are incubated under long day condition for 2 weeks.

Positive selected seedlings are transferred into soil for seed production and protein expression check. In this thesis seeds of the survivors (T2) are utilized for functional and interaction analyses. In some cases, T2 seeds are further selected on hygromycin plates to identify homozygous lines.

#### 2.2.3.7 PAMP assays for interaction studies and MS analysis

Additional to untreated plants different treatments with elicitors are performed, e.g. to study the potential change of phosphorylation sites of BIR2, or the release behavior of BIR2-BAK1 complex. Seedlings are incubated in 1 µM flg22/elf18 for 2.5 min, 5 min or in a mixture of 10 nM epi-brassinolide (BL) for 90 min and 1 µM of flg22, elf18, pep1 for 5 min.

#### 2.2.3.8 Oxidative burst

Leaves of 5-6 week old *Arabidopsis* plants are cut into square pieces, and floated in MilliQ water filled plastic dishes for 18-22 h. A master mix containing 20 µM luminol L-012 and 2 µg/ml peroxidase are prepared and pipetted into a white 96-well plate. The leaf pieces are carefully added to the mixture, and the whole apparatus are transferred into a plate reader/ luminometer (Centro LB 960, Berthold Technologies). The running program is stopped and checked for an equal blank run after 15 min. Then flg22 or elf18 are added to the system to final concentrations of 100 nM, and finally in a 30 min run the production of reactive oxygen species (ROS) are measured in each sample (45 samples per run).

#### 2.2.3.9 Infections with *Alternaria brassicicola*

5-6 week old *Arabidopsis* plants are treated with a spore solution of the *A. brassicicola* strain MUCL20297 (Thomma et al., 1999). 5 µl droplets of a concentration of 1 x $10^6$ spores/ml are pipetted on two leaves per plant with 2 drops per leaf. Optimal six replicates per line are treated and randomly distributed on three trays. These trays are well watered, and the lids are tightly closed to increase the humidity to 100 %. The experiment is implemented under short day conditions, and after 7, 10 and 13 days the spreading of the necrotrophic fungi is calculated after a bonitation method with following values: 1: no symptoms, 2: light brown spots at infection site, 3: dark brown spots at infection site, 4: spreading necrosis, 5: leaf maceration, 6: sporulation. The disease index (DI) is calculated with the following formula: $DI = \Sigma\, i * n_i$. 'i' is the symptom category, and '$n_i$' is the percentage of leaves in 'i'.

#### 2.2.3.10 Kinase inhibition assay

To study the general importance of the kinase activity for the BIR2-BAK1 complex, a kinase inhibitor called K252a is used. As described in Macho et al., (2014) 2 week old seedlings are 1 h pretreated with 2 µM K252a (in DMSO) or equal amounts of only dimethyl sulfoxide (DMSO). Immune responses are afterwards triggered by adding 1 µM flg22 for 5 min. Frozen samples are further used for Co-IPs.

### 2.2.4 Yeast-two-hydride assay

Direct protein-protein interaction studies are performed with the yeast two-hybrid (Y2H) system. For that assay only the intracellular domains of membrane proteins are used because transmembrane regions inhibit transport into the nucleus. The yeast strain PJ69-4A is cultivated on YPD plates and medium at 30 °C. The transformation of the vectors (pGADT7 and pGBKT7), carrying the proteins of interest into the competent yeast cells are reached in a two-step assay. First the yeast culture is incubated at 30 °C till a final $OD_{600nm}$ of 2 ($OD_{600nm}$ = 0.1 -> $10^6$ cells/ml) 50 ml is obtained. The culture is centrifuged (5 min, 4000 rpm at 15 °C) and the pellet suspended in 20 ml 1 x TE/LiAc (pH 7.5). After a further centrifugation step the yeast pellet is resolved in 1 ml of 1 x TE/LiAc and incubated for 30 min at 30 °C. In the meantime, 2 mg/ml salmon sperm DNA (ssDNA) is denatured at 95 °C for 5 min and then placed on ice. A mixture of 240 µl 50 % PEG 3350, 30 µl 10 x TE, 30 µl 10 x LiAc, 40 µl ssDNA and 50 µl competent cells ($5*10^7$ cells) which are obtained after the 30 min incubation, are prepared and added to approximately 100 ng of pGBKT7 plasmid DNA. Samples are again incubated at 30 °C for 30 min and then heat shocked at 42 °C for 10 min. Transformed yeast cells are then spun down for 15 sec at 13000 rpm and carefully resolved in 2 % glucose solution. Yeast culture is plated on SD-T (dropout -tryptophan) and incubated for 2-3 days at 30 °C. Grown colonies indicate the successful first transformation. Those cells are picked and cultivated in SD-T medium overnight at 30 °C. For the second step of transformation (pGADT7) the same procedure as described above are performed whereas the transformed cells are then plated on SD-LT plated (dropout -tryptophan and -leucine). Those yeast colonies which grown on SD-LT medium are transformed with both vectors and could be used for protein-protein interaction studies. For that purpose, the cells are incubated 24 h in SD-LT medium at 30 °C. The density of cells at an OD of 600nm are measured, and the samples are diluted to equal concentrations (optimum $OD_{600nm}$=1). 1.5-2 ml of culture is used for protein expression checks later on by boiling the cells in 70 µl 2.5 x SDS-loading dye for 5 min and the performance of a western blot. The remaining cells are diluted in a 1:10 serial dilution up to 1:1000. 7.5 µl droplets are dropped on SD-LT (as controls of equal distribution/dilution) and SD-HALT (dropout -Trp, -Leu, -histidine, -adenine), and are incubated at 30 °C for several days (3-10 d). Growth can indicate a direct interaction between intracellular domains of proteins.

### 2.2.5 Microscopy

Epifluorescence approaches are carried out with a microscope Eclipse 80i, Nikon and the images are processed with the Lucia Image software. The Leica SP2 confocal microscope are used for subcellular studies.

### 2.2.6 Protein modeling

PyMOL software (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC) is used for graphical modeling and editing of potential phosphorylation sites into BIR2 crystal structure are received by Blaum et al. (2014).

### 2.2.7 Quantification tools

Signal intensity of co-immunoprecipitated BIR2 in the kinase inhibition assay is quantified using ImageJ software, and relativized to the precipitated BAK1 signal.

### 2.2.8 Graphical presentation of plants

Pictures of plants are taken by a Nikon camera (Digital-Sight DS-U1), and are modified in Adobe Photoshop CS5.

### 2.2.9 Statistical analysis

Statistical significance of two groups are analyzed by using Student's t-test of excel (Microsoft). Asterisks represent significant differences (*p <0.05; **p <0.01; ***p <0.001).

# 3. Results

Plasma membrane-localized RLKs are fundamental for the first layer of plant innate immunity. Many RLKs act as PRRs, whereas other RLKs function in a regulatory manner. Regulatory kinases have often a reduced number of extracellular LRRs such as BAK1 and BIR2. The phenotype of knocked out-BIR2 in *Arabidopsis* is visible by eye (Halter et al., 2014a). The plants are reduced in their growth, and premature senescence of leaves is detectable. Moreover, in typical PTI responses, such as ROS burst, *bir2* plants are hyperresponsive to PAMPs. These phenotypical readouts are stronger in a knock down line of BIR2 in Col-0 (*bir2-1*). However, due to the partial expression of BIR2 in the Col-0 allele, the WS-0 null allele, *bir2-2,* is preferentially analyzed in this thesis. Moreover, BIR2 is identified as a negative regulator of BAK1 in PAMP-triggered-signaling pathways (Halter et al., 2014a). The interaction of BAK1 and BIR2 is constitutive (Figure 3-1 B), but after stimulation of PRRs BIR2 is released from BAK1 (Domínguez-Ferreras et al., 2015; Halter et al., 2014a; Imkampe, 2015). BAK1 is a catalytically functional kinase, and regulates other kinases and itself via transphosphorylation and autophosphorylation, respectively (Figure 3-1 A). By contrast, BIR2 seems to be an atypical kinase, and kinase activity is not observed (Figure 3-1 A) (Blaum et al., 2014; Halter et al., 2014a). Moreover, BIR2 was identified as a kinase substrate for BAK1 (Figure 3-1 A) (Halter et al., 2014a). Schwessinger et al. (2011) have published the identification of the novel BAK1 allele *bak1-5*, a hypoactive kinase, due to a single mutation (C408Y), just before the catalytic loop (subdomain VIa). This line, which is not affected in BR-signaling and cell death control, is used for Co-IPs (assay performed by Dr. Julia Imkampe). The amount of BIR2 proteins, bound to BAK1, is strongly decreased in *bak1-5* plants compared to wild type (Figure 3-1 B), indicating that BAK1 kinase activity has an impact on BIR2-BAK1 complex formation.

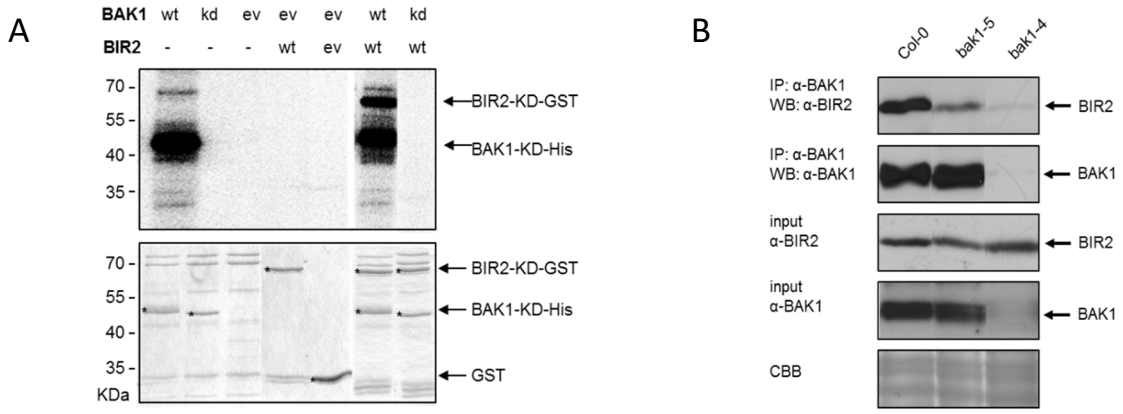



**Figure 3-1: BIR2, a substrate of BAK1, and the interaction of BIR2 and BAK1 is dependent on BAK1 kinase activity (Halter et al., 2014a).**
**A**: *In vitro* kinase assay with recombinantly expressed kinase domains of BAK1 wild type (wt) or kinase-dead (kd) mutant K317E fused to His6, and BIR2 fused to GST (ev, empty vector control). The upper panel shows the autoradiogram, the lower panel shows the Coomassie stained gel, and expressed proteins are marked with asterisks. **B:** *Arabidopsis* seedlings of the indicated genotypes are used for an immunoprecipitation (IP) of BAK1. Co-immunoprecipitated BIR2 is detected with BIR2 antibody on western blot (WB). Total BIR2 and BAK1 protein amount is given as input. Coomassie brilliant blue (CBB) staining of the membrane shows protein loading.

Phosphorylation is a general mechanism for activation and deactivation of processes in cells. In chapter 1 the importance of kinase activity and phosphorylation for the stability of the BIR2-BAK1 complex is investigated. Chapter 2 is focused on the identification of *in vivo* BIR2 P-sites by using MS technology, and in chapter 3 those identified P-sites are studied for their functional relevance.

## 3.1 BIR2-BAK1 complex behavior, and relevance of kinase activity for the complex stability

### 3.1.1 BIR2 is partially released from BAK1 after elf18 perception

In Halter et al. (2014a) it is shown that BAK1 is partially released from the BIR2-BAK1 complex after flg22 perception to support FLS2-signaling. A similar observation is made for pep1 (a DAMP) and brassinolide (BL)-treated *Arabidopsis* plants. Another well studied PAMP-signaling pathway involves elf18, sensed by EFR. In a previous PhD thesis (Imkampe, 2015) the author has confirmed a dissociation of BIR2 from BAK1 after treatment with a mixture containing flg22, elf18, pep1, and BL. Individual interactional effects of elf18 on BIR2-BAK1 complex is not tested yet. Here, it is shown that elf18 triggers a similar partial release of BIR2 from BAK1 (Figure 3-2). Due to the lack of functional EFR-specific antibodies, it is not possible to support this result by showing a stronger interaction of BAK1 with EFR after ligand perception. However, this outcome indicates that BIR2 is a negative regulator in EFR-signaling, which is previously confirmed by functional assays, such as ROS burst and growth inhibition (Halter et al., 2014a).

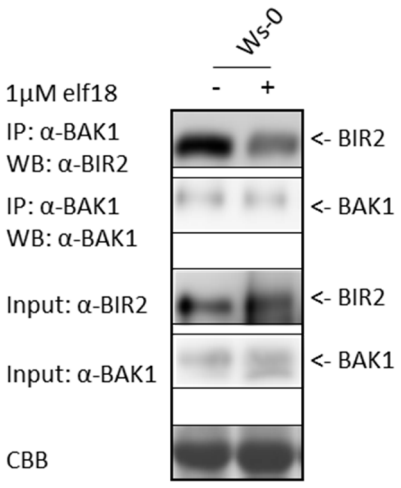



**Figure 3-2: Treatment with the PAMP elf18 leads to a partial release of BIR2 from the BIR2-BAK1 complex.** Ws-0 seedlings are treated with 1 µM elf18 for 5 min (+), or kept untreated (-). Protein extractions are equalized in the total protein amount, and BAK1 protein is immunoprecipitated. A western blot is performed, and proteins of interest (BIR2 and BAK1) are detected by using specific antibodies. Coomassie brilliant blue (CBB) staining is used as loading control.

### 3.1.2 Mutation of the catalytically important lysine has no effect on BIR2 functionality

Most kinases carry a catalytically important lysine (K) in their KD. In BIR2, the lysine is located at the amino acid position 335, and substitution to glutamic acid (E) (created by Dr. Sara Mazzotta, Mazzotta, 2012) are further investigated in this thesis. First of all, co-immunoprecipitation (Co-IP) and further assays are performed in stably transformed *Arabidopsis* plants. The mutation of the residue K335E does not affect the release ability of BAK1 from BIR2 after elf18-sensing compared to wild type (Figure 3-3 B). Further, the constitutive interaction quantities of BIR2 and BAK1 proteins are in a similar range like wild type (Figure 3-3 A). Summing up, K335 seems to have no impact on the complex stability of BIR2-BAK1.

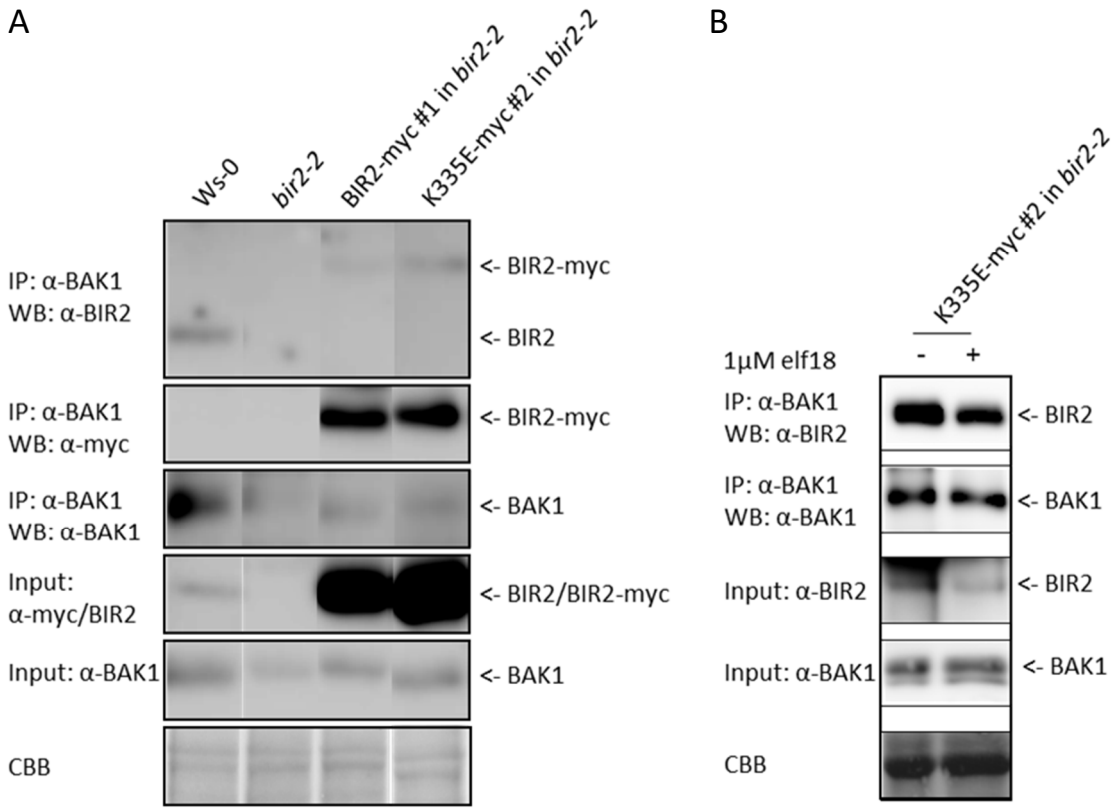



**Figure 3-3: A mutation in a conserved lysine residue of BIR2 KD does not change the interaction of BIR2 and BAK1.**
Stably transformed *Arabidopsis* seedlings of BIR2-myc and K335E-myc under the native BIR2 promotor, as well as Ws-0 and *bir2-2*, are used for Co-IPs. IP is performed by pulling down BAK1, and a western blot by using specific protein antibodies and/or myc antibodies. **A:** Untreated seedlings are used. **B:** Seedlings are treated 5 min with 1 µM elf18 (+), compared to untreated (-), and then further proceed as described above. Coomassie brilliant blue (CBB) staining is used as a loading control.

A complementation line with mutated K335E rescues the *bir2-2* growth phenotype (Figure 3-4 A), as well as the ROS burst output (Figure 3-4 B). These results show that this lysine is not necessarily essential for the function of BIR2. This and the previously observed unaffected integrity of the BIR2-BAK1 complex show that this residue is not required for BIR2 function and interaction. Kinase activity seems to be not necessary for BIR2 function as this mutant is a surely kinase dead variant. In

conclusion, the conserved K335 has no remarkable influence on the PTI-related performance of BIR2 and BAK1. These observations show that the atypically inactive kinase BIR2 exerts its function independent of any kinase activity (Blaum et al., 2014; Halter et al., 2014a).

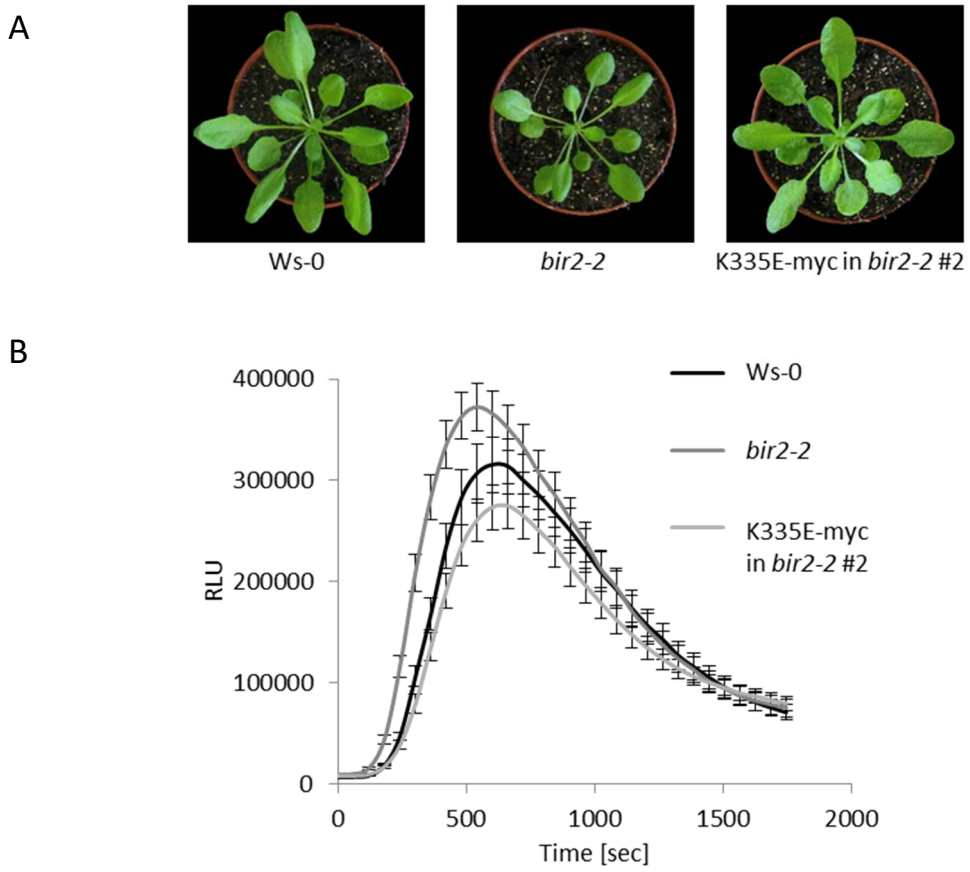



**Figure 3-4: K335E mutation in BIR2 KD does not inhibit functional complementation of *bir2*.**
**A:** Growth phenotype of 5 week-old *Arabidopsis* plants, grown under short day conditions. **B:** ROS burst assay. Overnight in water adjusted leaf pieces are treated with 100 nM elf18. The oxidative burst within the first 30 min is measured. n= 9, 3 biological replicates. Standard error is given.

### 3.1.3 Proper kinase activity is crucial for dissociation of BIR2-BAK1 complex

After the observation of a potential correlation between BAK1 kinase activity and BIR2-BAK1 complex formation (Figure 3-1 B), the question has raised, whether kinase activity can trigger a dissociation of the complex. To study this issue, a general kinase inhibitor, K252a, that acts by preventing ATP binding to kinases is tested (Figure 3-5 A). *Arabidopsis* seedlings are either pretreated with 2 µM K252a or DMSO (mock control solution), and are further inoculated with 1 µM flg22 for 5 min or directly frozen. A BAK1 IP is performed, and binding abilities of BIR2 and FLS2 are examined (Figure 3-5 B). Intriguingly, more BIR2 is bound to BAK1 after treatment with the inhibitor. FLS2 bound to BAK1 is detectable after flg22 treatment, and here a reduced amount of FLS2 is linked to BAK1 after treatment with K252a. Surprisingly, a weak band of FLS2 could be detect after kinase inhibitor treatment but without flg22 co-treatment. This observation is similar to the results of Schwessinger et al. (2011), however this finding is only once spotted, and thus has to be repeated and investigated in more detail. Quantitative

analyses of the protein bands show that in an inhibitor-free context slightly less BIR2 is bound to BAK1 as shown in interaction assays in Halter et al. (2014a) (Figure 3-5 C). The kinase inhibitor results in an overall increase of BIR2 bound to BAK1, whereas this complex stability is only to some extent weaker after flg22 perception (Figure 3-5 C). The influence of flg22 in this context is not that clear, by having still a BIR2 releasing effect after supplementing K252a (Figure 3-5 C). However, this finding might hint to an involvement of kinase activity to trigger the release of BAK1 from BIR2. Summing up, functional kinase activity of BAK1 and/or other kinases could be an important property to trigger mobility of the BIR2-BAK1 complex.

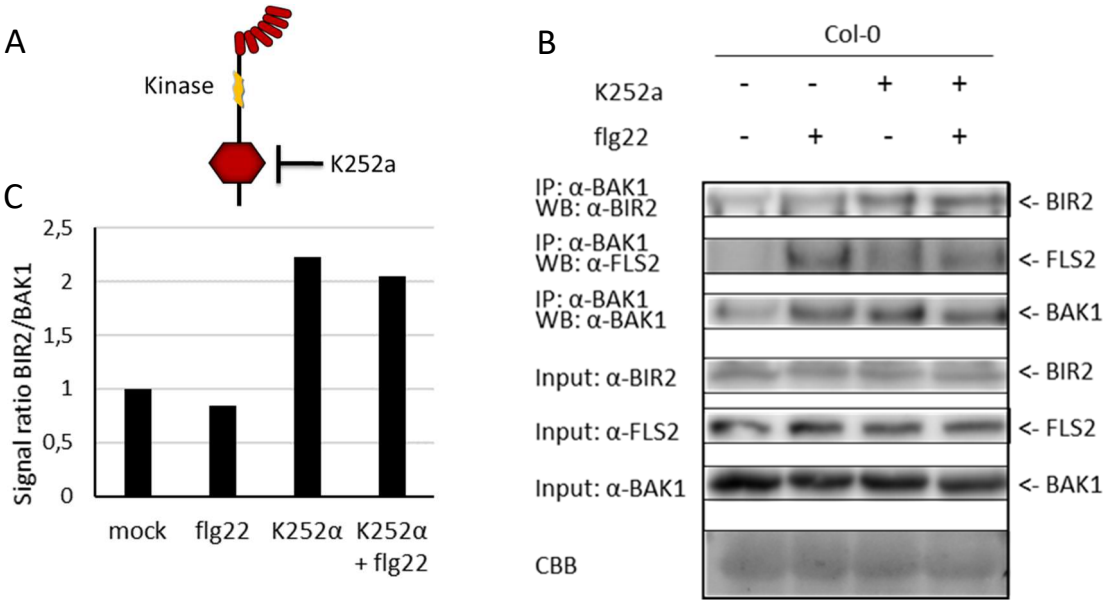



**Figure 3-5: Kinase activity could be an important feature for release of BIR2-BAK1 complex.**
**A:** A general kinase inhibitor called K252a is used. It can interfere with the ATP-binding site of kinase domains. **B:** 1 hour pretreatment of 2 week-old *Arabidopsis* seedlings with 2 µM K252a or DMSO (mock solvent control). Immune response is afterwards triggered by adding 1 µM flg22 for 5 min (+), or untreated (-). Co-IP is performed by pulling down BAK1, and checking for BIR2- and FLS2-binding in WB (specific antibodies are used). Equal loading pattern are checked by Coomassie brilliant blue (CBB) staining. **C:** Signal intensity of co-immunoprecipitated BIR2 is quantified by using ImageJ software relative to the precipitated BAK1 signal. Quantification represents the average of four independent assays.

## 3.2 Identification of *in vivo* P-sites

### 3.2.1 Phenotypical changes base on overexpressed BIR2 protein levels

In this work 35S::BIR2-YFP lines in *Arabidopsis* (Col-0) are used for the identification of *in vivo* P-sites, because of the need of high quantities of BIR2 protein. According to the regulatory function of BIR2, increased protein amounts lead to morphological and functional phenotypes (Halter, 2014; Imkampe, 2015). Those plants are smaller and denser (Figure 3-6 A) than wild type (wt, here Col-0). The correct BIR2-YFP localization is confirmed by epi-fluorescence microscopy (Figure 3-6 B). As previously shown (Halter, 2014; Imkampe, 2015), increased BIR2 levels result in reduced flg22 sensitivity (Figure 3-6 C), caused by enhanced complex formation of BIR2 and BAK1 (Halter et al., 2014a). These data show that BIR2-YFP fusion proteins are functional.

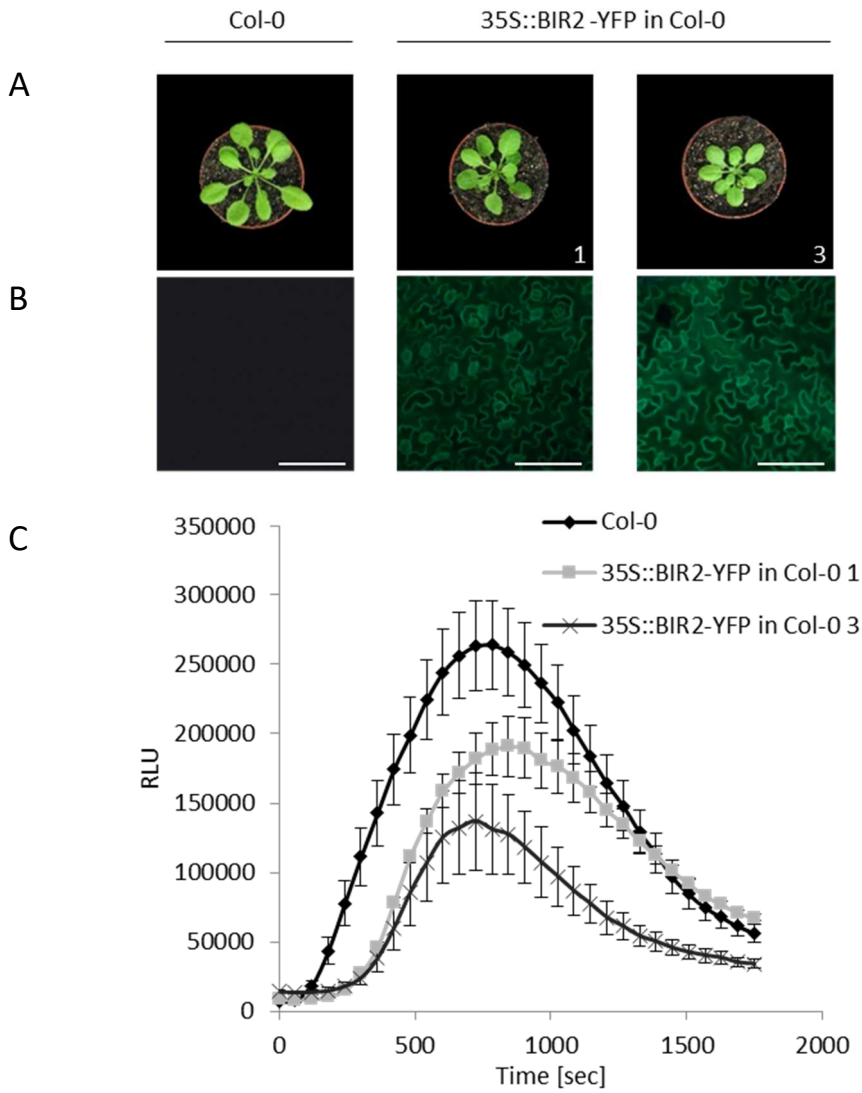



**Figure 3-6: Overexpressed BIR2-YFP in Col-0 background leads to reduced flg22 sensitivity.**
**A:** Growth phenotype of 5 week-old plants under short day conditions. Col-0 (wt) and two independent BIR2 OE lines 1 and 3. **B:** BIR2-YFP expression is detected with fluorescence microscopy. Bar represents 100 µm. **C:** ROS burst assay of leaf pieces, treated with 100 nM flg22, n= 2-6, and standard error is given. RLU= relative light units.

### 3.2.2 Processing of samples for the identification of *in vivo* P-sites via MS

Kinase activities, and the resulting phosphorylation events could be essential for binding affinities of BIR2 to BAK1, as shown in chapter 1 of the results. In a previous PhD thesis (Mazzotta, 2012), the KD of BIR2 and BAK1 are co-expressed in *E. coli*, and mass spectrometry has revealed potential P-sites of BIR2, transphosphorylated by BAK1. This *in vitro* method is suitable to achieve high amounts of proteins, required for the significant identification of P-sites via MS analysis. But it still represents an artificial system, and thus the identification and confirmation of P-sites of BIR2 in an *in vivo* system is the concerning issue. To execute this task, a workflow for the discovery of *in vivo* P-sites is established (Figure 3-7).

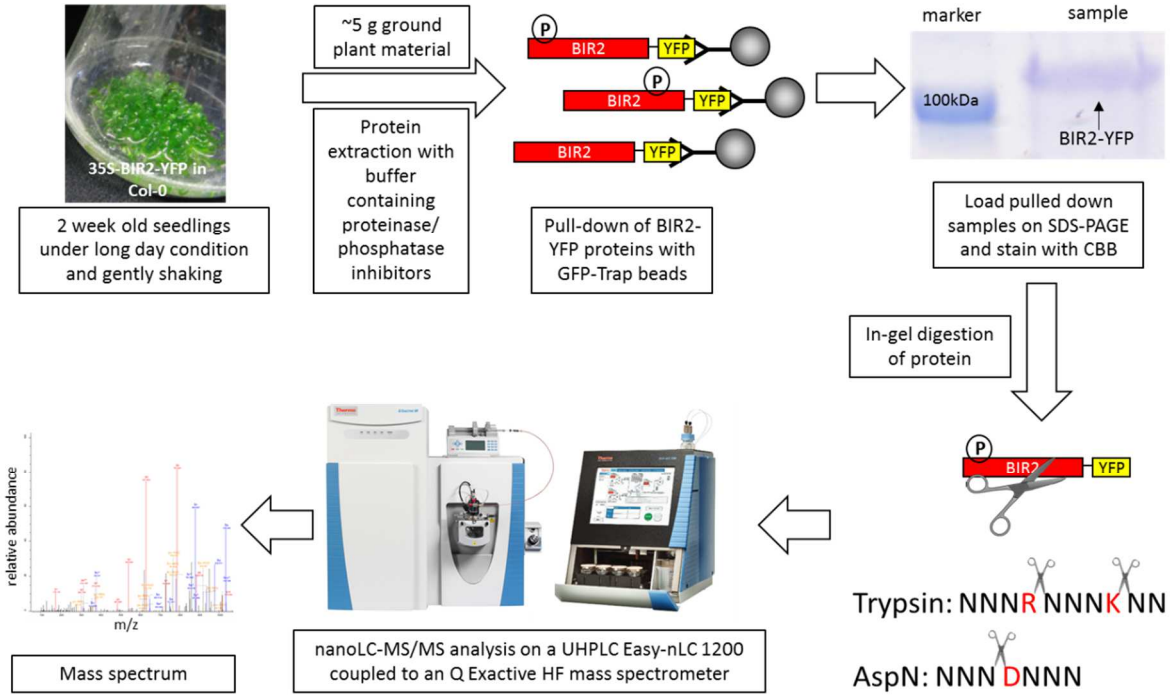



**Figure 3-7: Workflow of preparation and procession of samples for *in vivo* LC-MS/MS analysis.**
2 week-old BIR2-YFP overexpressed *Arabidopsis* seedlings are frozen and ground. Around 5 g of plant powder is used for protein extraction, and pull down of BIR2-YFP protein is performed with GFP-Trap beads. Separation and purification is done on an 8 % SDS-PAGE. Observed BIR2-YFP band is sent to Proteome Center for in-gel digestion, and mass spectrometry (MS).

Here, by using BIR2 overexpression lines, about 5 g of materials are extracted, and BIR2-YFP is pulled down by GFP-Trap beads. Separation and purification is performed on an 8 % SDS-PAGE (Figure 3-7/8), and stained BIR2-YFP band (~105 kDa) is sent to Proteome Center for in-gel digestion and mass spectrometry. Leftovers of immunoprecipitated BIR2-YFP is further proceeded on a western blot, and BIR2-YFP protein is detected with specific BIR2 antibodies (Figure 3-8). This additional step is performed as a control to confirm the correct spotted band on the stained SDS-PAGE for MS analysis.

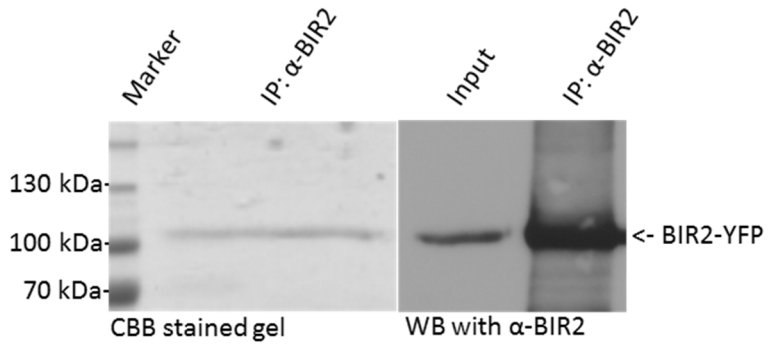



**Figure 3-8: Immunoprecipitated BIR2-YFP on CBB stained gel, and confirmation of BIR2 detection via WB for *in vivo* LC-MS/MS analysis.**
Around 5 g of 2 week-old BIR2-YFP overexpressing *Arabidopsis* seedlings are used for protein extraction, and BIR2-YFP is pulled down. Separation and purification is performed on an 8 % SDS-PAGE. Western blot (WB) is done to confirm BIR2-YFP detection on gel. Observed BIR2-YFP band on gel is sent to Proteome Center for in-gel digestion and mass spectrometry.

### 3.2.3 Identification of *in vivo* P-sites of BIR2

Several runs on a nanoLC-MS/MS are performed. The prepared protein bands on SDS-PAGE are classically digested with trypsin, and further processed as previously described. Table 3-1 (A) sums up the identified phosphopeptides, and the corresponding P-sites. Due to the lack of lysine (K) and arginine (R) in the activation loop of BIR2, a rather long phosphopeptide (LMVPSDNNESSFMTGDLGEFGYVAPEYSTTMLASLK) is detected, making the precise identification of a P-site complicated. In this context, another digestion enzyme, Asp-N is used (Table 3-1 B), and has partially helped to receive a significant confirmation of P-sites.

**Table 3-1: Summary of identified *in vivo* phosphorylation sites of BIR2.**
2 week-old untreated *Arabidopsis* seedling overexpressing BIR2 in Col-0 are sent for MS analysis, and identification of phosphorylation sites **A:** Observed P-sites after in-gel digestion with trypsin or in **B:** after Asp-N digestion. Numbers in brackets indicate no significant values (related to PEP and localization values, not shown) for a safe identification of potential P-sites. Juxtamembrane= JM, kinase domain= KD, and C-terminus= CT.

A

| Domain | Amino acid position | Phosphopeptides, trypsin digestion | Number of identification |
|---|---|---|---|
| JM | 263 | pSGLTEVGVSGLAQR | 7 |
| | 266 | SGLpTEVGVSGLAQR | 4 |
| | 271 | SGLTEVGVpSGLAQR | 1 |
| | 279 | LRpSHKLTQVSLFQK | 3 |
| | 286 | LTQVpSLFQKPLVK | 7 |
| KD | 349 | EFRpYEMNQLWELR | 1 |
| | 379 | FLVpYKYMSNGTLHSLLDSNR | 1 |
| | 381 | FLVYKpYMSNGTLHSLLDSNR | 1 |
| | 462 | LMVPSDNNEpSSFMTGDLGEFGYVAPEYSTTMLASLK | (1) |
| | 463 | LMVPSDNNESpSFMTGDLGEFGYVAPEYSTTMLASLK | (3) |
| | 466 | LMVPSDNNESSFMpTGDLGEFGYVAPEYSTTMLASLK | (1) |
| CT | 585 | QGYpSFSEQDDDFPLIFDTQENEK | (1) |

B

| Domain | Amino acid position | Phosphopeptides, Asp-N digestion | Number of identification |
|---|---|---|---|
| KD | 462 | DNNEpSSFMTG | (1) |
| | 463 | DNNESpSFMTG | (2) |
| | 466 | DNNESSFMpTG | 1 |
| | 492 | DVpYGLGVVLLELATGLKAVGGEGFKGSLV | 2 |

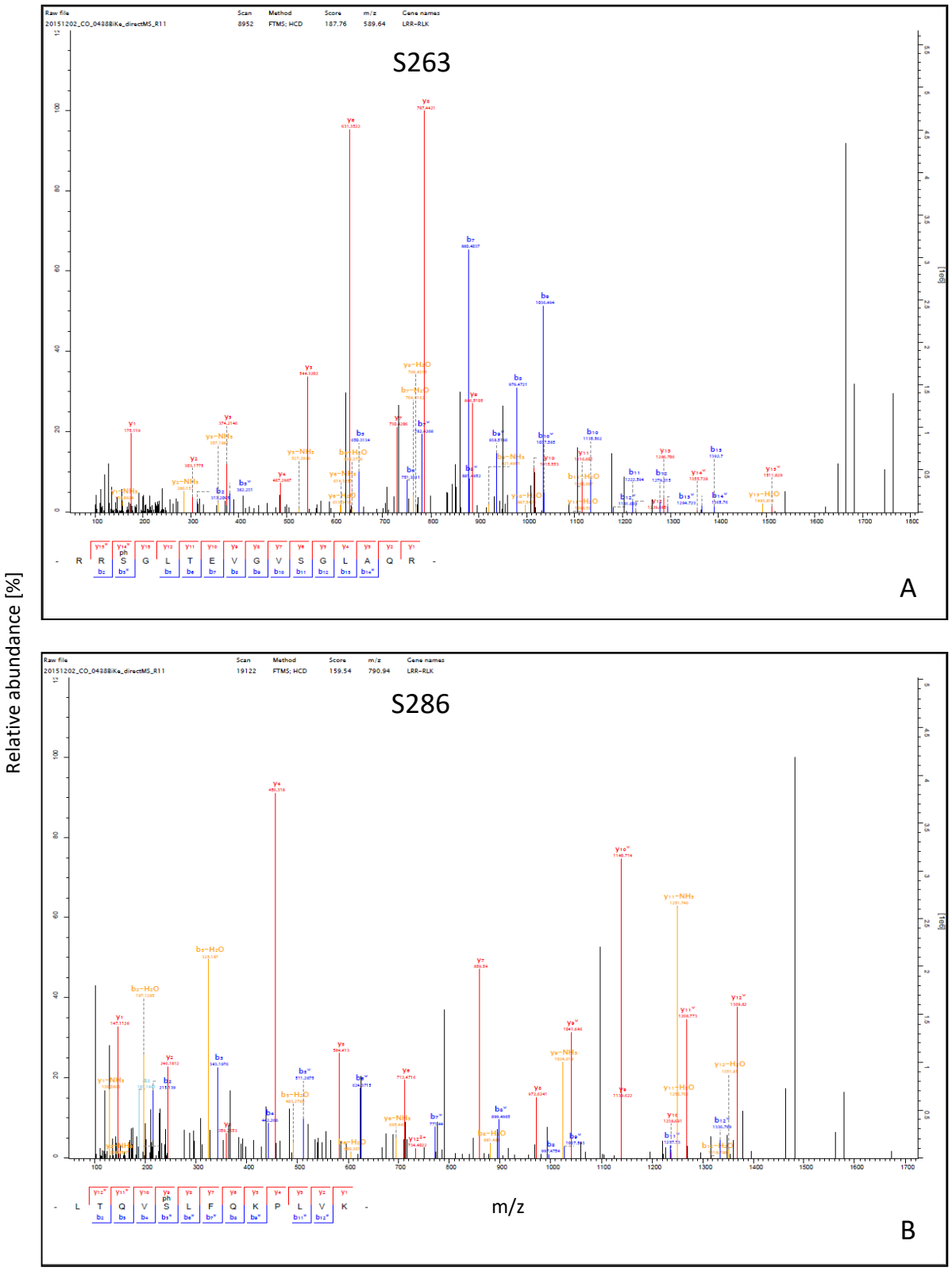
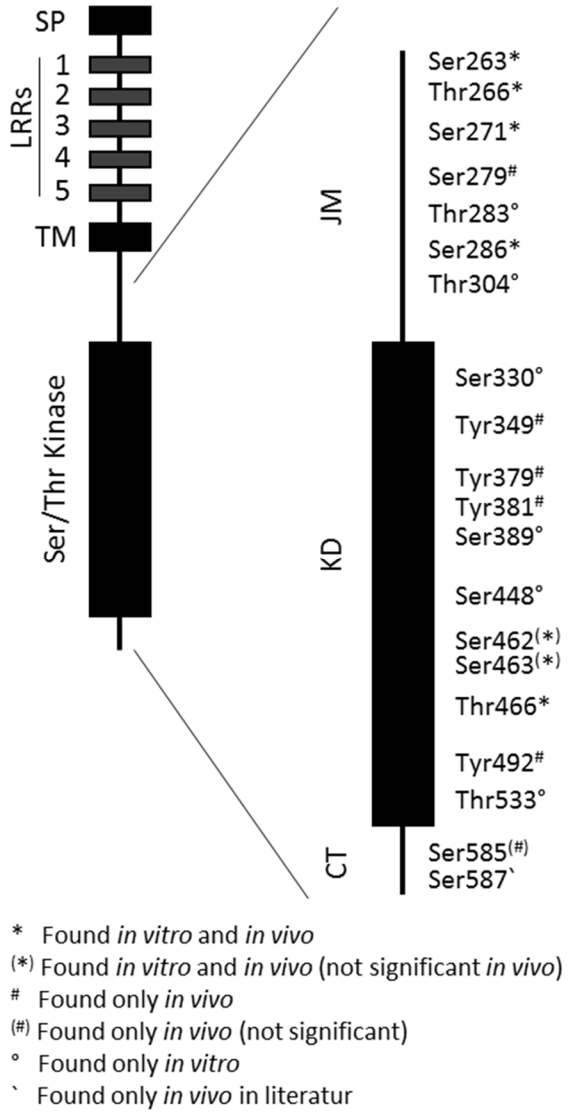



**Figure 3-9: MS spectra of two abundantly identified phosphopeptides of BIR2.**
**A:** Spectra of BIR2 peptides containing phosphorylated S263 and in **B:** phosphorylated S286. Mass spectra are given in relative abundance (%) vs. mass to charge (m/z).

Overall 13 P-sites are detected, located in the JM (five P-sites), in the KD (seven P-sites), and in the C-terminal section (one P-site) of BIR2. The potential P-sites in the JM are S263, T266, S271, S279 and S286, which are discovered several times (except S271) in independent repetitions, pointing to a significance of the JM as a specific target for phosphorylation events. Especially S263 and S286 are found in almost all assays, pointing to a major role of these two P-sites for BIR2. Thus, Figure 3-9 (A, B) represents the MS spectra of these phosphopeptides, including phosphorylated S263 (Figure 3-9 A) and phosphorylated S286 (Figure 3-9 B), respectively, and confirms the identification of these potential BIR2 P-sites. All other MS spectra of observed phosphopeptides of BIR2, carrying a potential BIR2 P-sites, are added as supplements (Figure 8-1-6). Seven BIR2 P-sites are discovered in the KD, which are Y349, Y379, Y381, S462, S463, T466 and Y492. These *in vivo* P-sites are rather rarely observed, and S462 and S463 are not significantly identified (related to PEP and localization values, not shown), yet. T466 is detected as a true P-site after changing the digestion enzyme. Surprisingly, a high concentration of tyrosine P-sites are located in the KD, whereas in the JM serine is the dominant P-site. S462, S463 and T466 are located in a catalytically important section of kinases, in the activation loop. A single P-site is situated in the CT, S585, but this potential site is not significantly observed yet, and only found once.

Comparing and summarizing the *in vivo* P-sites with the previously discovered *in vitro* P-sites of BIR2 (Blaum et al., 2014; Mazzotta, 2012) and results from literature research, a general overview can be established (Figure 3-10 A and Table 8-1). A total of 20 potential P-sites of BIR2 are identified, whereby the five sites, S263, T266, S271, S286 and T466 are significantly found *in vitro* and *in vivo*. The two adjacent residues S462 and S463 are identified *in vitro* but not significantly *in vivo*, although this observation still indicates a possible action of one or both serines as potential P-sites of BIR2. The JM-located sites T283 and T304, and the KD located sites S330, S389, S448 and T533 are only identified in *in vitro* assays. Whereas, the P-sites S279, Y349, Y379, Y381 and Y492 are exclusively detected *in vivo*. The C-terminal P-sites S585 and S587 are identified *in vivo* assays, whereby S587 is only discovered after literature studies (Nühse et al., 2004). Summing up, 25 % of the potential BIR2 P-sites are confirmed *in vitro* and *in vivo*, and are almost completely positioned in the JM, with the exception of T466 within the activation loop of the KD. Moreover, the residue tyrosine is only confirmed as a phosphoacceptor *in vivo*. The overall distribution of the 20 P-sites shows that 35 % of P-sites are within the JM, 55 % in the KD and 10 % in the CT. 55 % of the P-sites are the residue serine, 25 % threonine and 20 % tyrosine residues (Figure 3-10 B).

A




B

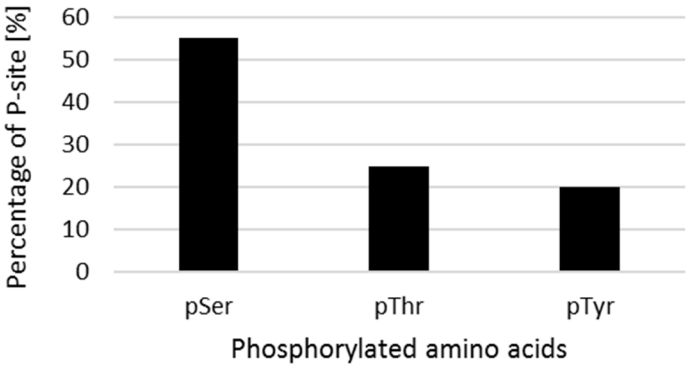



**Figure 3-10: Schematic overview of all *in vitro* and *in vivo* identified P-sites of BIR2.**
**A:** SP= signal peptide, TM= transmembrane region, JM= juxtamembrane domain, KD= kinase domain, CT= C-terminus. Ser= serine, Thr= threonine, Tyr= tyrosine. **B:** Relative distribution (in %) of certain amino acids as potential BIR2 P-sites.

Based on the crystal structure of BIR2 (accession number: PDB:4L68) (Blaum et al., 2014), a 3D model of its intracellular part is created with PyMOL (Figure 3-11). The color coding represents in orange the JM, in blue the KD and in red the CT. The 20 likely P-sites are modulated into the crystal structures, and are labeled yellow. Interestingly, many of the residues are located on the surface of the protein structure, allowing an easy access for other proteins, such as kinases to transphosphorylate BIR2, and might further act as interaction platforms for downstream signaling. Nevertheless, the P-sites are rather widely distributed in the 3D model, but show some central groups, created by two or more P-sites, such as S263, T266 and S271, and S462, S463 and T466.

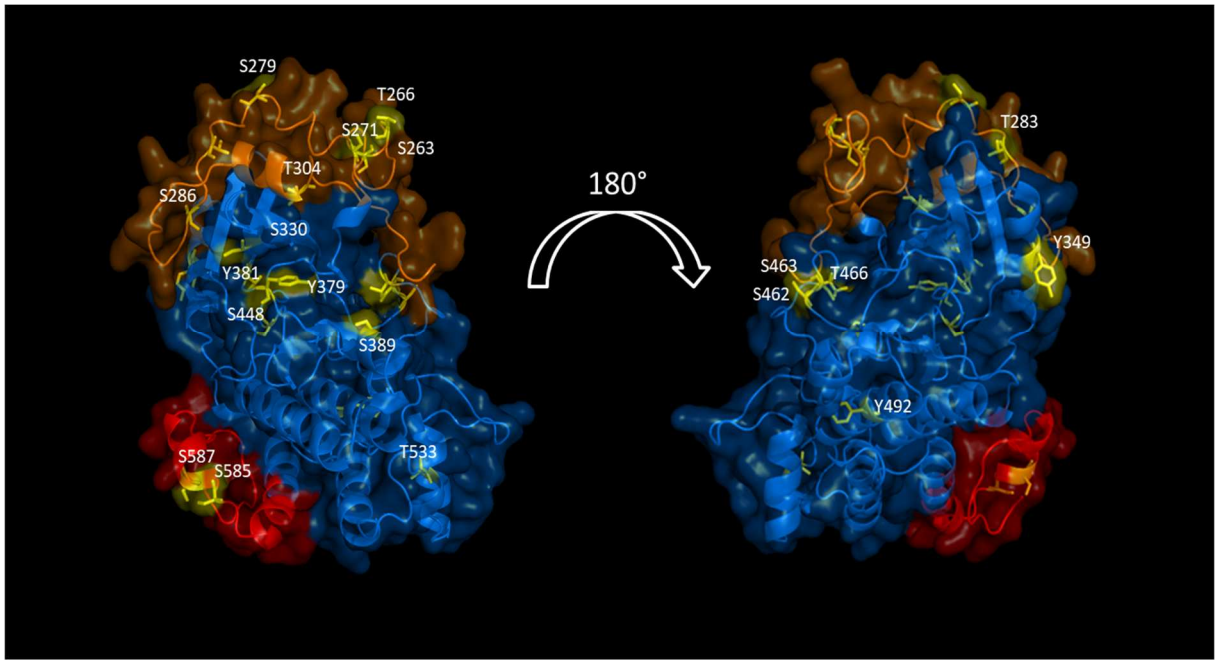



**Figure 3-11: 3D model of intracellular domains of BIR2.**
Orange correlating with juxtamembrane domain, blue represents kinase domain, and red the C-terminus. Highlighted amino acids in yellow show identified P-sites. S= serine, T= threonine and Y= tyrosine. Created with PyMOL, and crystal structure is based on Blaum et al. (2014).

### 3.2.4 Identification of *in vivo* P-sites of BIR2 in a PAMP/DAMP/BL-dependent manner.

As mentioned in chapter 3.1, kinase activity and resulting phosphorylation might be a mechanism for functional activation or deactivation of proteins. In the case of BIR2 and BAK1, their interaction behavior is a key tool for the further activation of PTI, triggered by ligand perception of the corresponding PRR. The flg22-FLS2 signaling pathways is well described (Couto & Zipfel, 2016; Gómez-Gómez & Boller, 2000), as is the meaning of BIR2 and BAK1 in this pathway (Halter et al., 2014a). The perception of a PAMP/DAMP by a PRR triggers the release of BAK1 from its negative regulatory-acting interaction partner BIR2. This might influence certain BIR2 residues, which are phosphorylated or non-phosphorylated, respectively. Thus, 35S::BIR2-YFP seedlings are treated with flg22 or a mix of flg22, elf18, pep1 and BL, and treatments are stopped at different time points (2.5 min or 5 min). Samples are processed as previously descripted, and are sent for MS analysis. Untreated samples are processed in the same way as the treated once for comparison reasons. The JM-located sites S263 and S286 are found in high consensus in untreated and treated samples (Table 3-2). The outcome of phosphorylated T266 and S279 is different. Both are only found in untreated samples after 2.5 min flg22 and mix treatment (5 min PAMP/DAMP, 90 min BL), but surprisingly detected in treated samples after 5 min, supplementing only flg22. This could indicate that not only the duration of treatment is important for specificity of phosphorylation events, but also the complexity of treatment. However, these two sites are overrepresented in an untreated environment. The P-sites in the KD are much harder to be significantly detect in MS, and the outcome does not show any specific outline.

**Table 3-2: Summary of identified *in vivo* phosphorylation sites of BIR2 after treatment with elicitors.**
2 week-old *Arabidopsis* seedling overexpressing BIR2 are treated with 1 µM flg22 for 2.5 min up to 5 min, or with a mix containing 1 µM flg22, elf18, pep1 (5 min) and 10 nM BL (90 min). As a negative control, untreated (untr.) materials are used. All samples are sent for MS analysis, and identification of phosphorylation sites. Numbers in brackets indicating no significant values (related to PEP and localization values, not shown). Juxtamembrane = JM, kinase domain= KD, and C-terminus= CT.

| Domain | Amino acid position | Number of identification | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | untr. | 2.5 min flg22 | | untr. | 5 min flg22 | | untr. | mix |
| JM | 263 | 2 | 2 | | 4 | 3(1) | | 2 | 2 |
| | 266 | 2 | | | 1 | 3 | | 2 | |
| | 279 | 1 | | | 2 | 2(1) | | 1 | |
| | 286 | 2 | 2 | | 4 | 4 | | 2 | 2 |
| KD | 349 | | 1 | | 1 | | | | |
| | 379 | 1 | | | | (1) | | 1 | 1 |
| | 381 | (1) | 1 | | | (1) | | (1) | |
| | 462 | | | | | (1) | | | (1) |
| | 463 | (1) | | | (1) | (1) | | (1) | (1) |
| | 466 | (1) | | | | (1) | | (1) | (1) |

Similar starting materials, which should be confirmed regarding total protein amount, total peptide amount, and similar quality of the chromatography, can be employed to achieve an estimation when aiming for a rough comparison of the abundance of certain phosphopeptides in different samples. Thus, the intensity values of S263 and S286 are further investigated after checking and approving the

above-mentioned comparability of the material (Figure 8-7/8), here related to untreated versus treated material. Two individual MS assays are used and compared in the total intensity values of their phosphopeptides (Figure 3-12 A, C), and the fold changes related to the intensity values of treated versus untreated samples (Figure 3-12 B, D) are calculated for the previously named treatments. The intensities of the phosphopeptide, containing S263 show no differences in treated versus untreated material after 2.5 min flg22 treatment (Figure 3-12 A, B). After an incubation time of 5 min of flg22 the two independent assays behave rather different (Figure 3-12 A). Assay 1 indicates almost no increase, whereas assay 2 shows an enhanced phosphopeptide intensity after treatment. Clearer is the output of mix treated samples, leading to an increase of the phosphopeptide after incubation with the mixture (Figure 3-12 B). Similar observation is made for the intensities of the S286-included phosphopeptide (Figure 3-12 C, D). Already 2.5 min flg22 treatment results in an enhanced intensity of the phosphopeptide, and this result is even more intense after 5 min flg22, and highest after mix treatment (Figure 3-12 D). Overall, an incubation time of 5 min seems to be suitable to trigger intensity changes of phosphopeptides. Treatments with several ligands at once seem mostly to strengthen the effect.

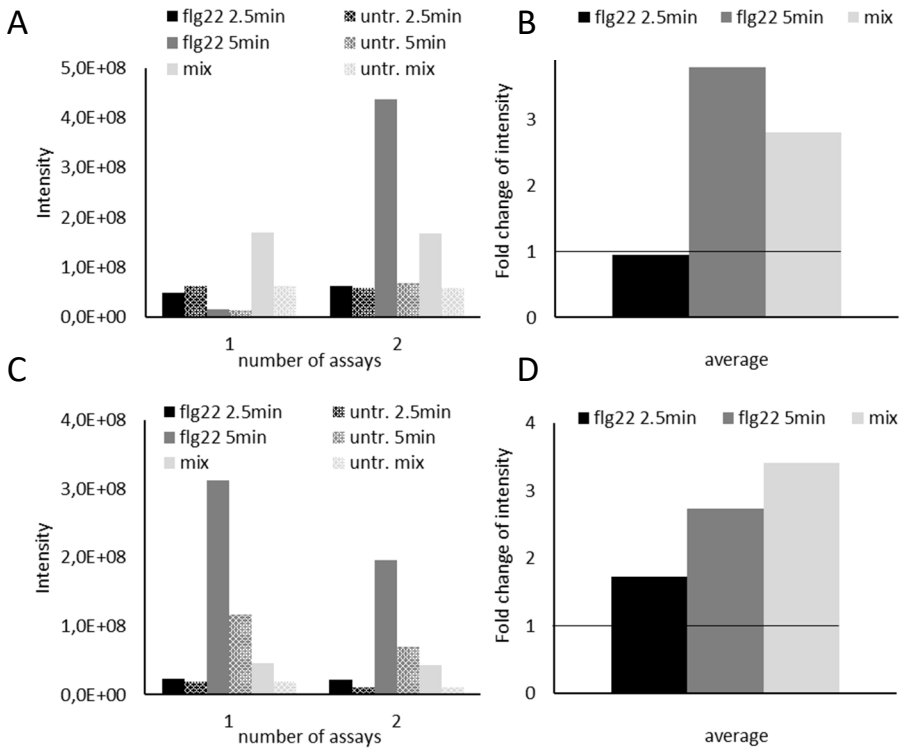



**Figure 3-12: Ligand treatments have an increasing effect on intensities of identified phosphopeptides.**
2 week-old *Arabidopsis* seedling are treated with 1 µM flg22 for 2.5 min up to 5 min or with a mix containing 1 µM of flg22, elf18, pep1 (5 min) and 10 nM BL (90 min). As a negative control, untreated (untr.) materials are used. All samples are sent for MS analysis to the proteome center, and identification of phosphorylation sites. Based on equal chromatogram patterns within treated versus untreated samples as well as equal total BIR2 peptide intensities and amounts (see Figure 8-7/8), the intensities of phosphopeptides could be compared in a semi-quantitative manner. **A:** Total intensity of phosphopeptides containing S263. **B:** Fold change of intensity (calculated out of **A**) of P-site S263 treated versus untreated (whereas the black line (=1) represents no differences between treated versus untr.). **C:** Total intensity of phosphopeptides containing S286. **D:** Fold change of intensity (calculated out of **C**) of P-site S286 treated versus untreated (whereas the black line (=1) represents no differences between treated versus untr.).

### 3.2.5 Overexpressed BIR2 in the absence of BAK1 leads to flg22 insensitivity

*In vitro* assays for the identification of BIR2 P-sites are made by co-expressing BIR2-KD and BAK1-KD in *E. coli* (Mazzotta, 2012); thus identified *in vitro* P-sites are transphosphorylated by BAK1. However, the discovery of the only *in vivo* occurring P-site S279 has raised the question whether some BIR2 P-sites are BAK1-independent or additional targets of other kinases. To resolve this issue, 35S::BIR2-YFP lines in *bak1-4* background are studied (Figure 3-13 B). Growth phenotypes of these plants (Figure 3-13 A) already show that these plants are much smaller and denser than the *bak1* knock out line (three independent lines 1, 4 and 5). Especially, line 4 and 5 show slightly premature senescence of leaves. ROS burst assays reveal flg22 insensitivity of these lines, showing the negative regulatory effect of BIR2 on the flg22 pathway (Figure 3-13 C).

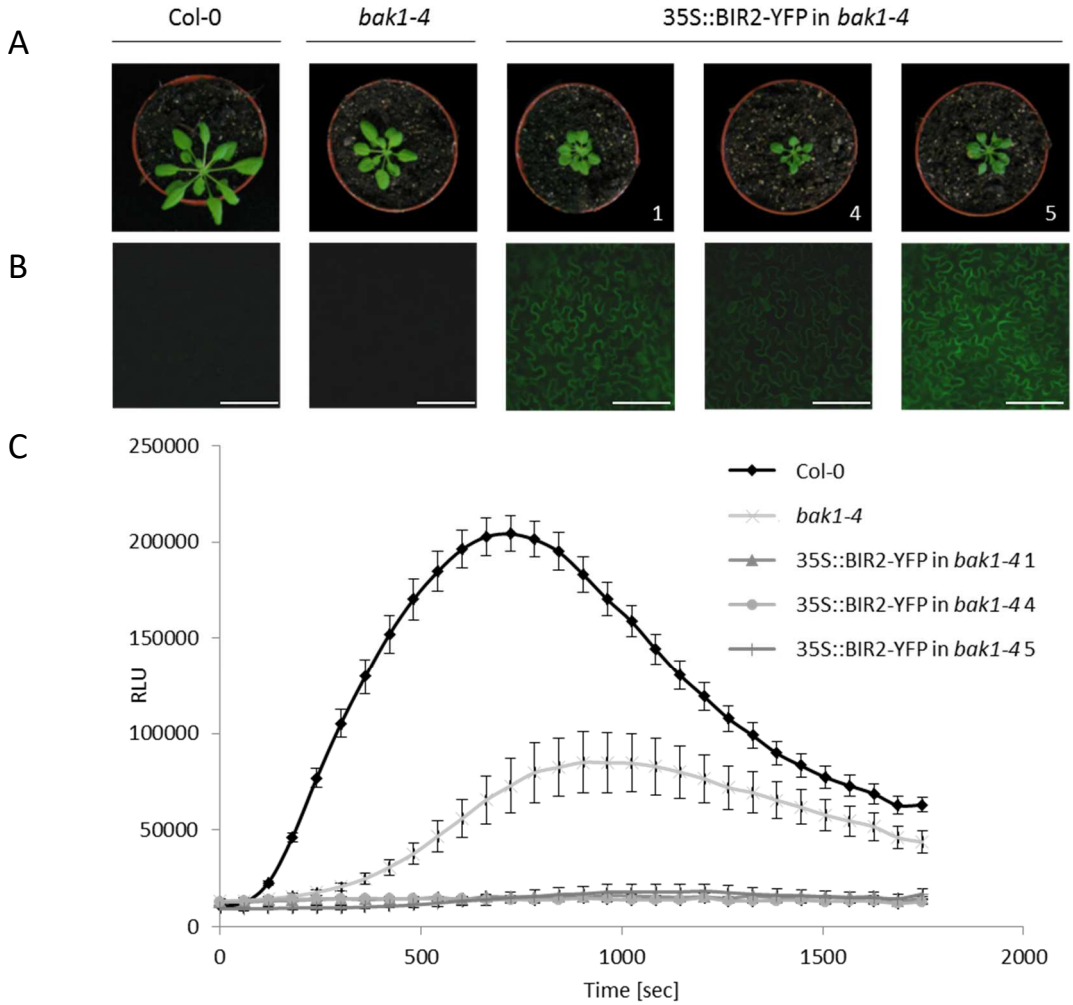



**Figure 3-13: Overexpressed BIR2-YFP in *bak1-4* background leads to flg22 insensitivity.**
**A:** Growth phenotype of 5 week-old plants under short day condition. Col-0 (wt), *bak1-4* (BAK1 null mutant) and three independent 35S::BIR2-YFP in bak1-4 lines (1, 2 and 5) **B:** BIR2-YFP expression detected with fluorescence microscopy. Bar represents 100 µm. **C:** Time course ROS production by leaf pieces treated with 100 nM flg22, n= 5-7, and standard error is given.

### 3.2.6 Identification of *in vivo* P-sites of BIR2 in a BAK1-independent manner

Due to the growth phenotype of overexpressed BIR2 in *bak1-4*, the seedlings of the line are rather difficult to cultivate to obtain sufficient amounts for MS analysis. However, two MS runs are performed and summed in Table 3-3. Surprisingly, only two P-sites are identified, S263 and S279. The observation of S263 in a BAK1-deficient background, points to a multi-targeted site not only for BAK1 but also for other kinases. The S279 is only found *in vivo* so far. The additional information that this site is not detectable *in vitro*, but *in vivo* in *bak1-4* plants, opens the discussion that S279 is BAK1-independent and a target of other kinases. However, the reduced number of identified BIR2 P-sites in plants lacking BAK1 expression, show that BAK1 is the main kinase for BIR2 transphosphorylation.

**Table 3-3: Summary of identified *in vivo* phosphorylation sites of BIR2 in *bak1-4* background.**
2 week-old *Arabidopsis* seedling overexpressing BIR2 in *bak1-4* background are sent for MS analysis, and identification of phosphorylation sites. Numbers in brackets indicating no significant values (related to PEP and localization values, not shown). Juxtamembrane= JM.

| Domain | Amino acid position | Number of identification |
|---|---|---|
| JM | 263 | 1(1) |
| | 279 | 1 |

## 3.3 Functional analysis of P-sites

In the following chapter, the functional importance of certain BIR2 P-sites is tested. For that purpose, single phosphorylated residues are mutated either to alanine (A) or to aspartate (D) via site-directed mutagenesis. This works is partly done by Dr. Sara Mazzotta (Mazzotta, 2012). Alanine has a hydrophobic side chain, and is typically used as a basic amino acid, preventing phosphorylation due to its characteristics. In contrast, substitution of aspartate at specific residues mimics constitutive phosphorylation under certain circumstances, thus the negative charged side chain of aspartate can behave like a phosphate group. Stably transformed *Arabidopsis* plants (*bir2-2* background, T2) with the BIR2 mutant constructs are tested regarding their ability to interact with BAK1, and further their skills to rescue the PAMP and cell death phenotype.

### 3.3.1 Mutations of potential BIR2 P-sites have no effect on subcellular localization of expressed protein

Some mutated P-sites (S263, S286 and T304) are picked for testing the subcellular localization of the expressed protein to exclude a possible negative impact of the mutation. These mutated BIR2 proteins are fused to the yellow fluorescence protein (YFP), and transiently expressed in *Nicotiana benthamiana* leaves. Confocal images are taken 2-3 days after inoculation, and BIR2 expression is confirmed on western blot (Figure 3-13). As controls, unmutated BIR2 and P19 (negative control) are included (Figure

3-14 A). BIR2-YFP is located in the plasma membrane as shown by Halter et al. (2014a)**.** The changed residues S263, S286 and T304 to alanine and aspartate, show analogous YFP signals at the plasma membrane region as published and investigated for BIR2-YFP (Figure 3-14 B-D). This outcome indicates that the site-directed mutagenesis has no effect on the subcellular localization of the protein, allowing further studies of the mutant lines.

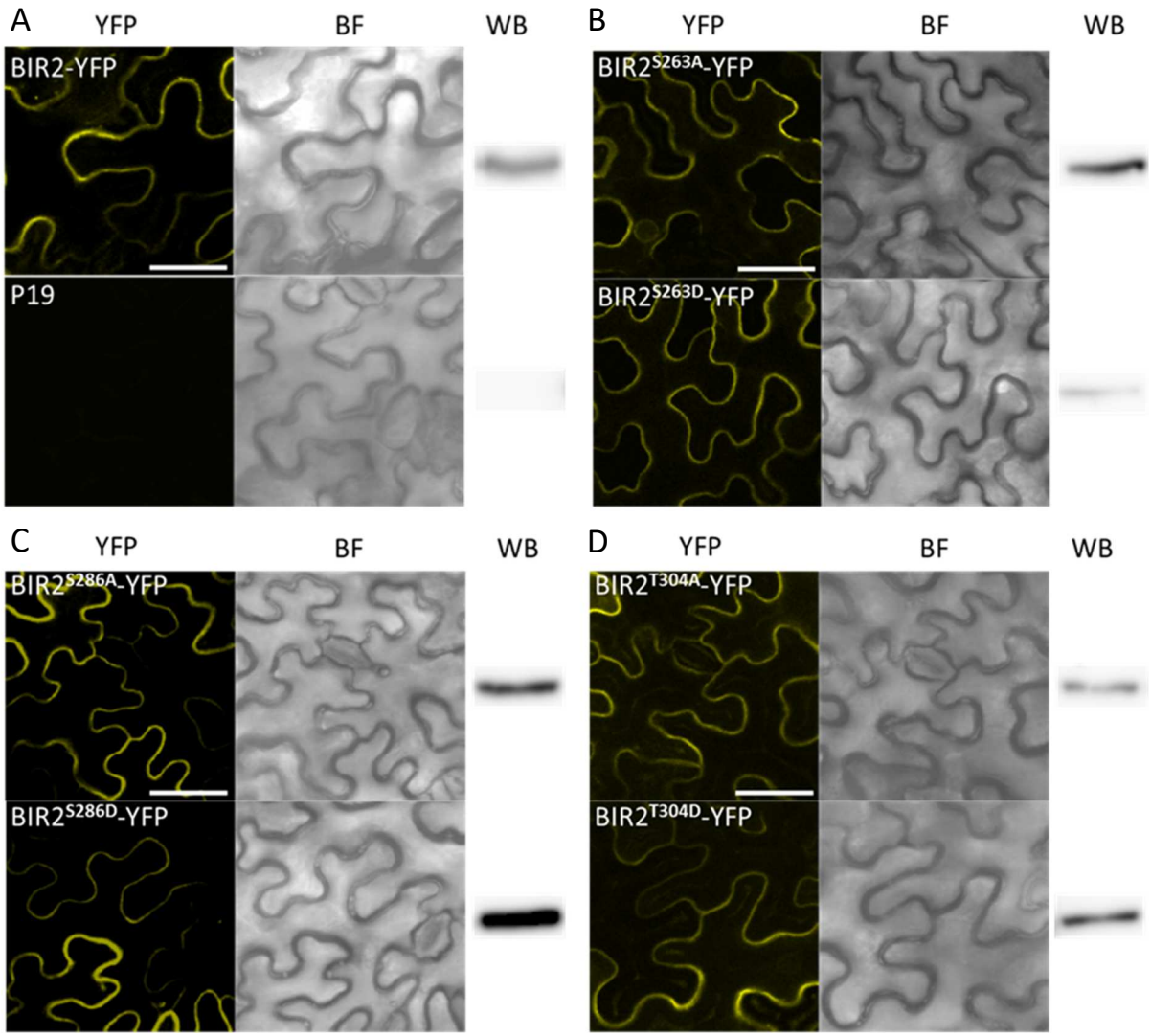



**Figure 3-14: The S263A/D, S286A/D and T304A/D mutations do not effect BIR2-YFP subcellular localization.** Confocal images of BIR2 and BIR2 P-site mutants fused to yellow fluorescence protein (YFP) which are transiently expressed in *N. benthamiana* leaves. White bar represents 45 µm. Western blot (WB) is performed to confirm BIR2 expression. **A**: Controls; BIR2-YFP and P19. **B**: S263A/D-YFP. **C**: S286A/D-YFP. **D**: T304A/D-YFP. BF=Brightfield.

### 3.3.2 Wild type BIR2 constructs complement *bir2* phenotype in stably transformed plants

Investigations of certain BIR2 P-sites are performed by classical complementation assays. Thus, unspecific side effects of myc-tag fused BIR2 proteins should be excluded. Two independent BIR2-myc lines in *bir2-2* are generated, and further explored (Figure 3-15).

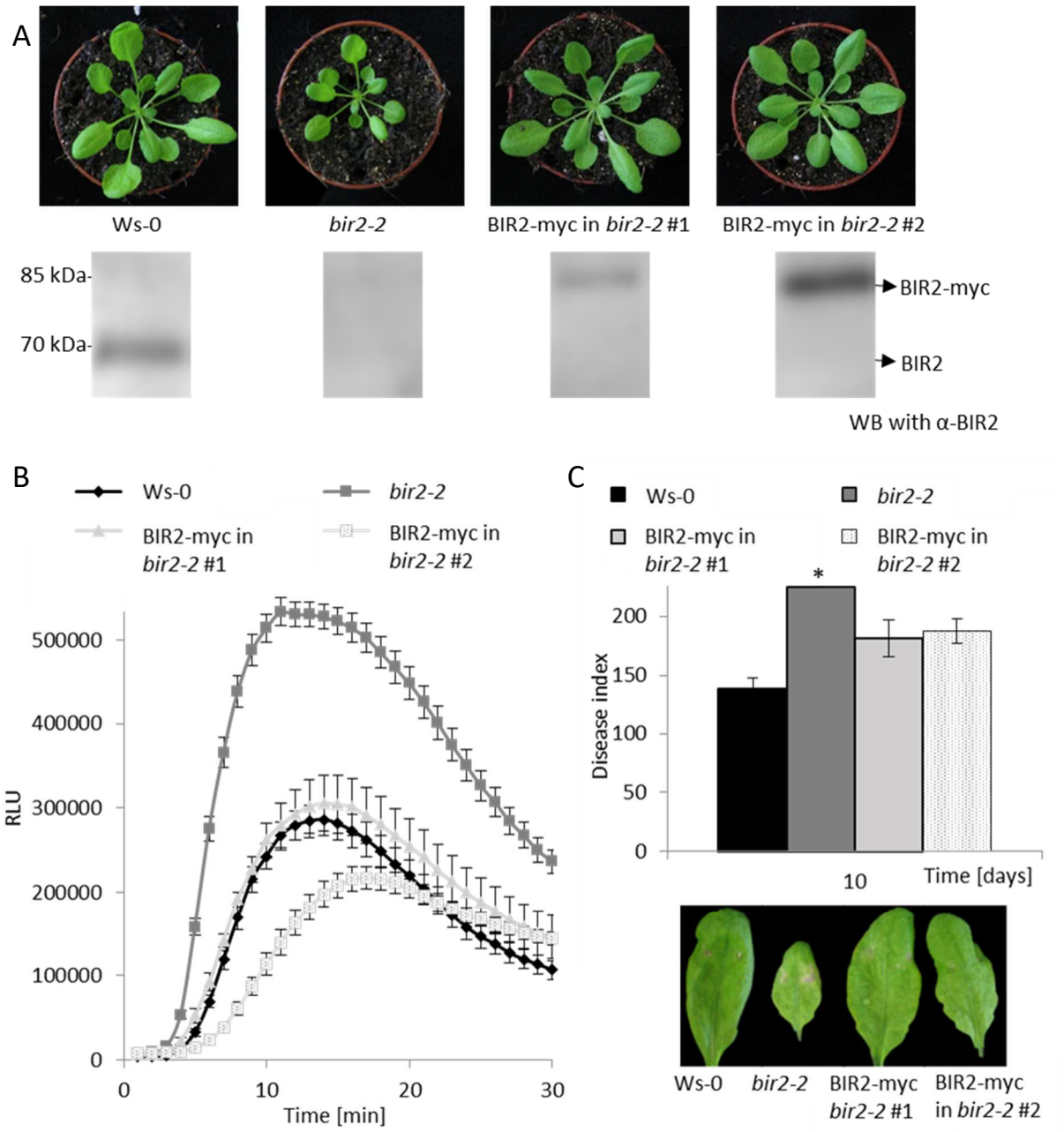



**Figure 3-15: BIR2-myc fusion proteins complement *bir2-2* PAMP-, growth and Alternaria phenotypes.**
**A:** 5-6 week-old *Arabidopsis* plants are grown under short day conditions. Western blot is performed to confirm BIR2-myc expression. Detection with BIR2 specific antibody. **B:** ROS burst assay. Overnight in water adjusted leaf pieces are treated with 100 nM elf18. The oxidative burst within the first 30 min are measured. n= 9, 3 biological replicates. Standard error is given. **C:** 2 leaves per plant are inoculated with 2 5 µl droplets of *Alternaria* spores (1:20 diluted from stock solution $2*10^7$ spores/ml). Plants are kept under 100 % humidity in a short day chamber, and bonitated after 10 days. Pictures of disease symptoms are taken. n= 8-16, 4-8 biological replicates. Standard error is given. Student's t test is related to Ws-0 *p<0.05, **p<0.01, ***p<0.001.

The growth phenotype of 5-6 week-old plants indicate a rescue of the *bir2-2* phenotype, and expression is confirmed on western blot (Figure 3-15 A). Complementation is also observed in functional assays, such as ROS burst after eliciting with elf18 (Figure 3-15 B), and after inoculation with the necrotrophic fungus *Alternaria brassicicola*. Bonitation of disease symptoms and the corresponding pictures of infected leaves confirm complementation of the *bir2* mutant phenotypes in lines expressing BIR2 under its endogenous promotor (Figure 3-15 C).

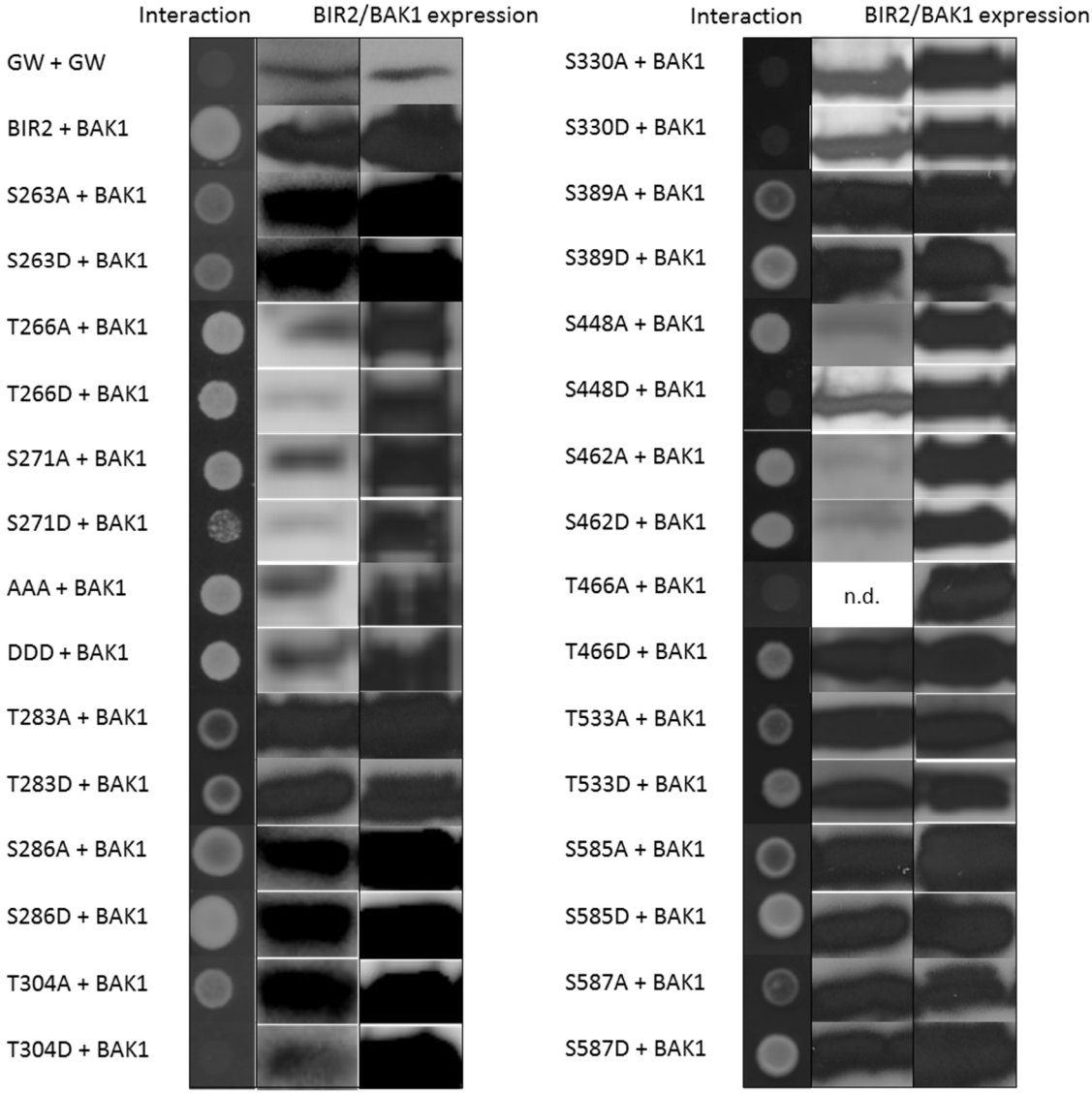
In all cases, BIR2-myc insertion rescues the BIR2 knock out phenotypes, whereas an expression-dependent complementation is visible. Line #2 shows stronger BIR2-myc levels, and thus the phenotypes of growth and ROS burst partially behave in a similar way like BIR2 overexpression lines (Figure 3-6). In conclusion, BIR2-myc shows complementation in a dose-dependent manner, as expected for a regulatory protein, where certain amounts of protein are relevant for precisely monitored processes in plant cells.

### 3.3.3 Y2H screen of BIR2 P-site mutants

Most of the BIR2 P-sites are mutated to alanine and aspartate, and firstly checked in a Y2H assay to get a rough overview of their interaction behavior with its interaction partner BAK1 (Table 3-4), and to confirm Y2H data accumulated by Dr. Sara Mazzotta. Autoactivation probabilities of BIR2 P-site mutants with empty corresponding Y2H vector (GW) are checked and excluded (data not shown). A screen of 14 P-sites plus the triple P-sites (S263, T266 and S271) with the alanine triple mutants labeled as AAA and the aspartate variants with DDD, respectively, are performed and revealed that most constructs are able to interact with BAK1 as known for unmutated BIR2 and BAK1. There are a few exceptions; T304D, S330A, S330D, S448D and possibly T466A, where no interaction is detectable. T304 is the only P-site in the JM, whereas the other sites are located in the KD. S330 is near the catalytically important K335, which is not relevant for BIR2 function (Table 3-4). The other two P-sites S448 and T466 are located in the activation segment of the KD. S448 is part of the magnesium binding motif (DSG), while T466 is further downstream in the activation loop. Alterations of these mutants in interaction with BAK1 could hint to a functional importance of these P-sites. T466 is the only sites, which is *in vivo* found, too, but protein expression data in the Y2H assay are not clarified yet. However, a loss of interaction due to a substitution to alanine could point to an involvement of this phosphorylaation site to support interaction to BAK1. Both mutated versions of S330 have lost their abilities to interfere with BAK1. Under these circumstances, aspartate might not act as a phospho-mimic residue, and phosphorylation of this site support the BIR2-BAK1 complex formation, too, or there is a structural clash. Residue switches for T304 and S448 to aspartate have the effect of loss of interaction, and could explained a function of this P-sites to inhibit BIR2-BAK1 interaction. However,

Y2H is an artificial system, using only the intracellular parts of the protein of interest, and structural problems cannot be excluded. Consequently, some interesting P-sites are further explored in planta.

**Table 3-4: Yeast-two-hybrid screen to investigate interaction ability of BIR2-BAK1.**
The intracellular domains of BIR2 (JM, KD, CT) in pGBKT7 and the intracellular domains of BAK1 (JM, KD, CT) in pGADT7 are co-expressed in yeast strain PJ69-4a. Material is dropped on SD-LT medium as loading control (data not shown) and on SD-HALT plates to investigate interaction behavior. Pictures are taken after 2-6 days. Expression of proteins are confirmed via WB by using antibodies against BIR2/myc or HA (detecting BAK1). n.d.= not determinated.



### 3.3.4 Investigations of BIR2 P-site S263

One of the most abundant P-site, which is discovered in MS studies, is S263 within the JM. Several independent lines of alanine- or aspartate-mutated S263 are investigated. The growth phenotype of S263A is smaller than observed for S263D, but still not *bir2-2*-like (Figure 3-16 A). The aspartate mutation seems even to increase slightly the growth performance compared to wild type. However, functional assays such as ROS burst and treatment with *A. brassicicola* show a complementation of the *bir2* phenotype for both mutations (Figure 3-16 B, C).

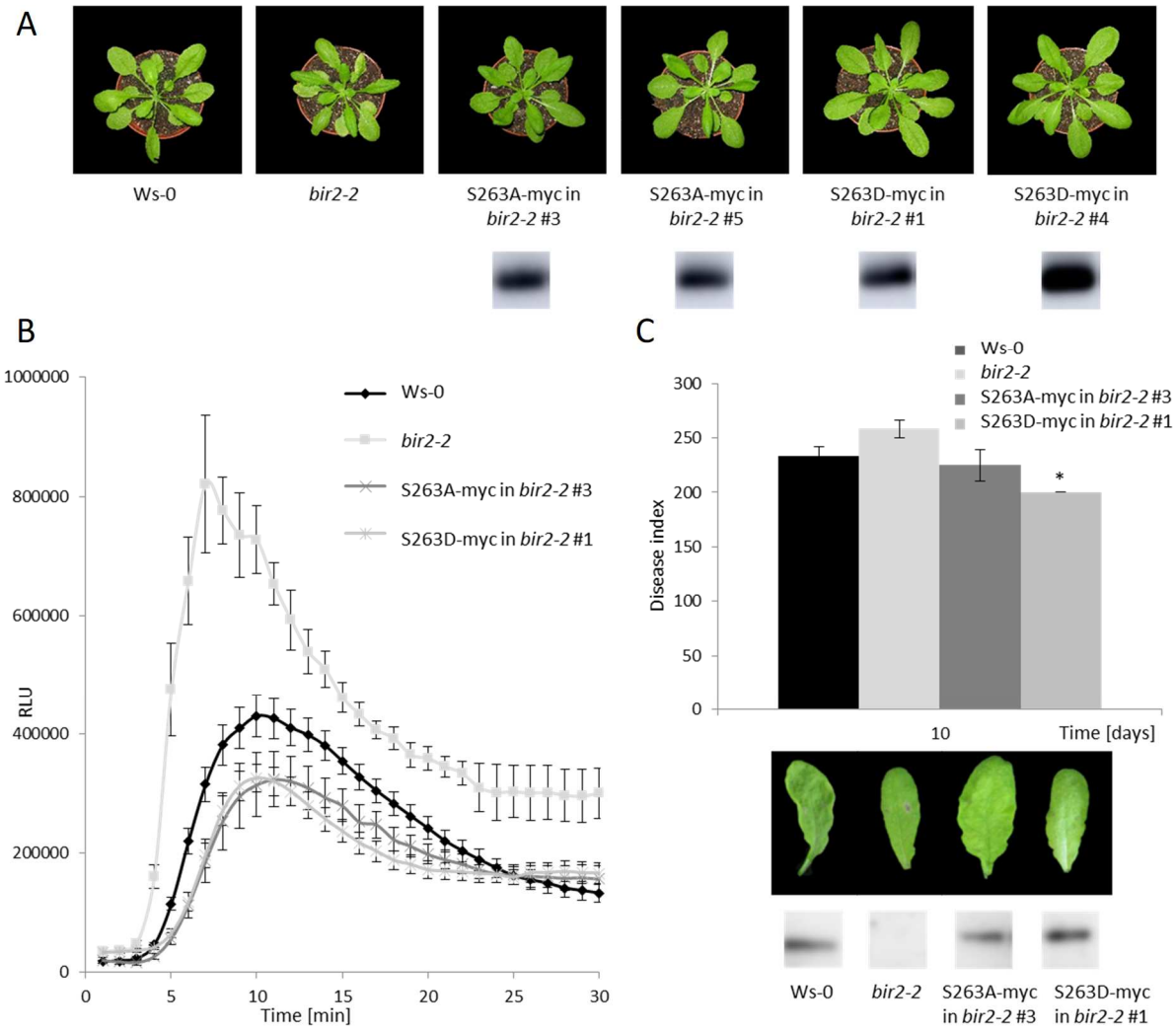



**Figure 3-16: S263A/D-myc fusion lines show complementation in *bir2-2* defected growth and signaling pathways.**
**A:** 5-6 week-old *Arabidopsis* plants are grown under short day conditions. Western blot is performed to confirm BIR2-myc expression. Detection with myc antibodies. **B:** ROS burst assay. Overnight in water adjusted leaf pieces are treated with 100 nM elf18. The oxidative burst within the first 30 min are measured. n= 3-6, 1-2 biological replicates. Standard error is given. **C:** 2 leaves per plant are inoculated with 2 5 µl droplets of *Alternaria* spores (1:20 diluted from stock solution $2*10^7$ spores/ml). Plants are kept under 100 % humidity in a short day chamber, and bonitated after 10 days. Pictures of disease symptoms are taken. n= 12, 6 biological replicates. Standard error is given. Western blot is performed to confirm BIR2-myc expression. Detection with BIR2 specific antibodies. Student's t test is related to Ws-0 *p<0.05, **p<0.01, ***p<0.001.

The previous Y2H assay already indicates that both mutations do not influence the interaction skills to BAK1. To investigate this observation on a quantitative level, a serial dilution of transformed yeast cells is dropped on selection plates (Figure 3-17). Both lines grow a bit better than the positive control (unmutated BIR2 and BAK1), but in a similar range to each other.

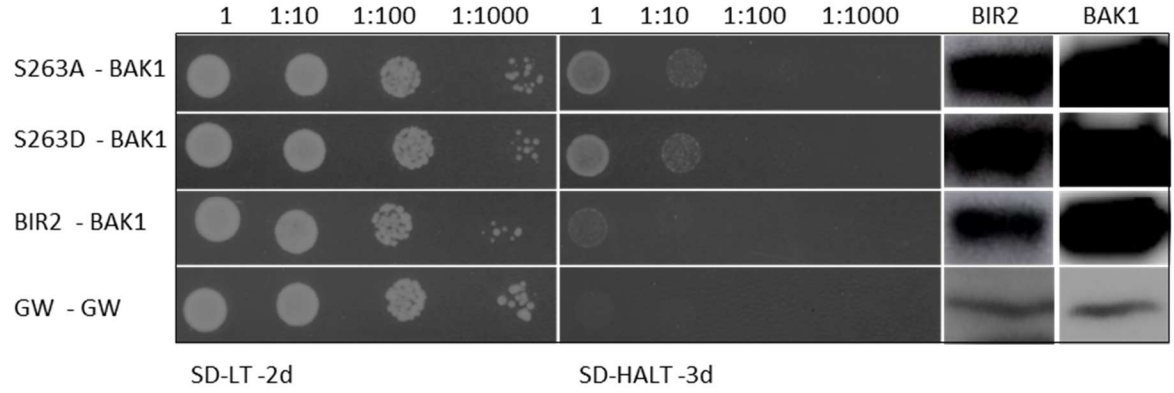



**Figure 3-17: Yeast-two-hybrid assay confirms ability of S263A/D-myc fusion line to interact with BAK1.** The intracellular domains of BIR2 (JM, KD, CT) in pGBKT7 and the intracellular domains of BAK1 (JM, KD, CT) in pGADT7 are co-expressed in yeast strain PJ69-4a. Material is diluted in a 1:10 ratio and dropped on SD-LT medium as loading control, and on SD-HALT plates to investigate interaction ability. Pictures are taken after 2-3 days. Expression of proteins are confirmed via WB. Empty Gateway vector=GW.

Interaction behavior of BIR2-BAK1 is tested in stable *Arabidopsis* lines by Co-IP. Surprisingly both mutant lines do not show a reduced interaction of BIR2 with BAK1 after ligand perception, as known for wild type (Figure 3-18). This preliminary data could indicate that the mutations lead to ligand insensitivity. In conclusion, the mutation of the single P-site S263 has no direct influence on the functionality of the complemented lines, but show a possible defect in the ligand perception system.

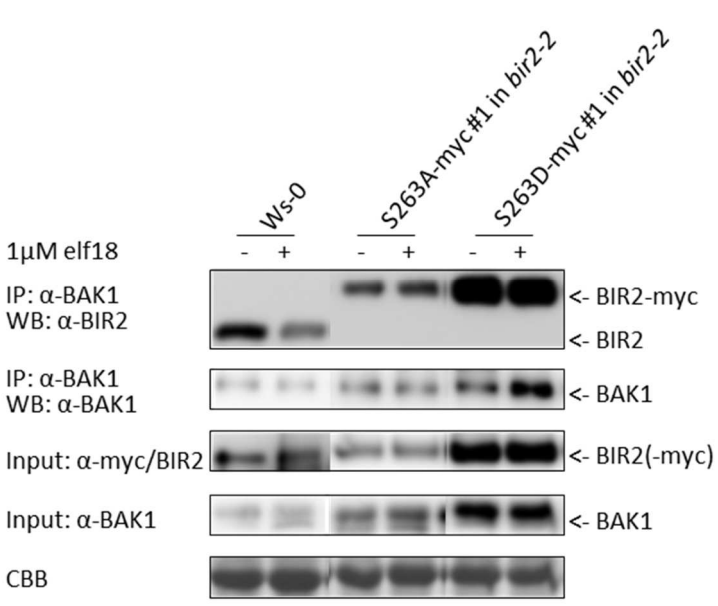



**Figure 3-18**: **Co-IP confirms ability of S263A/D-myc fusion lines to interact with BAK1, whereas elicitor treatment shows no strong effect on interaction stability.**
2 week-old seedlings are treated with 1 µM elf18 for 5 min. Untreated seedlings are used as a control. In equal amounts of protein extract, BAK1 protein is immunoprecipitated with an anti-BAK1 antibody and co-iped BIR2 is detected with an anti-BIR2 antibodies. A western blot is performed, and proteins of interest are detected by using specific or anti-myc antibodies. Expression of the proteins are detetcted with the respective protein specific antibodies (input). CBB shows equal loading of the input protein extracts.

### 3.3.5 Investigations of triple BIR2 P-sites S263/T266 and S271

S263 is closely located to two other P-sites, T266 and S271, all found *in vivo* as significant BIR2 P-sites. Thus, in a previous work (Mazzotta, 2012) all three sites are mutated to alanine and aspartate (AAA/DDD), and stably transformed into BIR2-deficient plants. Complementation assays show a clear rescue of the *bir2* phenotype in all tested assays (Figure 3-19).

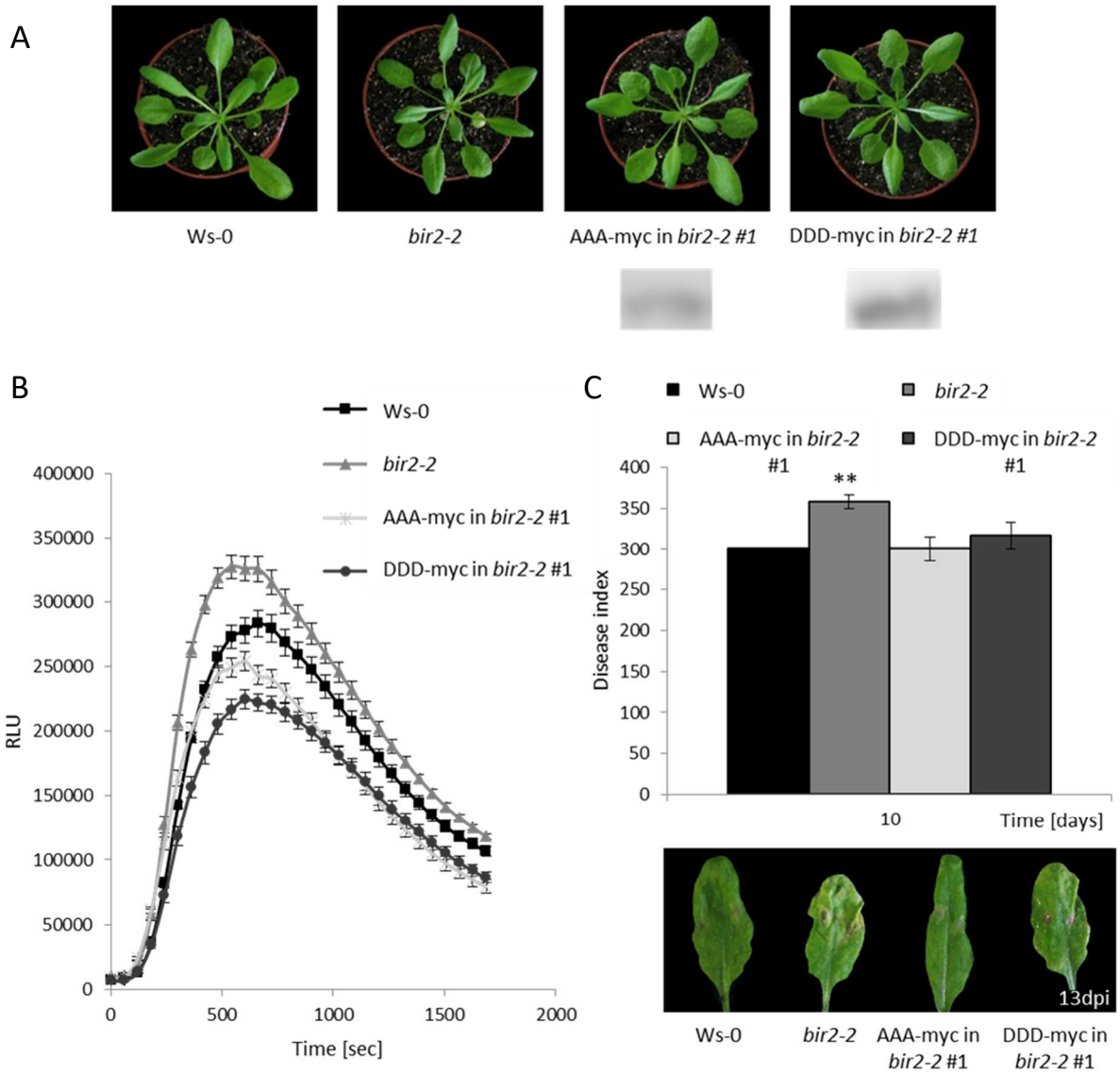



**Figure 3-19: AAA/DDD-myc fusion lines show complementation in *bir2-2* defected growth and signaling pathways.**
**A:** 5-6 week-old *Arabidopsis* plants are grown under short day conditions. Western blot is performed to confirm BIR2-myc expression. Detection with myc antibodies. **B:** ROS burst assay. Overnight in water adjusted leaf pieces are treated with 100 nM elf18. The oxidative burst within the first 30 min are measured. n= 6-7, 3 biological replicates. Standard error is given. **C:** 2 leaves per plant are inoculated with 2 5 µl droplets of *Alternaria* spores (1:20 diluted from stock solution $2*10^7$ spores/ml). Plants are kept under 100 % humidity in a short day chamber, and bonitated after 10 days. Pictures of disease symptoms are taken after 13 days. n= 12, 6 biological replicates. Standard error given. Student's t test is related to Ws-0 *p<0.05, **p<0.01, ***p<0.001.

Serial dilutions of a Y2H assay is performed for the single as well as for the triple mutants (Figure 3-20). All BIR2 constructs can still interact with BAK1, whereby alanine mutations lead to an increased growth behavior compared to aspartate mutants. The phospho-preventing residue seems to strengthen the BIR2-BAK1 complex. S271 substitution to aspartate has even a weaker interaction performance than the positive control. Summing up, mutated T266 and S271 show a changed growth behaviors to BAK1. However, the triple mutations confirm the tendency of alanine-mutated T266 and S271. In planta, preliminary Co-IP data reveal a proper interaction ability of BIR2-BAK1 in a ligand-dependent manner (Figure 3-21).

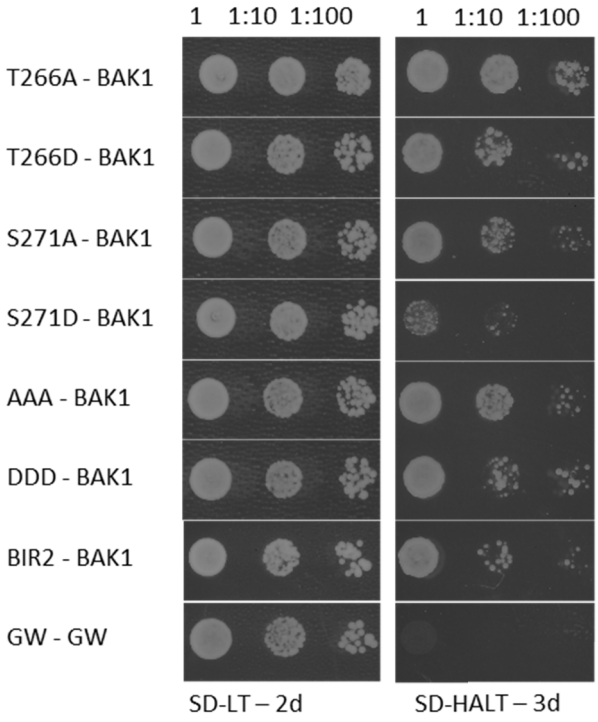



**Figure 3-20: Yeast-two-hybrid assay confirms ability of AAA/DDD-myc fusion lines and single P-site lines to interact with BAK1.**
The intracellular domains of BIR2 (JM, KD, CT) in pGBKT7 and the intracellular domains of BAK1 (JM, KD, CT) in pGADT7 are co-expressed in yeast strain PJ69-4a. Material is diluted in a 1:10 ratio and dropped on SD-LT medium as loading control and on SD-HALT plates to investigate interaction ability. Pictures are taken after 2-3 days. Expression of proteins are confirmed via WB (data not shown).

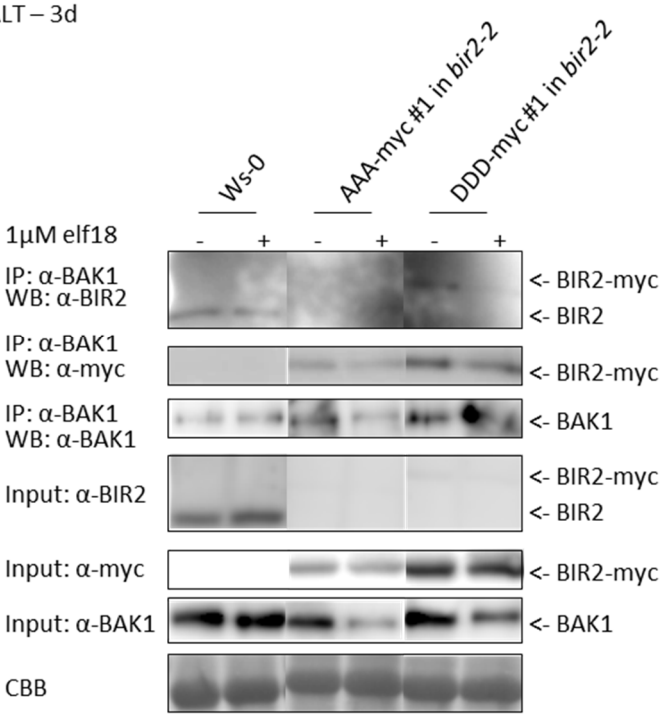



**Figure 3-21: Co-IP confirms ability of AAA/DDD-myc fusion lines to interact with BAK1, whereas elicitor treatment leads to a weak destabilization effect of the BIR2-BAK1 complex.**
2 week-old seedlings are treated with 1 µM elf18 for 5 min. Untreated seedlings are used as a control. In equal amounts of protein extract, BAK1 protein is immunoprecipitated. A western blot is performed and proteins of interest are detected by using specific protein antibodies or myc antibodies.

### 3.3.6 Investigations of BIR2 P-site S286

Another very abundant *in vivo* P-site of the MS results is S286, also allocated with the JM. Stable mutation lines show complementation of *bir2-2* growth defects and signaling phenotypes (Figure 3-22). Alanine and aspartate-mutated lines have a slightly enhanced growth phenotype than wild type (Figure 3-22 A). Due to the rather strong BIR2 expression both mutation lines partially over-complement the wild type phenotype in ROS burst and *A. brassicicola* (Figure 3-22 B, C). Surprisingly, the aspartate-mutated line shows a stronger over-complementation than the alanine-mutated line, although the corresponding expression pattern do not show such a different (Figure 3-22 A).

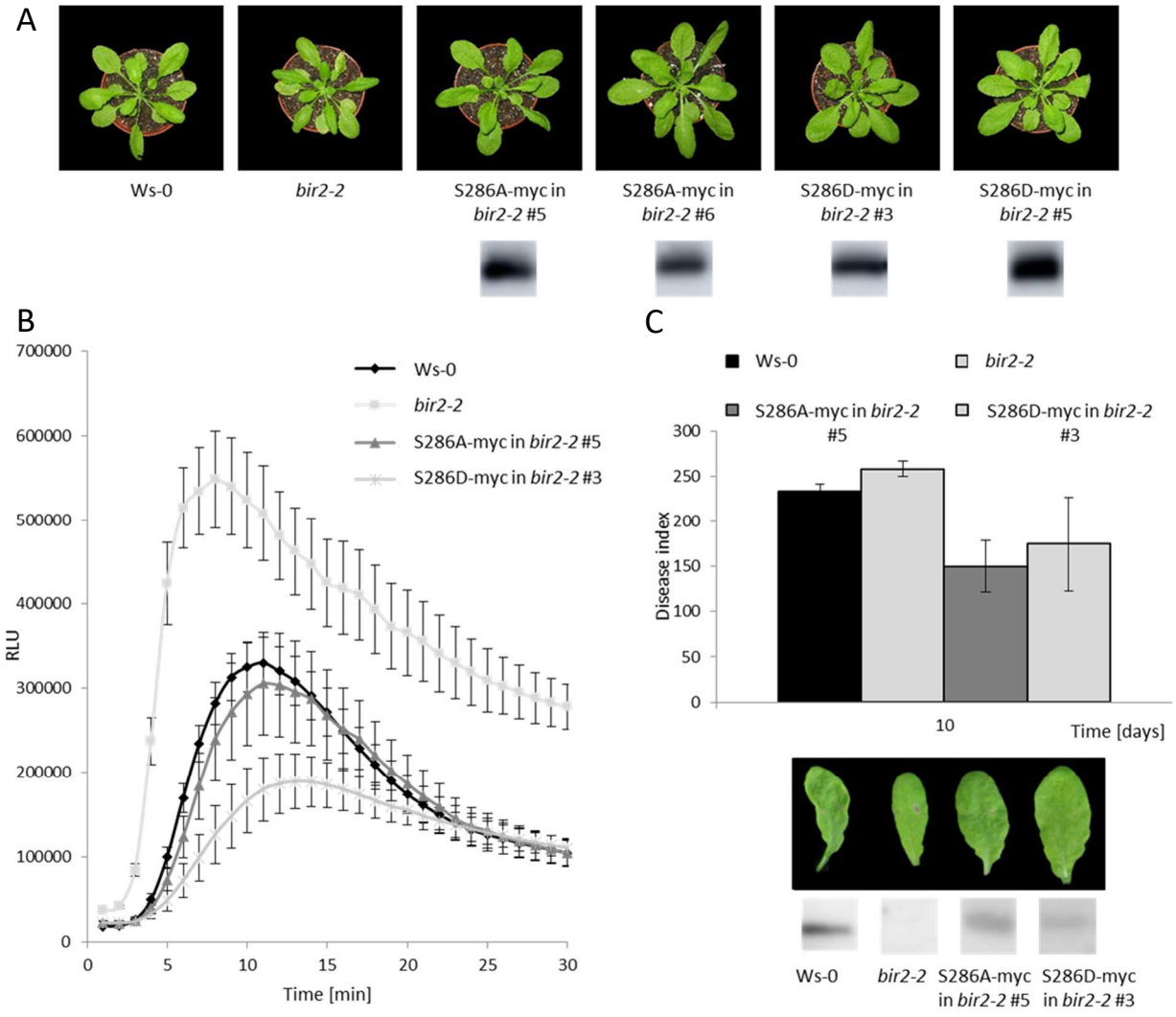



**Figure 3-22: S286A/D-myc fusion lines show complementation in *bir2-2* defected growth and signaling pathways.**
**A:** 5-6 week old *Arabidopsis* plants are grown under short day conditions. Western blot is performed to confirm BIR2-myc expression. Detection with myc antibodies. **B:** ROS burst assay. Overnight in water adjusted leaf pieces are treated with 100 nM elf18. The oxidative burst within the first 30 min are measured. n= 3-6, 1-2 biological replicates. Standard error is given. **C:** 2 leaves per plant were inoculated with 2 5 µl droplets of *Alternaria* spores (1:20 diluted from stock solution $2*10^7$ spores/ml). Plants are kept under 100 % humidity in a short day chamber, and bonitated after 10 days. Pictures of disease symptoms are taken. n= 12, 6 biological replicates. Standard error is given. Western blot is performed to confirm BIR2-myc expression. Detection with BIR2 specific antibodies. Standard error is given.

Interaction assays of S286A/D and BAK1 in yeast show differences in the growth skills due to the certain mutations (Figure 3-23). Yeast expressing alanine-mutated S286 has a weaker growth ability than the positive control, and S286D shows an enhanced phenotype. The inhibition of a phosphorylated S286 might trigger a release of BIR2-BAK1.

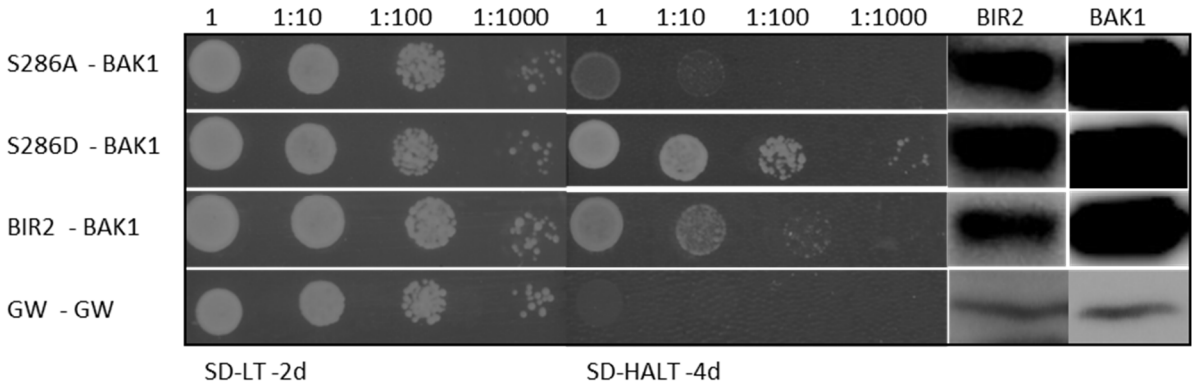
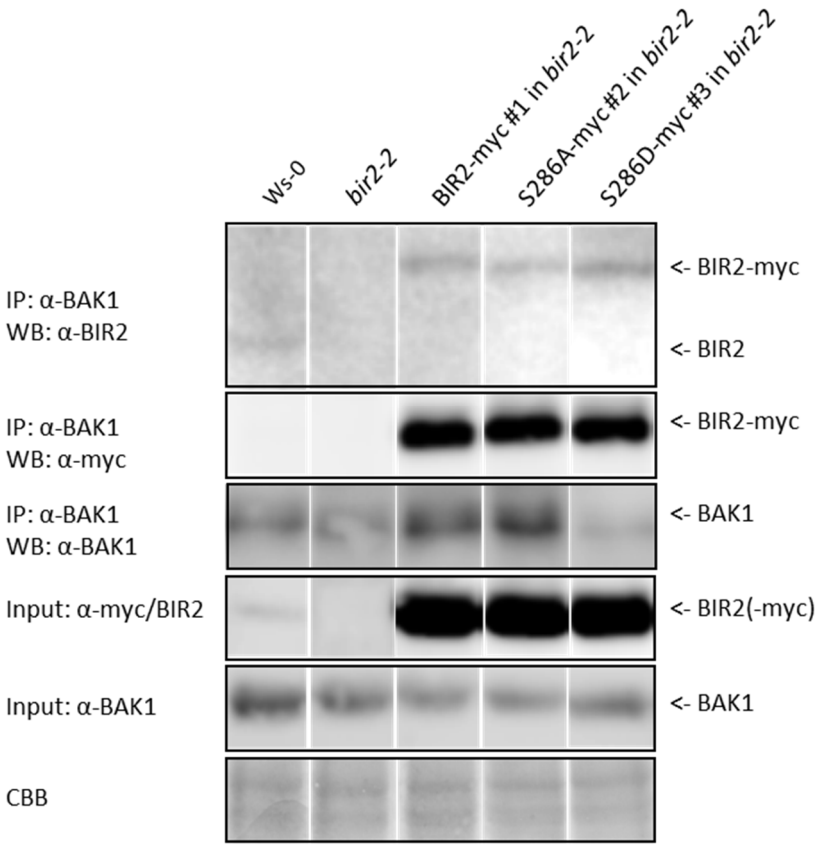



**Figure 3-23: Yeast-two-hybrid assay confirms ability of S286A/D-myc fusion line to interact with BAK1.** The intracellular domain of BIR2 (JM, KD, CT) in pGBKT7 and the intracellular domain of BAK1 (JM, KD, CT) in pGADT7 are co-expressed in yeast strain PJ69-4a. Cell suspension is serially diluted in a 1:10 ratio and dropped on SD-LT medium as loading control and on SD-HALT plates to investigate interaction ability. Pictures are taken after 2-4 days. Expression of proteins are confirmed via WB.

Interaction assays in *Arabidopsis* do not indicate a quantitative difference of interaction strength caused by the different mutations (Figure 3-24 A). However, ligand-triggered Co-IPs of BIR2-BAK1 interaction reveal that alanine mutants are still sensitive to ligand-dependent release of BAK1 from BIR2. In contrast, S286D is unaffected by this treatment (Figure 3-24 B). These preliminary results of ROS burst assays and interaction studies point to an important function of phosphorylated S286 for the stabilization of the BIR2-BAK1 complex.

A




B

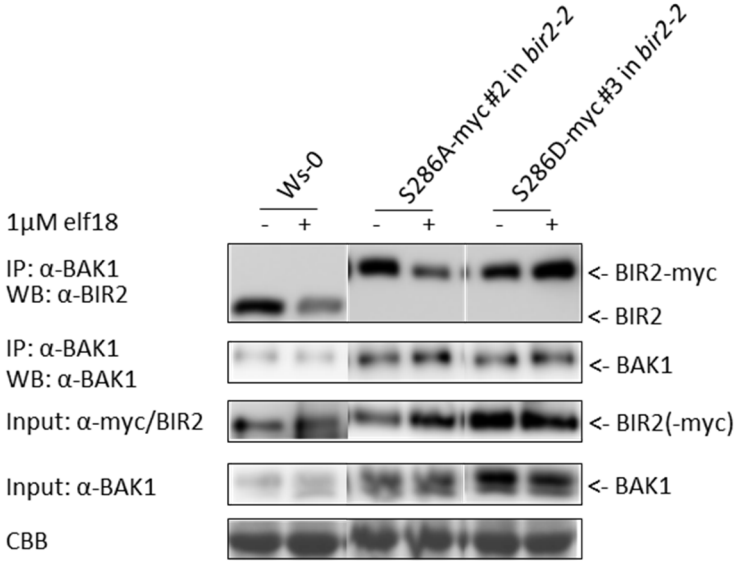



**Figure 3-24: Co-IP confirms ability of S286A/D-myc fusion lines to interact with BAK1, whereas elicitor treatment shows release of S286A-myc fusion line from BAK1 which is not visible in S286D-myc fusion line.** 2 week old seedlings are untreated (**A,B**) or treated with 1 µM elf18 for 5 min (**B**). In equal amounts of protein extract, BAK1 protein is immunoprecipitated. A western blot is performed, and proteins of interest are detected by using specific antibodies or myc antibodies.

### 3.3.7 Investigations of BIR2 P-site T304

In the Y2H screen T304 is detected as an interesting *in vitro* P-site due to the inability of the aspartate-mutated construct to interact with BAK1 (Table 3-4, Mazzotta, 2012). Moreover, T304A/D are not affected by their subcellular localization as previously shown by confocal images. Growth phenotypes of substitution to alanine show BIR2 overexpression phenotypes (Figure 3-25 A), whereas this dose-depending observation is not generally made (Table 8-2). However, T304D lines rescue the *bir2* dwarfism and cell death phenotype (Figure 3-25 A). ROS burst assays of mutated T304 hint to an antagonistic behavior of alanine and aspartate switches (Figure 3-25 B).

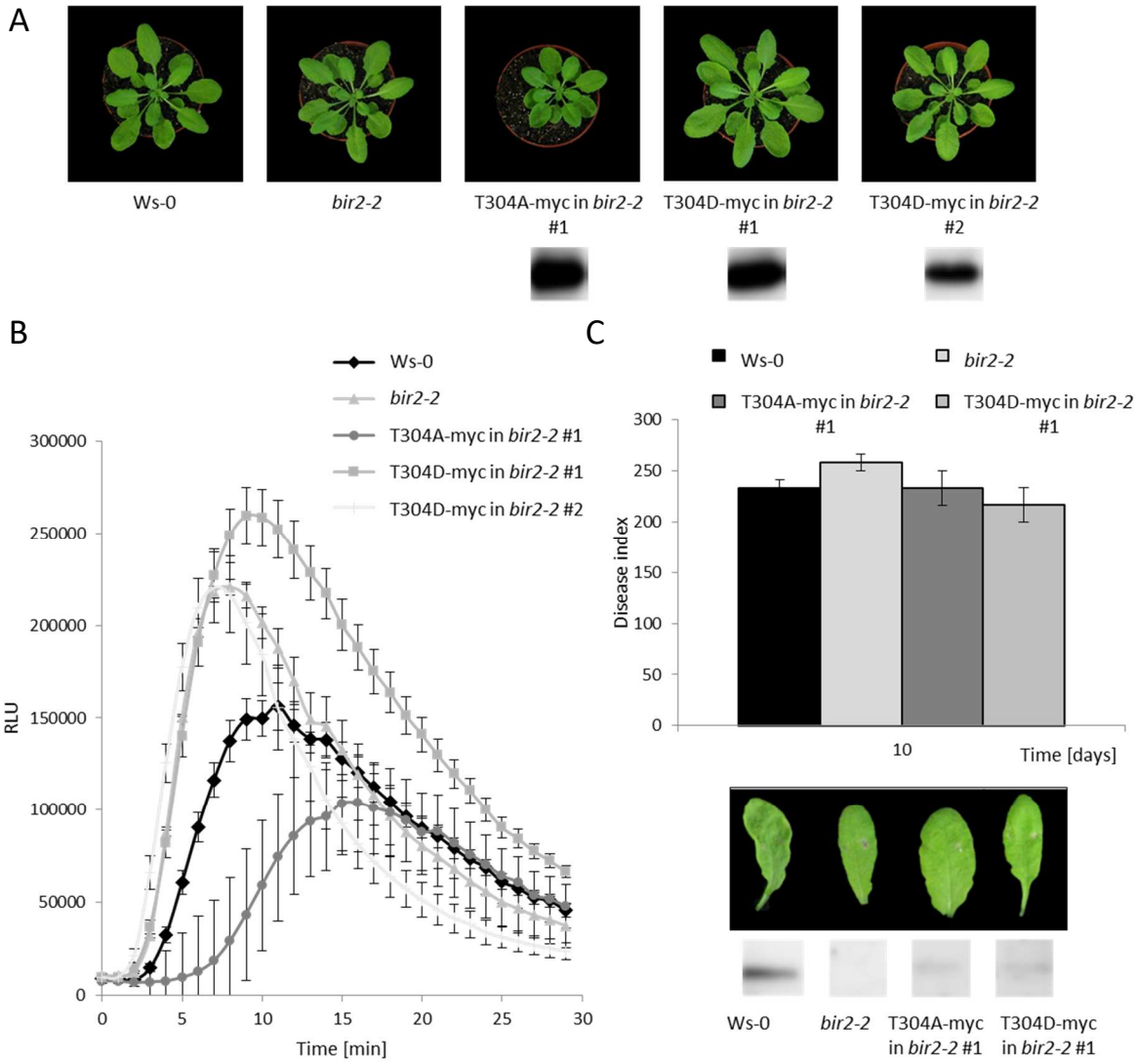



**Figure 3-25: T304D-myc fusion line shows no complementation in *bir2-2* defected PAMP signaling pathways.**
**A:** 5-6 week-old *Arabidopsis* plants are grown under short day conditions: Western blot is performed to confirm BIR2-myc expression. Detection with myc antibodies. **B:** ROS burst assay. Overnight in water adjusted leaf pieces are treated with 100 nM elf18. The oxidative burst within the first 30 min are measured. n= 6, 3 biological replicates. Standard error is given. **C:** 2 leaves per plant are inoculated with 2 5 µl droplets of *Alternaria* spores (1:20 diluted from stock solution $2*10^7$ spores/ml). Plants are kept under 100 % humidity in a short day chamber, and bonitated after 10 days. Pictures of disease symptoms are taken. n= 12, 6 biological replicates. Standard error given. Western blot is performed to confirm BIR2-myc expression. Detection with BIR2 specific antibodies.

The alanine-mutated line over-complement the wild type phenotype, because of the strong protein levels. In contrast, two independent T304D lines are tested, and elf18-triggered ROS burst is not rescued. On the other hand, both mutant lines have equal fungal symptom development (Figure 3-25 C), hinting to a possible specificity of P-sites for certain signaling pathways.

Quantification of the Y2H is made by serial dilution, and confirms a loss of interaction due to the aspartate mutation, as well as a stronger growth performance of alanine-mutated T304 containing yeast compared to the positive control (Figure 3-26). In planta, this finding is reproduced. Co-IP assays confirm a weaker interaction of T304D to BAK1 compared to T304A and wild type complementation line (#1) (Figure 3-27 A). Preliminary data of ligand-induced release of BAK1 from BIR2 are not observed for both mutant lines (Figure 3-27 B), indicating a potential elf18 insensitivity of complex release of these lines. However, interaction is detectable for the full length T304D mutated BIR2 protein, and hints to an additional action of the transmembrane and/or ectodomain for proper binding of BIR2 to BAK1. In conclusion, phosphorylated T304 seems to induce the release of BAK1 from BIR2.

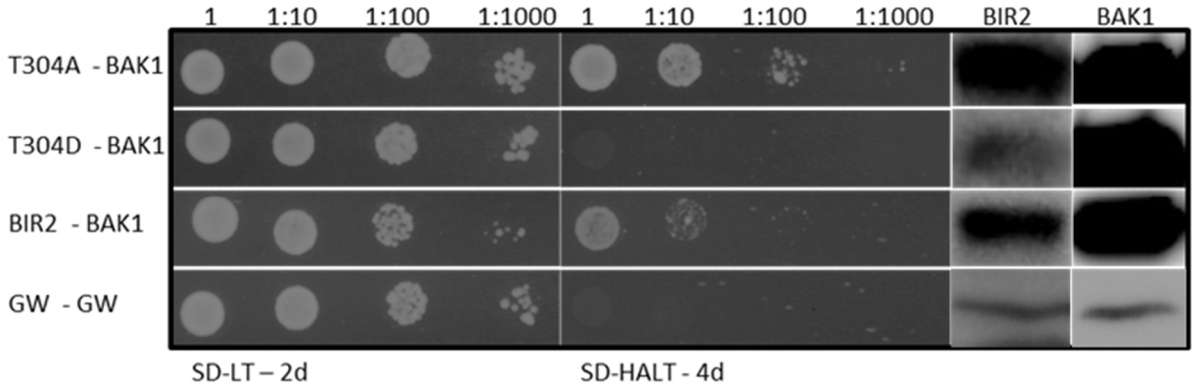
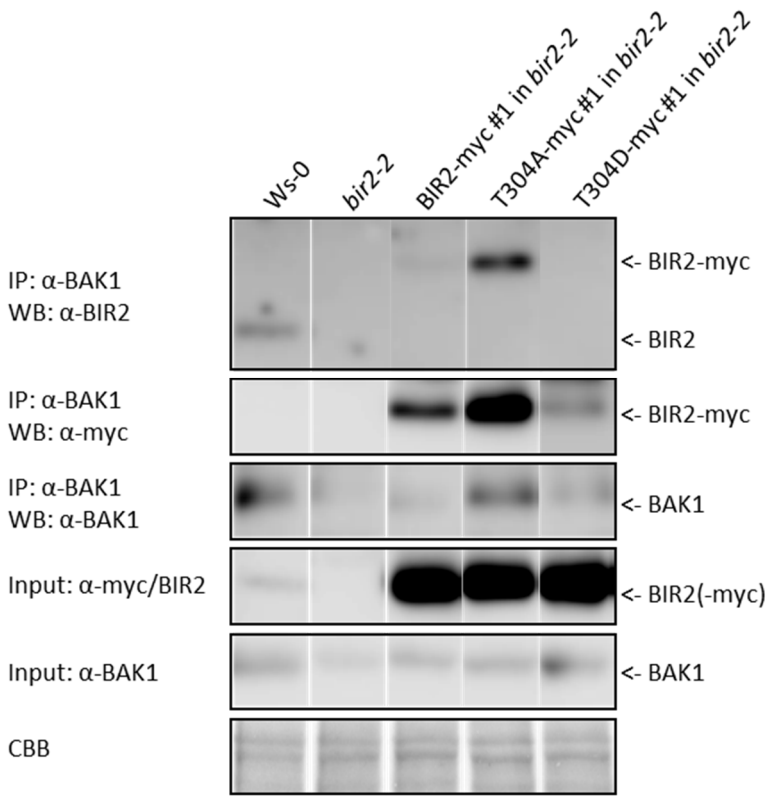



**Figure 3-26: Yeast-two-hybrid assay confirms ability of T304A-myc fusion line to interact with BAK1 whereas T304D -BAK1 interaction is blocked.**
The intracellular domains of BIR2 (JM, KD, CT) in pGBKT7 and the intracellular domains of BAK1 (JM, KD, CT) in pGADT7 are co-expressed in yeast strain PJ69-4a. Material is diluted in a 1:10 ratio and dropped on SD-LT medium as loading control and on SD-HALT plates to investigate interaction ability. Pictures are taken after 2-4 days. Expression of proteins are confirmed via WB.

A




B

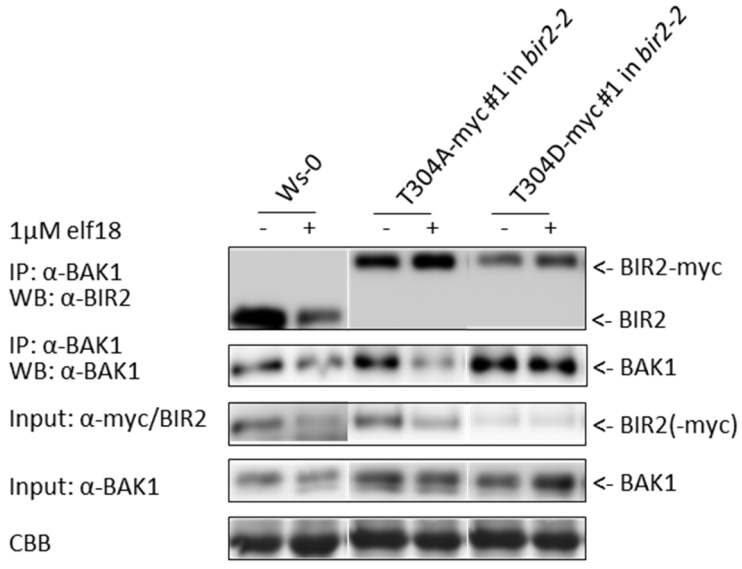



**Figure 3-27: Co-IP confirms ability of T304A/D-myc fusion lines to interact with BAK1. Interaction of T304D -BAK1 is weaker compared to T304A. Elicitor treatment has no visible release effect on BIR2-BAK1 complex.** 2 week-old seedlings are untreated (**A, B**) or treated with 1 µM elf18 for 5 min (**B**). In equal amounts of protein extract, BAK1 protein is immunoprecipitated. A western blot is performed, and proteins of interest are detected by using specific/myc antibodies.

### 3.3.8 Investigations of BIR2 P-site S448

Similar Y2H pattern as shown for T304A/D are found for S448A/D, an *in vitro* P-site in the magnesium binding motif within the KD. Stable lines of mutated S448 are generated by Dr. Sara Mazzotta, and are available in an untagged version (pGWB1). Thus, confirmation of complementation of unmutated BIR2 in this vector system is necessary. The tested *Arabidopsis* line rescues *bir2* phenotypes in the functionally relevant assays (Figure 3-28) (Halter, 2014). The alanine- and aspartate-mutated lines of S448 are able to complement the *bir2* phenotypes, too (Figure 3-29). The growth phenotype shows a slight BIR2 overexpression phenotype of the S448A line (Figure 3-29 A). However, in functional assays both lines perform like wild type (Figure 3-29 B, C), even so that S448D is unable to interact with BAK1 in yeast (Table 3-4).

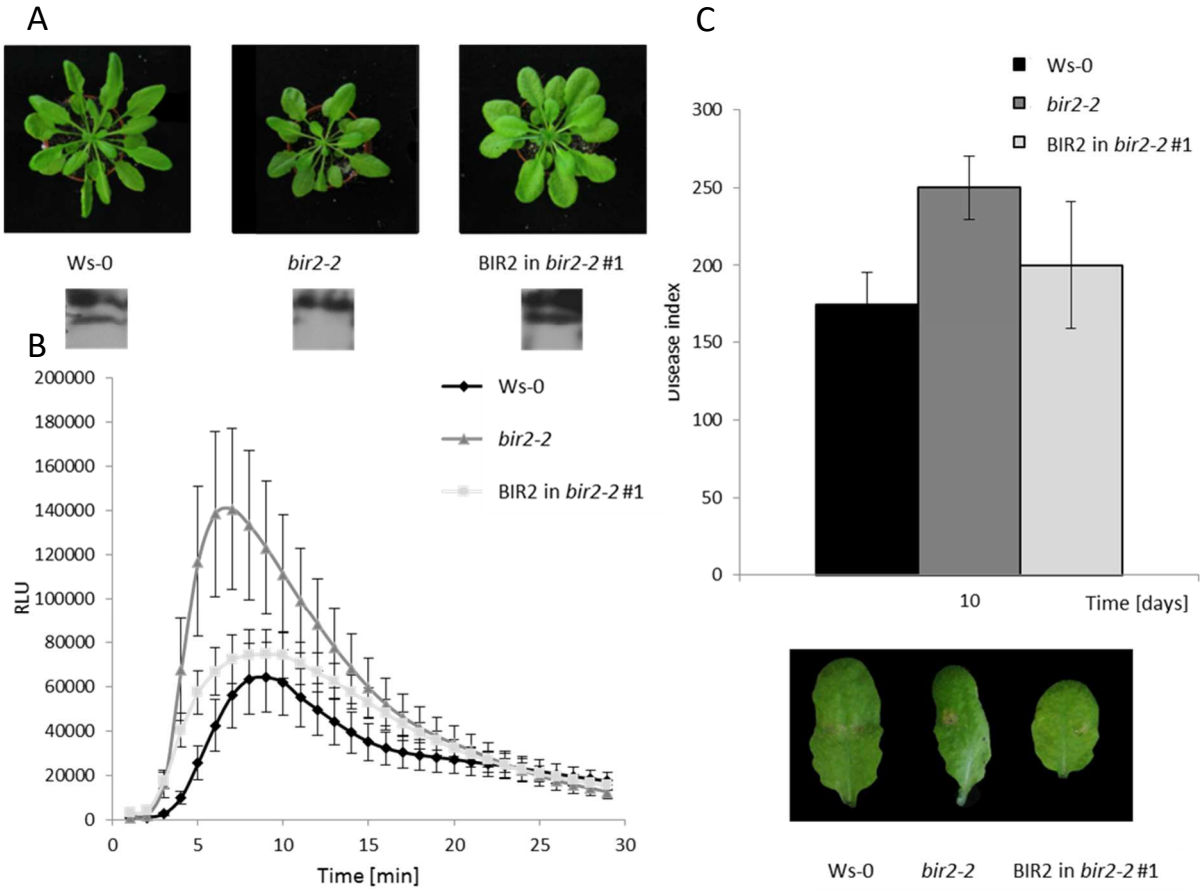



**Figure 3-28: BIR2 protein complements *bir2-2* PAMP-, growth- and partial Alternaria phenotypes.**
**A:** 5 -6week-old *Arabidopsis* plants are grown under short day conditions. Western blot is performed to confirm BIR2 expression. Detection with BIR2 specific antibody. **B:** ROS burst assay. Overnight in water adjusted leaf pieces are treated with 100nM elf18. The oxidative burst within the first 30 min are measured. n= 7, 2 biological replicates. Standard error is given. **C:** 2 leaves per plant are inoculated with 2 5µl droplets of *Alternaria* spores (1:20 diluted from stock solution $2*10^7$ spores/ml). Plants are kept under 100 % humidity in a short day chamber, and bonitated after 10 days. Pictures of disease symptoms are taken. n= 8, 4 biological replicates. Standard error given. Western blot is performed to confirm BIR2 expression. Detection with BIR2 specific antibodies.

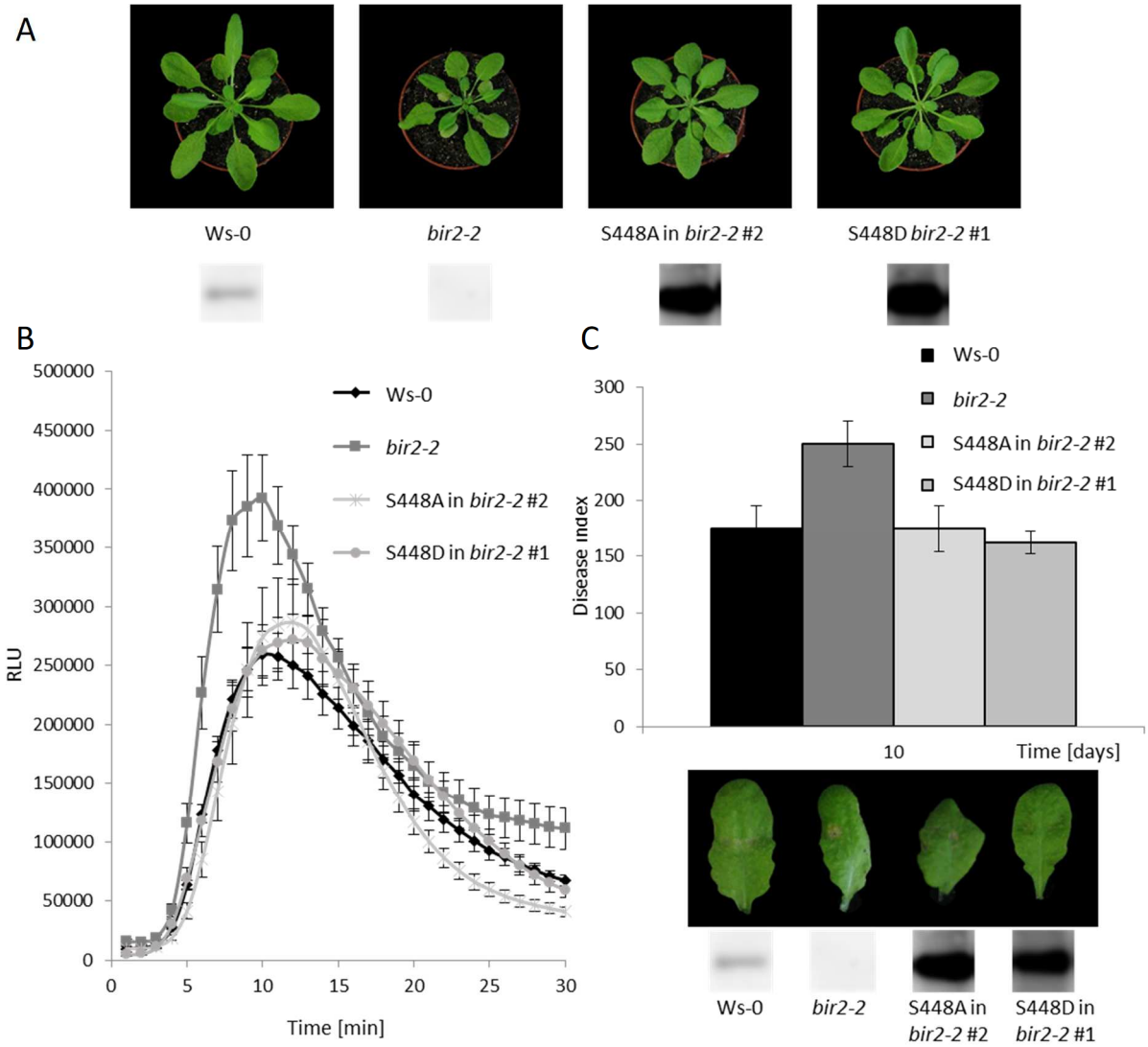



**Figure 3-29: S448A/D lines complement *bir2-2* PAMP-, growth and Alternaria phenotypes.**
**A:** 5-6 week-old *Arabidopsis* plants are grown under short day conditions. Western blot is performed to confirm BIR2 expression. Detection with BIR2 specific antibody. **B:** ROS burst assay. Overnight in water adjusted leaf pieces are treated with 100 nM elf18. The oxidative burst within the first 30 min are measured. n= 2-6, 1-3 biological replicates. Standard error is given. **C:** 2 leaves per plant are inoculated with 2 5 µl droplets of *Alternaria* spores (1:20 diluted from stock solution $2*10^7$ spores/ml). Plants are kept under 100 % humidity in a short day chamber, and bonitated after 10 days. Pictures of disease symptoms are taken. n= 8, 4 biological replicates. Standard error given. Western blot is performed to confirm BIR2 expression. Detection with BIR2 specific antibodies.

### 3.3.9 Phosphorylated residues of BIR2 can influence the interaction stability of BIR2-BAK1 complexes

A general overview of the above described BIR2 P-sites is summed up in Table 3-5 and Figure 3-30 (Table 8-2). In most cases a complementation on a functional and interaction level is obtained. However, some differences in the degree of complementing are visible, and allowing a careful interpretation of the relevance of certain BIR2 P-sites.

**Table 3-5: Summary of functional and interaction analyses of potential P-sites.**
*Only mutated intracellular part of BIR2 is investigated in a Y2H. (+)/yellow indicating phenotypes comparable to Ws-0 levels and interaction ability to BAK1, respectively. (-)/blue indicating phenotypes comparable to *bir2-2,* and no interaction to BAK1 detectable. (+/-)/green partial complementation, and weak interaction stability to BAK1. (++)/orange over-complementation phenotypes. Grey growth phenotype is bigger than Ws-0. n.d. (not determined).

| | Functional analyses | | | Interaction assays | |
|---|---|---|---|---|---|
| Investigated P-sites in *bir2-2* background, T2 generation | Growth phenotype | ROS burst | *Alternaria* symptoms | Y2H | Co-IP (IP@BAK1) |
| BIR2-myc | + | + | + | + | + |
| S263A-myc | + | + | + | ++ | + |
| S263D-myc | + (bigger) | + | ++ | ++ | + |
| T266A* | n.d. | n.d. | n.d. | ++ | n.d. |
| T266D* | n.d. | n.d. | n.d. | + | n.d. |
| S271A* | n.d. | n.d. | n.d. | + | n.d. |
| S271D* | n.d. | n.d. | n.d. | +/- | n.d. |
| S263A/T266A/S271A-myc | + | + | + | ++ | + |
| S263D/T266D/S271D-myc | + | + | + | + | + |
| S286A-myc | + (bigger) | + | ++ | +/- | + |
| S286D-myc | + (bigger) | ++ | + | + | + |
| T304A-myc | ++ (OE-like) | ++ | + | ++ | + |
| T304D-myc | + | - | + | - | +/- |
| BIR2 | ++ (OE-like) | + | + | + | + |
| S448A | ++ (OE-like) | + | + | + | + |
| S448D | + | + | + | - | + |

S263 mutant lines are functionally unobtrusive, but Y2H assays reveal a stronger interaction stability, leading to elf18 insensitivity in Co-IPs. This is true for both mutations, pointing to a not constitutively phospho-mimic aspartate-mutated line. However, this outcome hints to a function of phosphorylated S263 to support release of the complex. Alanine-mutated T266 and S271 show increased interaction formation of BIR2 and BAK1 in yeast, and might function in a similar way than S263. Studies of the residue S286 reveal that this residue could support the interaction of BIR2 with BAK1 in a ligand-absent

environment. This conclusion is supported by functional and interaction data. The incapability of elf18 treatment to trigger release of aspartate-mutated S286 from BAK1, could underline that the substitution to aspartate leads to an increased affinity of BIR2-BAK1 complex. Both *in vitro* P-sites T304 and S448, mutated to aspartate are unable to interact with BAK1 in yeast. This finding is partially confirmed in T304D by *in vivo* Co-IPs. ROS burst assays promote this observation, and point to an involvement of phosphorylated T304 to induce dissociation of the BIR2-BAK1 complex. Functional assays of mutated S448 are wild type-like, and by now, only the loss of interaction in Y2H assays might hint to an action of this P-site to support release of BIR2-BAK1 complex, too. The location of S448 within a catalytically important motif, connected to the full complementation in all functional assays, supports that BIR2 acts as an atypical kinase without the need of enzymtatic activity. Overall, these functional assays do not show strong phenotype differences, possibly because of the necessity of several active P-sites to trigger strong visible effects. Moreover, interaction performance can be directly connected to PTI-signaling, where ROS burst is a suitable readout. In contrast the cell death control, which could be related to *Alternaria*-induced phenotypes, may not be linked to BIR2-BAK1 complex formation. Thus, *Alternaria* spreading output has to be interpreted in an interaction independent-manner. Figure 3-30 sums up the interaction and functional results of the investigated BIR2 P-sites, and shows a model of their action regarding a reinforcment or weakening of the BIR2-BAK1 interaction in an elicitor-sensing context. So far, only for phosphorylated S286 a function in strengthening of the complex formation could be shown, whereas the other studied P-sites imply a potential effect on dissociation of the BIR2-BAK1 complex. However, the results open the discussion for the importance of BIR2 P-sites to induce release and to support the interaction of BIR2-BAK1 complexes, and consequently suppress further downstream immune responses.

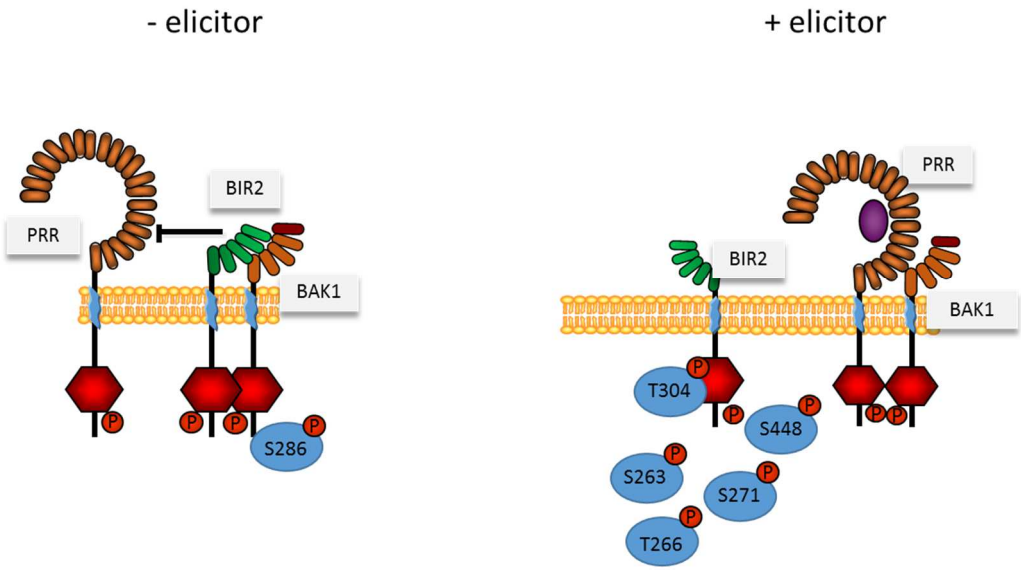



**Figure 3-30: Model indicating potential role of certain identified BIR2 P-sites for BIR2-BAK1 interaction and functionality in related signaling pathways.**
P= phosphorylated, S= serine, T= threonine.

# 4. Discussion

## 4.1 Function and mechanism of the kinase BIR2

### 4.1.1 BIR2, a regulator in multiple PAMP-signaling pathways

A small group of LRR-RLKs in the model organism *Arabidopsis thaliana* are subject of this thesis. BIR proteins are involved in multiple plant immune signaling pathways, as central regulators. The high sequence homology within the BIR family, and the action of the proteins, indicate a partially functional redundancy within this group. Previous PhD theses (Halter, 2014; Imkampe, 2015) have dealt with the description of these proteins, especially focused on BIR2 and BIR3, both BAK1 interactors. BIR2 is a RLK with five leucine rich repeats, a transmembrane domain, and an intracellular section, containing a juxtamembrane domain, a kinase domain as well as a short C-terminal tail. BIR2 is described as a negative regulator in flg22-signaling by controlling BAK1-FLS2 complex formation (Halter et al., 2014a). Treatments with other PAMPs or DAMPs have revealed a fundamental role of BIR2 as an inhibitor of BAK1-complex formation with ligand binding receptors in a ligand-dependent manner. So far, the influence of elf18, a bacterial PAMP conserved in the elongation factor Tu, on BIR2-BAK1 complex stability is not tested yet. Here the evidence is given that BIR2 also negatively affects the BIR2-BAK1 interaction, when elf18 is sensed by EFR, and to trigger a partial release of BIR2-BAK1 in Ws-0 plants (Figure 3-2). Summing up, the BIR2-BAK1 complex is important for several PAMP-signaling pathways, displaying its central role for the first layer of plant innate immunity.

### 4.1.2 Expression levels, functional tool of regulatory proteins?

Regulatory proteins, such as BIR2 and BAK1, are essential for fine-tuned cell responses, and thus changed expression levels of these proteins can have critical consequences for sensing and signaling. Elicitor-induced ROS burst is increased in *bir2* mutants compared to wild type, showing hypersensitivity of *bir2* plants regarding these treatments (Halter et al., 2014a). Interestingly, increased BIR2 protein levels in a BAK1-deficient background have striking impact on plant performance. These lines are completely insensitive to flg22 treatment as shown in a ROS burst assay (Figure 3-13 C). BAK1 knock out lines show an already reduced ROS burst effect after PAMP treatment, but generally less drastic as described above. This could indicate that other SERK proteins might redundantly act in the FLS2-signaling pathway, as shown in Roux et al. (2011) (SERK1, SERK2, BKK1). The increased BIR2 protein level could lead to an enhanced inhibition of SERKs to interact with FLS2, and thus influences the ROS formation in plants. On the other hand, the growth of BIR2 overexpression lines in *bak1-4* background is severely impaired (Figure 3-13 A). The plants are small, a growth phenotype which could hint to an affect on BR or cell death pathways. In cell death control assays, mutant lines (OE or KO) of BIR2 and BAK1, have comparable phenotypes, for example after treatments with the necrotrophic fungus

*Alternaria brassicicola*. No protein expression leads to increased spreading on fungal spores, whereby overexpressed levels enhance the fungal induced symptom development, too (Domínguez-Ferreras et al., 2015; Imkampe, 2015). These results show that protein amounts of BIR2 and BAK1 are crucial for correct cell death control, and the findings of BIR2 OE in *bak1-4* underline the importance of expression levels. These lines (Figure 3-13 A) have phenotypical similarities to *bak1-3 bkk1* plants, a double knock out strongly impaired in cell death (Albrecht et al., 2012; He et al., 2007). However, the phenotypical observations of overexpressed BIR2 in *bak1-4* plants are preliminary results, and have to be confirmed and further investigated, especially according to the cell death performance and potential involvement in BR or other BAK1-mediated pathways. Whether BIR2 and BAK1 act as a complex in the cell death control is not clarified yet. An alternative detection system might function in this case. In Imkampe (2015) the involvement of a guard is discussed. This downstream component could be a R-protein, needed for the sensing of BIR2 and BAK1 protein levels. Protein levels, probably linked to affinity changes, have a strong impact on the cellular action, and could be a special feature of the fine-tuned performance of regulatory proteins.

### 4.1.3 BIR2, an atypical kinase

Functional kinases have specific amino acid motifs conserved in the kinase domain (KD), which are critical for the catalytic performance of the protein. Most important residues/motifs are the lysine in subdomain II and the DFG motif in subdomain VII. These motifs are crucial for ATP fixation, correct folding of the catalytic apparatus, substrate stabilization, and finally the transfer of a phosphate (Kannan & Taylor, 2008; Mukherjee et al., 2008). The pseudokinase $Ca^{2+}$/calmodulin-activated serine kinase (CASK) shows altered amino acids in the φRDXKXXN motif and the DFG motif, but can still perform phosphotransfer in the absence of $Mg^{2+}$ ions (Mukherjee et al., 2008). This finding underlines that even an atypical amino acid sequence can still result in a fully functional kinase.

BIR2 is a special type of kinase due to its amino acid sequence, lacking most of these important residues/motifs (Figure 8-9). A hydrophobic network of side chains build a barrier that occlude the ATP binding site. Furthermore, saturation transfer difference (STD)-nuclear magnetic resonance (NMR) experiments are performed and confirmed no specific binding of the ATP analogue AMP-PNP to BIR2 KD in solution (Blaum et al., 2014). Likewise, the pseudokinase vaccinia-related kinase 3 (VRK3) is completely inactive due to a hydrophobic side chain, occupying the ATP docking site (Scheeff et al., 2009). Another famous kinase, lacking enzymatic phosphotransfer activity, is strubbelig (SUB) (Chevalier et al., 2005), a LRR-RLK with functions in organ development. Comparing amino acid sequences (Figure 8-9) of BIR2, SUB and BAK1 (as an active kinase) underline the classification of BIR2 as a pseudokinase.

Here in this thesis the conserved lysine at amino acid position 335 is studied in more detail. Mutations of this residue leads in active kinases to inactivation concerning the incapability to fix the α- and β-phosphate of ATP. This observation is made for many animal and plant kinases such as PKA (K72, Iyer, Moore, & Taylor, 2005), BAK1 (K317, Schulze et al., 2010), BIR1 (K331, Gao et al., 2009), BRI1 (K911, Oh et al., 2000), and XA21 (K736, Chen et al., 2010). Intriguingly, there is an example of a small human and plant kinase group naturally lacking this lysine, with no lysine (K) (WNK) (Min et al., 2004; Urano et al., 2015; Xu et al., 2000, 2002). The conserved lysine is replaced by a cysteine, but another lysine (K233, Min et al., 2004) further upstream in the sequence possesses the catalytic functionality, and keeps WNK proteins kinase active. BIR2 lacks this further upstream lysine. In a former PhD thesis (Mazzotta, 2012) mutated BIR2 with the K335 replaced by E (K335E) are studied related to BAK1 interaction, and *in vitro* transphosphorylation. In Y2H no interaction of BIR2-BAK1 is observed, whereby in a transient expression system with full length constructs of both, a proper interaction is confirmed. This could mean that either the LRR domain and TM are essential for stable interaction, or that the mutation causes structural problems in yeast cells. Further, it is shown that the transphosphorylation of BIR2 K335E is slightly reduced compared to unmutated BIR2 (Mazzotta, 2012), maybe due to a structural blockade of suitable substrate binding. However, here in this work K335E mutated BIR2 is investigated in stably transformed *Arabidopsis* plants. Interaction assays (Co-IPs) confirm an unaffected complex formation of BIR2-BAK1 as previously shown in transient expression systems. Moreover, the ligand-induced dissociation of BIR2-BAK1 is not changed in this line (Figure 3-3 B). Functional assays of BIR2 K335E in *bir2* background indicate a complete rescue of the knock out phenotypes.

Summing up, the conserved BIR2 K335 seems to play an unobtrusive role, meaning that BIR2 kinase activity is certainly not necessary for BIR2 function. However, the findings of CASK and WNK should keep in mind the complication of summing up pseudokinases as always catalytically dead kinases.

### 4.1.4 Kinase activity, an important feature for BIR2-BAK1 complex stability

In PTI, BIR2-BAK1 interaction behavior is essential for regulation of downstream signaling processes. To address this issue, the involvement of kinase activity in complex formation is explored. Published *in vitro* kinase assays (Halter et al., 2014a) reveal that BIR2 has no autophosphorylation activity as shown for the active kinase BAK1 (Li et al., 2002). Further, BIR2 acts as a substrate for BAK1. In Halter et al. (2014a), the authors claim an influence of BAK1 kinase activity on BIR2-BAK1 complex formation. A reduced BAK1 kinase activity, as obtained in the *bak1-5* mutant line (Schwessinger et al., 2011) reduces the interaction solidity of BIR2-BAK1 complexes. In conclusion, BAK1 kinase activity, and thus transphosphorylation of BIR2, seems to be fundamental for the interaction of these proteins. As discussed above, BIR2 kinase activity is not detectable, and therefore BIR2 seems to be unable to

regulate itself via autophosphorylation. The question is raised how BIR2-BAK1 complexes can partially separate after ligand perception. To investigate this point, a general kinase inhibitor, named K252a, is used. It is known that this chemical, interfering with the ATP binding pocket, blocks PTI responses (Macho et al., 2014). In Chinchilla et al. (2007a) *Arabidopsis* seedlings are pretreated for 5 min with 1 µM K252a, and further inoculated for 5 min with 10 mM flg22. A α-FLS2 IP is performed, and revealed that less BAK1 protein is bound to FLS2 after these above-mentioned treatments. Here in this thesis the assay is switched, and binding properties of BIR2 and FLS2 to immunoprecipitated BAK1 are investigated. First of all, 1 h kinase inhibitor pretreatment causes increased affinities of BIR2 to BAK1, and reduced interaction of FLS2 to BAK1, corresponding to the published data of Chinchilla et al. (2007a)**.** Blocked or strongly diminished kinase activity seems to decrease the release of BIR2 from BAK1, even in a ligand-independent manner. This could mean that there is a general and constitutive turnover of BIR2-BAK1 complex formations, for example to keep enough BAK1 available for its multiple involved signaling pathways, or to remain the sensitivity and plasticity of the system. However, the chemically manipulated reduction of kinase activities has a clear impact on the interaction ability of BAK1 to FLS2 and BIR2. The increased BIR2 levels bound to BAK1 after K252a treatment points to the importance of kinase activity, and the occurring phosphorylation events for a BIR2-BAK1 dissociation.
The detection of a weak FLS2 band in the BAK1-immunoprecipitated assay after K252a treatment, but without ligand induction, is an interesting discovery. This preliminary observation could hint to an involvement of phosphorylation to trigger affinity changes of proteins. Similar findings are made in assays with *bak1-5*, where the authors show that the hypoactive BAK1-5 has an increased affinity to FLS2 in a ligand-absent environment (Schwessinger et al., 2011). However, K252a can inhibit all active kinases in the system, and thus the functional action of other or several kinases (e.g. FLS2 and BIK1) for the partial dissociation of BIR2-BAK1 complex, cannot be excluded.
The above-mentioned observations underline the importance of kinase activity and phosphorylation for protein-protein interaction. Published data (Halter et al., 2014a) have previously confirmed phosphorylation as a tool to stabilize BIR2-BAK1 interaction. In this work, studies point to an additional action of phosphorylation to trigger release of the BIR2-BAK1 complex. That phosphorylation can modulate interaction in both directions, strengthening and weaking, is hypothesized for BIK1 (Lu et al., 2010). Ligand-perception leads to BIK1 transphosphorylation, which enables BIK1 to enhance activity of FLS2 and BAK1. A further transphosphorylation event of BIK1 might finally cause the release of BIK1 from the FLS2-BAK1 complex. The BRI1-BAK1 complex is also activated by phosphorylation (Wang et al., 2005b, 2008). The full capacity of BR-signaling is realized when ligand-activated BRI1 interacts with BAK1. This leads to kinase activation of BAK1 by BRI1-mediated transphosphorylation of its activation-loop residues. Next, active BAK1 transphosphorylates BRI1 on JM and CT residues, and quantitatively increasing BR-signaling by enhancing the phosphorylation of specific BRI1 substrates (Wang et al., 2008).

## 4.2 Identification of *in vivo* BIR2 phosphorylation sites

### 4.2.1 *In vitro*/*in vivo* P-sites of BIR2 discovered via mass spectrometry

The general function of kinases is the transfer of a phosphate to a substrate, which could be another kinase. This most common post-translational modification can cause several changes within the phosphorylated protein, such as alteration of activity and structure, by producing for example docking sites for downstream signaling components. Only certain amino acids are phosphorylated, in eukaryotes mostly serine (S), threonine (T) and tyrosine (Y), due to their hydroxyl groups (-OH). For a better understanding of the BIR2-BAK1 interaction mechanisms, a former PhD student (Mazzotta, 2012) has analyzed *in vitro* BIR2 phosphorylation sites (P-sites). Co-expressed BIR2 and BAK1 (only intracellular part of proteins) in *E. coli* have led to the discovery of 13 *in vitro* BIR2 P-sites; S263, T266, S271, T283, S286, T304, S330, S389, S448, S462, S463, T466 and T533 (Figure 4-1).


**Figure 4-1: Cytoplasmic amino acid sequence of BIR2 with all 20 potential P-sites after *in vitro*/*in vivo* MS analyses, as well as data from the literature.**
Numbers indicate the position in the amino acid sequence. S= serine, T= threonine and Y= tyrosine.

Here in this work, BIR2-overexpressing *Arabidopsis* lines are used to identify *in vivo* P-sites. The idea is to confirm on the one hand the *in vitro* P-sites, and on the other hand to reduce the number of P-sites to the essential ones. But surprisingly, *in vivo* mass spectrometry (MS) assays have even resulted in the identification of novel BIR2 P-sites. For the processing of the plant material for MS, a new procedure is established in this work (Figure 3-6). The following 13 *in vivo* BIR2 P-sites are discovered; S263, T266, S271, S279, S286, Y349, Y379, Y381, S462, S463, T466, Y492 and S585 (Figure 4-1). There is an overlap of *in vitro*/*in vivo* P-sites of seven residues, namely; S263, T266, S271, S286, S462, S463 and T466. The first four amino acids are located in the juxtamembrane domain (JM), whereas S462, S463 and T466 are in the activation loop of the kinase domain (KD). Some *in vitro* P-sites could not be reproduced in *in vivo* assays. First of all, *E. coli* expressed proteins lead to an increased amount of available protein which can positively influence the MS outcome. To exclude phosphorylation triggered by bacterial kinases, a kinase-inactive BAK1 mutant (K335E) is co-expressed with BIR2, and S263 is the only BIR2 P-site observed in this control assay (Mazzotta, 2012), although it is found *in vivo* in this work and in the literature (Benschop et al., 2007; Nakagami et al., 2010; Roitinger et al., 2015; Sugiyama et al., 2008).

Interestingly, phosphorylated tyrosine is exclusively found *in vivo*, as well as S279, and are discussed later in this thesis. *In vitro* and *in vivo* MS analyses, as well as screening the literature, resulted in a total of 20 potential BIR2 P-sites (Figure 4-1). These residues are placed on all intracellular sections of BIR2; JM, KD and CT, but mostly concentrated on the KD (55%) and JM (35%). Large-scale phosphoproteome profiling of the *Arabidopsis* plasma membrane unveils that three-quarters of the identified phosphopeptides are connected to the JM or CT (Nühse et al., 2004). The use of different methods and approaches could explain such variations. The JM is a linker between the TM and the kinase core domain, and phosphorylation of this linker can strongly influence the kinase behavior. Hubbard (2001, 2004) has reviewed that the JM in an unphosphorylated state can suppress kinase activity, whereas phosphorylated JM can serve as a docking site for other proteins. The CT might have similar functions (Nühse et al., 2004; Pawson, 2002, 2004). Wang et al. (2005a) could show the autoinhibitory function of the unphosphorylated CT of BRI1. The KD itself is the core of the catalytic activity, and phosphorylation can alter the activation state, and can affect binding of kinase substrates (Adams, 2003). A common way to induce this event is triggered by autophosphorylation, described for many plant kinases. SERK1 has at least 24 autophosphorylation sites, almost exclusively located in the KD, and most of the phosphorylated P-sites, often threonine, are conserved in the other SERK proteins (Karlova et al., 2009). Investigations of single potential P-sites in the KD have shown that their phosphorylation is required for full kinase activity, in a ligand in- and dependent manner. For instance, SERK1 S562 is a crucial P-site for controlling kinase activity (Karlova et al., 2009); a similar observation is made for BAK1, by phosphorylation of T455 (Wang et al., 2008). Several publications deal with the importance of phosphorylated tyrosine residue for kinase activity and functionality (more details in next section) (Jaillais et al., 2011; Lin et al., 2014; Macho, Lozano-Durán, & Zipfel, 2015; Oh, Clouse, & Huber, 2009b; Oh et al., 2011). BIR2 has an unexpected high number of P-sites located in its KD (Figure 4-1), which is an unexpected observation because of the atypical nature of this RLK. The KD-located P-sites could be evolutionary relics of a previous active kinase, or these P-sites are still employed for structural features such as the creation of docking sites for other interaction partners.

Wang et al. (2013) have published a table summarizing the overall distribution of phosphorylated serine, threonine and tyrosine in animal and plant species. Amazingly, these distribution patterns are quite equal within the different organisms; 87-89 % of all P-sites is a serine, followed by 9-19 % threonine, and up to 4 % tyrosine. BIR2 P-sites are separated into 55 % serine, 25 % threonine, and 20 % tyrosine (Figure 3-10 B), and showing comparable distribution patterns. A higher consensus is not expected, because of the different comparison parameters (P-sites of one kinase compared to the results of more than 1000 kinases). Anyway, some P-sites in BIR2 may still remain undetected.

Large-scale phosphoproteomics have investigated the existence of specific phosphorylation motifs (Wang et al., 2013; Van Wijk et al., 2014). Wide-spread motifs of phosphorylated serine are SP, SF, SD and DS, and longer amino acid sequences such as Sx[D/E], RxxS and Sxx[D/E], with x as any possible

amino acid. Phosphorylated threonine is often found in motifs like TP and TDD, and modified tyrosine in the combination KY and RY. Wang et al. (2013) have described many proline-directed motifs of serine and threonine as phosphorylation targets. BIR2 has nine prolines in its intracellular domains, whereas none of the detected BIR2 P-sites is situated next to such a residue. Screening the BIR2 P-sites some overlaps of published motifs are identified (Figure 4-1). For instance, S463 and S585 have a SF motif, S448 has a DS motif, S263 and S279 have a RxxS motif, and S389 and S585 show the motif SxxD and SxxE, respectively. Furthermore, some new amino acid combinations connected to P-site serine are found in this work, such as SG (S263, S271 and S448) and SxL (S330, S389 and S448). The BIR2 P-site threonine is not directly found in one of the above-mentioned motifs, but twice as TxD (T466 and T533). T266 and T283 have a LTxV motif, not described as a typical motif for threonine phosphorylation, yet. The BIR2 tyrosine residues Y349 and Y381 have a RY and KY motif, respectively, and the other observed tyrosine P-sites, Y379 and Y492, show the sequence VY, not published as a conserved motif. The classification of motifs with a high likelihood to carry a P-site, should help to speed up the identification of important residues, but there are always exceptions that should be kept in mind. However, some observed BIR2 P-sites fit nicely in this motif-based tool, and supporting their potential of being true BIR2 P-sites.

### 4.2.2 Importance of tyrosine P-sites of kinases

Tyrosine phosphorylation is a very common protein modification in the animal system, but rarely in plants. In the last years, the identifications of plant tyrosine P-sites have dramatically increased, and are reviewed by Macho et al. (2015)**.** PRRs, such as EFR (Macho et al., 2014) and BRI1 (Oh et al., 2009a; Oh, Clouse, & Huber, 2009b) have those P-sites. Same is observed for RLCKs, such as BIK1 (Jaillais et al., 2011; Lin et al., 2014; Zhang et al., 2010b). Moreover, the small LRR-RLK BAK1 (Oh et al., 2010) can be phosphorylated on a tyrosine residue. Mutations of certain tyrosine sites have revealed their functional importance. For instance, BRI1 Y831 is crucial for BR signaling, whereas Y956 and Y1072 are involved in kinase activity (Oh et al., 2009a). A single EFR tyrosine site (Y836) is found as a specific elf18-induced P-site (Macho et al., 2014). The potential tyrosine P-sites of BAK1 (Y610) and BKI1 (Y211) are linked to BL-induced phosphorylation events (Jaillais et al., 2011; Oh et al., 2010). These findings underline that tyrosine phosphorylation appears to be a fundamental regulatory modification, and occurs on several signaling layers in the plant cell, responsible for kinase activation, initiation of subsequent signaling or downstream signal transduction. Their essential role for protein functionality is further confirmed by the discovery of a bacterial effector HopAO1, which is an active tyrosine phosphatase (Macho et al., 2014).

Here in this work for the first time BIR2 tyrosine P-sites are identified; Y349, Y379, Y381 and Y492, all four located in the KD. First descriptions of phosphorylated tyrosine are obtained *in vitro* (Mu, Lee, &

Kao, 1994). Nowadays, improved MS technology has increased the sensitivity for the detection of *in vivo* tyrosine P-sites. BAK1 is classically described as a serine/threonine kinase (Li et al., 2002), but the detection of Y610, an autophosphorylation site of BAK1 (Oh et al., 2010) disrupts this previous characteristic. BAK1 seems to be a dual-specificity kinase; a new definition for many formerly termed serine/threonine kinases (Macho et al., 2015). Another member of the SERK family, SERK1, is classified as a dual-specificity kinase already in 2001 (Shah, Vervoort, & De Vries, 2001). However, the absence of *in vitro* BIR2 tyrosine P-sites cannot be explained by having only BIR2 and BAK1 in the tested *in vitro* system, and might hint to technically limiting reasons. The functional importance of many investigated tyrosine residues highlights the discovered BIR2 tyrosine sites. Time limitation has not allowed functional investigations of these sites, but already started site-directed mutagenesis might help to understand their action for BIR2 functionality in future. Two BIR2 tyrosine residues are highly conserved in other screened kinases. For instance, BIR2 Y379 corresponds to BRI1 Y956, EFR Y791, BAK1 Y363, BIK1 Y150 and BIR3 Y373, and BIR2 Y492 corresponds to BRI1 Y1070 (Oh et al., 2009a), EFR Y915, BIK1 Y263 and BIR3 Y489. Y956 in BRI1 is an autophoshorylation site, thus classifying BRI1 as a dual-specificity kinase, too**.** Further investigations of this P-site revealed that it is essential for kinase activity (Oh et al., 2009a). Similar observation of an additional tyrosine autophosphorylation ability is made for BIK1, and Y150 is likely catalytically important (Lin et al., 2014). Although BIR2 seems to have no phosphorylation activity, the overlap of BIR2 tyrosine sites with functionally important tyrosine sites of other kinases, could point to a relevant function of these sites.

### 4.2.3 BIR2 P-sites can be BAK1-independent and target of multiple kinases

In the *in vitro* kinase assays, only P-sites that are dependent on BIR2 and BAK1 could be detected. Whereas, in *in vivo* assays additional kinases might be able to transphosphorylate BIR2. Therefore, BIR2 P-sites, which are not found *in vitro* are potentially BAK1-independent. Technical problems and detection limits can also explain the absence of detectable phosphorylation in the different assays. However, S279 is a JM-located P-site only found in *in vivo* MS analyses. This residue is also detected to be phosphorylated in the *bak1-4* mutant background where BAK1 is absent. Thus, S279 might be a BAK1-independent BIR2 P-site, and important for a BAK1-unrelated signaling pathway. Based on time limitation, only site-directed mutagenesis could be performed so far, and keep this site as a promising candidate for functional and interaction investigation in future.

In addition to S279, a further P-site is detected in *bak1* mutant lines, the JM-located S263. This site is detected *in vitro* and *in vivo*, whereby it is also found in the negative control (*in vitro*, BAK1 K335E – kinase-dead mutant), which should show if there is unspecific phosphorylation action during the bacterial expression process e.g. by bacterial kinases (Mazzotta, 2012). *In vitro* MS analyses of SERK1 have revealed three P-sites in a kinase-dead SERK1 line (K330E), too (Karlova et al., 2009). One of those,

S303, is also found *in vivo*, and this suggests that S303 is not only transphosphorylated by *E. coli* kinases. That S263 is an autophosphorylation site of BIR2 can be excluded as it was clearly shown that BIR2 is not kinase active and unable to bind ATP. However, BIR2 S263 is not only found *in vivo* in this thesis; furthermore, several publications have detected this P-site (Benschop et al., 2007; Nakagami et al., 2010; Roitinger et al., 2015; Sugiyama et al., 2008), weighting S263 as a BIR2 P-site. The presence of phosphorylated S263 in a private BAK1 surrounding, and also in a BAK1-absent nature, implying to be a transphosphorylation target of BAK1 and other plant or bacterial kinases. The rare findings of BIR2 P-sites (only two) in *in vivo* MS assays of overexpressed BIR2 in *bak1-4* background could indicate that BAK1 is the primary kinase for BIR2 transphosphorylation. However, the reduced growth performance of the used plant line (Figure 3-13 A) could influence the outcome of the assay, and therefore the identification of further BAK1-unrelated or not BAK1 exclusive BIR2 P-sites cannot be excluded.

## 4.3 Functional studies of selected BIR2 phosphorylation sites

MS-based identification of potential P-sites of the protein of interest, is a common procedure. These detected P-sites might be relevant for certain protein functions, and therefore site-directed mutagenesis of the identified residues to residues that are unable to be phosphorylated or that are potentially mimicking phosphorylation can help to identify the functional impact of these sites. P-site modifications to alanine (A) and aspartate (D) can result in phospho-prevented and phospho-mimicked residues, respectively (Cao et al., 2013). A substitution to a phospho-mimicking residue, such as the mentioned aspartate or glutamic acid, does not consequentially reproduce the effect of phosphorylation, as shown in Paleologou et al. (2008). That the mutations are meaningless for the subcellular location of the protein, is tested and confirmed in transiently expressed *Nicotiana benthamiana* leaves. The BIR2 P-site mutants are further investigated in *in vitro* systems, or directly stably transformed into *bir2 Arabidopsis thaliana* lines. For complementation assays the null mutant allele *bir2-2* in the ecotype Ws-0 is used. In contrast to the published *bir2-1* allele it has no residual transcripts or protein expression of BIR2. However, flg22 responses are not detectable in this ecotype and cell death responses are usually less pronounced than in the Col-0 background. A likely explanation for these phenotypical differences could be the fact that the still expressed BIR2 in *bir2-1* has an inhibition effect, due to the blocking of signaling pathways, where similarly acting proteins cannot take over. This hypothesis is supported by the slightly weaker phenotypes of amiRNA BIR2 lines in Col-0 (Halter et al., 2014a).

Myc-fused BIR2 constructs are used, and complementation assays of unmutated wild type BIR2-myc are able to rescue the *bir2* phenotypes, in PTI-signaling and cell death control. The BIR2 mutant lines are expressed under the BIR2 native promotor, however enhanced expression is still detectable, likely

dependent on the position of insertion in the plant genome, and the number of T-DNA insertions. Thus, enhanced protein levels lead to a weak BIR2 overexpression phenotype, observed in some assays.
Pre-selected stably transformed BIR2 P-site mutants are used for complementation of the function in the different signaling pathways as well as in interaction studies. The MS data are utilized to select some residues of interest, mainly based on their MS detection as a BIR2 P-site, and further by showing first interaction phenotypes in Y2H assays. These residues are discussed in the following sections.

### 4.3.1 S263, T266 and S271, three BIR2 P-sites closely located to each other

The JM-located *in vitro* and *in vivo* identified BIR2 P-sites S263, T266 and S271, are closely located to each other, as shown in the three-dimensional BIR2 structure (Figure 3-11). Especially S263 is detected several times in MS runs (Table 8-1), and moreover in large-scale phosphoproteomics (Benschop et al., 2007; Nakagami et al., 2010; Roitinger et al., 2015; Sugiyama et al., 2008).
The question is raised whether ligand induction causes the phosphorylation of specific BIR2 P-sites. *Arabidopsis* seedlings are treated with certain concentrations of ligands such as flg22 or a mixture containing flg22, elf18, pep1 and BL. For the single flg22 inoculation, a timeframe of 2.5 min up to 5 min is chosen. These durations of treatment are selected according to published data (Chinchilla et al., 2007a; Halter et al., 2014a; Schulze et al., 2010). Ligand-induced FLS2-BAK1 association takes place after seconds of treatment, thus *de novo* phosphorylation of BAK1 is detectable relatively rapid (Chinchilla et al., 2007a; Schulze et al., 2010). However, these experiments are made in *Arabidopsis* cells, and supplemented ligands can be promptly recognized by PRRs. The timing for seedlings to take up the ligand, and to activate immune responses is likely longer. ROS burst assays and MAP kinase assays of adult plants, are further downstream PTI responses, and have the highest responses related to ligand treatment after approximately 10 min (Chinchilla et al., 2007a). Moreover, *Arabidopsis* seedlings show a BAK1 release from the BIR2-BAK1 complex after 5 min ligand treatment (Halter et al., 2014a). Summing up, 5 min ligand treatment should be sufficient to trigger dissociation responses, whereby *de novo* phosphorylation might be the previous step, and thus a shorter duration of treatment is additionally investigated (2.5 min). Phosphorylation can be also an indirect signal for degradation of the used protein, resulting in ligand-mediated receptor endocytosis as shown for FLS2 (Robatzek et al., 2006). However, phosphorylation is a sensitive modification, and unknown turnover rates of phosphorylated to unphosphorylated residues have to be taken into account. Here in this thesis, S263 is found, among others, before and after ligand treatment independent of the incubation time and the ligand. Due to comparable basic raw material of untreated versus treated seedlings, the observed intensities of identified phosphopeptides could be compared to give a rough estimation of changes of phosphorylation. The BIR2 phosphopeptide containing phosphorylated S263 is found with a higher intensity after 5 min flg22 and mix treatment, while 2.5 min flg22 inoculation has no effect on

the intensity. Benschop et al. (2007) have treated *Arabidopsis* cells with either 1 µM flg22 or 10 µg/ml fungal xylanase for 10 min, and used a quantitative phosphoproteomics approach based on $^{14}N/^{15}N$ metabolic labeling. The ratio between treatment versus mock is calculated, and values of <0.67 or >1.5 are accepted as indicators of quantitated differences of detected peptides. S263 is identified in this publication as a BIR2 P-site, but treatments with xylanase revealed a ratio of 1.03 ± 0.04, thus no effect on quantitative levels. Data with flg22 treatments are also listed. S263 is not significantly infected in this data set, whereby the ratio with 1.39 ± 0.12 shows that it is slightly stronger than in the untreated samples. Focusing on the accumulated data in this thesis, S263 could be a P-site which is influenced by ligand treatment, and consequently could play an important role in PTI-signaling. Therefore, this BIR2 P-site might be a candidate that could be altered after flg22 treatment. That the ligand handling might not directly guide to clear results is expected, because of the dynamics of the BIR2-BAK1 complex. For instance, ligand treatment leads only to a partial release of BAK1 from BIR2 (Halter, 2014; Imkampe, 2015). Interaction studies and functional complementation assays of mutated S263 are performed to investigate this point. 2-3 independent S263A/D mutant lines in T2 generation are checked for their complementation ability in *bir2* plants. Interestingly, Y2H studies reveal a stronger interaction of BAK1 with both BIR2 S263 mutant alleles compared to unmutated BIR2 control. This improved interaction could not be confirmed in Co-IPs, but surprisingly, this preliminary Co-IP data indicates an insensitivity of S263 mutants regarding elf18 treatment. The slightly over-complemented ROS burst phenotypes could be explained by the inability of BAK1 to be released from BIR2 S263A/D mutants, but this finding has to be investigated in more detail. The stronger interaction observed in yeast assays also support the idea that phosphorylated S263 is necessary for dissociation of BIR2-BAK1 complex formation, and again could fit to the weak over-complementation in ROS assays. The aspartate-mutated line might not present a phospho-mimic mutant, and simply avoids S263 transphosphorylation, too.

As mentioned in the beginning of this section S263 is within a group of two further BIR2 P-sites, T266 and S271. T266 is also identified after 5 min flg22 treatment, but not after mix treatment, which also includes flg22. Single T266A/D and S271A/D mutant constructs are only studied in Y2H assays. Here, all modified BIR2 constructs are still able to bind to BAK1, but on a quantitative level, differences are visible. As already described for S263A/D, T266A and S271A perform a stronger yeast growth, thus might better interact with BAK1, than the aspartate-mutated P-sites. The three BIR2 residues show equal performance in Y2H, pointing to a shared function in supporting release of BIR2 from BAK1. Therefore, two triple mutants containing S263, T266 and S271 mutated to A (called AAA) and D (called DDD) that have been previously generated by Dr. Sara Mazzotta are analyzed. Y2H confirms the slightly stronger growth skills of the alanine-mutated triple line. Functional assays of the triple lines demonstrate complementation in all tested experiments. Even though the Y2H assays hint to a similar action of these three residues, mutations of all three sites at once have no dramatic effect. There could be individual involvement in independent signaling pathways, or a time-dependent order of

phosphorylation as shown for fibroblast growth factor receptor 1 (FGFR1), activated in a three-step autophosphorylation procedure (Lew et al., 2009). The authors claim that the ordered autophosphorylation of the residues has a physiologically relevant role for the temporal recruitment of downstream signaling partners, a possible strategy of BIR2 too.

### 4.3.2 S286, a BIR2 P-site involved in enhancing stability of BIR2-BAK1 interaction?

A JM-located serine of BIR2 is identified as a potential P-site in *in vitro* and *in vivo* MS assays. Moreover, flg22 and mix treatments with followed MS analyses reveal increased intensities of phosphopeptides, as described for S263 in the previous section. Interestingly, a short ligand treatment of 2.5 min has already an increased effect on the intensity of phosphopeptides carrying S286, which could point to an early involvement of phosphorylated S286 in immune signaling. In Benschop et al. (2007) or other published phosphoproteomic data (Nakagami et al., 2010; Nühse et al., 2004; Roitinger et al., 2015; Sugiyama et al., 2008) this phosphopeptide is not observed. Functional complementation studies of mutated S286A/D show a rescue effect in ROS burst experiments and cell death control. The S286D line has a slight over-complementation outcome in ROS burst assays, which cannot be directly explained by high protein expression levels. Interaction assays of these mutant lines to BAK1 are investigated, and Y2H studies show an enhanced growth effect of S286D lines compared to wild type, and a weaker interaction in the case of S286A. The phospho-mimicking mutation seems to support BIR2-BAK1 complex formation. Preliminary Co-IP data cannot confirm the quantitative differences of the different substitutions of S286. However, the S286A line is still partially released from BAK1 after elf18 perception, whereby this effect is abolished in S286D mutated transgenic plants. This lack of BIR2 release from BAK1 after elf18 treatment in the aspartate-mutated line, and the slightly stronger over-complementation of this line in ROS assays, drawing a functional conclusion of S286, where a phosphorylation reinforces the BIR2-BAK1 stability.

### 4.3.3 T304, a BIR2 P-site involved in decreasing stability of BIR2-BAK1 interaction?

The exclusively *in vitro* found BIR2 P-site T304 is located in the JM. Even so it is not found in *in vivo* MS assays yet, the outcomes of a Y2H pre-screen of mutated BIR2 P-sites has added T304 to the list of interesting candidates. Alanine- and aspartate-mutated T304 residues show antagonistic yeast growth properties. The BIR2 T304D and BAK1-transformed yeast cells are completely unable to accumulate on selective medium, thus appears to fail interaction. In contrast, T304A and BAK1 containing yeast can grow stronger, than the wild type control. However, in *in vivo* Co-IPs an interaction of T304D with BAK1 is present, hinting to an additional action of TM and/or ECD in solid complex construction. For instance, crystalized ECDs of BAK1 and FLS2 are able to interact via some LRR motifs in a ligand-independent manner (Sun et al., 2013). Furthermore, several publications have confirmed that RLP interaction with

their corresponding co-receptor (e.g. SOBIR1) is induced through a conserved GxxxG motif (as single or several tandem repeats) in the TM domains (Bi et al., 2015; Gust & Felix, 2014). The LRR-RLK, SOBIR1 has a GxxxGxxxG motif, creating a flat and weakly hydrophobic surface in the α-helix of the TM, and mutations of those glycine residues result in loss of interaction to RLPs (Bi et al., 2015). BIR2 lacks such a charge-specific arrangement of amino acids in its TM domain (personal communication with Philippe Chatelain), but interaction affinities of the BIR2 TM to BAK1 TM cannot be excluded.

Subcellular localization of expressed aspartate-mutated T304 is tested to exclude negative side effects related to the mutation. Thus, the mutation does not influence the general protein performance. Indeed, the interaction quantity of T304D to BAK1 is reduced, and enhanced in relation to T304A, corresponding to the Y2H data. This observation should have an influence on PTI responses, and in fact, T304D lines are not able to complement the *bir2* phenotype. Ligand-induced partial dissociation of BIR2-BAK1 complex could not be observed, but this preliminary data should be repeated. Summing up, a phosphorylated BIR2 T304 could weaken the interaction of BIR2 to BAK1. The lack of identification of this P-site in *in vivo* MS experiments could be explained by the need of a certain trigger. This trigger could be one of the tested ligands, but the duration of treatment might be a crucial factor, too.

Surprisingly, the outcome of the *Alternaria* assay for both amino acid switches does not show differences such as seen for ROS burst. Both mutant lines can complement the *bir2* phenotype. This could have several reasons; for instance, T304 could be specific for PTI-signaling. It is not shown yet that BIR2-BAK1 interaction or lack of interaction can result in a similar cell death phenotype as observed in *bir2* and *bak1* mutants. Thus, effects of T304 on the interaction of BIR2 and BAK1 might not be relevant for the cell death phenotype. Further functional investigations might help to solve this issue.

A screen for equivalents of BIR2 T304 in other LRR-RLKs, including cytoplasmic proteins such as BIK1 and BKI1, revealed that this site is a highly-conserved residue with functional importance for those proteins. In rice, T705 of XA21 is important for autophosphorylation and interaction to other proteins (Chen et al., 2010). In BRI1 T880 is identified as an *in vivo* P-site (Wang et al., 2005b), and also EFR has a threonine equivalent to BIR2 T304 (EFR T709) (Chen et al., 2010). The functional relevance and ability to be phosphorylated of EFR T709 is not tested yet. Mutations of FLS2 T867 are impaired in FLS2 internalization and flg22-induced FLS2 response (Robatzek et al., 2006). Besides the mentioned PRRs, the co-receptor BAK1 has a conserved serine at this position, BAK1 S286. Constitutively phosphorylated BAK1 S286 (S286D) leads to loss of interaction with BIR2 in Y2H assays (Mazzotta, 2012), and Wang et al. (2008) have shown that the mutation BAK1 S286D results in a lack of BAK1 kinase activity, explaining the described impact on BIR2-BAK1 interaction. BAK1 S286 might be a phosphorylation-dependent regulatory site of BAK1 (Wang et al., 2008), and same could be the case for BIR2 T304. Intriguingly, phospho-preventing mutations of these conserved threonine or serine have

only a weak or even no impact on the protein performance as shown for BAK1 (Mazzotta, 2012; Wang et al., 2008), BRI1 (Wang et al., 2005b) and BIR2 (Figure 3-25-27). By contrast, modified plants with XA21 T705A (Chen et al., 2010) and FLS2 T867V (Robatzek et al., 2006) are functionally affected. For instance, transgenic FLS2 lines with a T867V mutation show an abolished flg22-induced ROS output and impaired flg22 downstream signaling (Robatzek et al., 2006).

The strong conservation of this potentially phosphorylated amino acid in RLKs, plus the remarkable findings in this thesis make BIR2 T304 to a functionally relevant residue, where phosphorylation could trigger dissociation, and the functional consequences of this release.

### 4.3.4 S448, a potential BIR2 P-site in an atypical Mg-binding motif

Kinases in the animal or plant kingdom have conserved motifs essential for proper kinase activity. BIR2 is a special case as it is an atypical kinase, that lacks several of these conserved motifs. Magnesium ($Mg^{2+}$) is a metal ion needed for the transfer of the phosphate to the substrate. In functional kinases, the ions are fixed by a conserved motif, DFG, located in the activation segment of the KD. In the case of BIR2, the aromatic phenylalanine (F) in the DFG motif is changed to a serine, S448. This residue is identified as a possible BIR2 P-site in *in vitro* assays, but undetected in *in vivo* MS experiments, as well as in the literature. The Y2H pre-screen has revealed similar patterns as shown for T304, no growth of yeast transformed with a BIR2 aspartate mutation of S448, but growth in yeast cells expressing BIR2 S448A. This first finding indicates an action of S448 regarding a support of release of BIR2-BAK1 complex. However, functional studies result in complementation of *bir2* phenotypes, and could not underline an involvement in the tested pathways. P-sites in the KD are known to be require for full kinase activity (Clouse, Goshe, & Huber, 2012; Karlova et al., 2009; Mitra et al., 2015; Wang et al., 2008), and concerning to the catalytically inactive nature of BIR2, a mutation of this KD-located residue might have no influence on the protein performance. These functionally unaffected outcomes of the performed assays could underline the pseudokinase functionality of BIR2. But the inhibition of interaction in Y2H could point to an important action of this P-site for docking purposes, which has to be proved by further *in vivo* experiments.

## 4.4 Conclusion: Phosphorylation, a powerful signaling tool for BIR2

*In vitro* and *in vivo* MS-based assays resulted in the identification of several potential BIR2 P-sites. The *in vivo* data are accumulated in this thesis, and could partially confirm the previously detected *in vitro* P-sites (Mazzotta, 2012). Moreover, new BIR2 P-sites are discovered such as four phosphorylated tyrosine residues. Kinase-triggered phosphorylation is a powerful tool for cell signaling as described in many publications (Cohen, 2000; Jensen, 2004; Park, Caddell, & Ronald, 2012; Pawson & Scott, 1997; Seet et al., 2006). Not only kinase activation is caused by adding a phosphate-group to a substrate of

interest, furthermore, important protein-protein interaction platforms are established for downstream signaling (Jaillais et al., 2011). Kim et al. (2009) deal with the BR-signaling pathway. Active BRI1 can phosphorylate BSK1 and this promotes BSK1 binding to the phosphatase BSU1. BSU1 dephosphorylates BIN2, resulting in increased amounts of unphosphorylated BZR treanscription factors in the plant nucleus. BIR2 could have a function as an important adaptor protein, building interaction platforms to further downstream acting proteins.

In this thesis, selected BIR2 P-sites are investigated in more detail, and functional assays indicated the potential importance of those P-sites for BIR2 action. The JM-located residues S263, T266, S271 and T304, and the KD-located S448 show a strong potential that their phosphorylation could be linked to release activation mechanism of BIR2-BAK1 complex. Dissociation of these constitutively interacting LRR-RLKs is crucial for breaking the obstructing impact on PTI-signaling, and other BIR2- and BAK1-dependent signaling pathways. Mutations of the *in vitro* P-site T304 show the strongest effects on PAMP-involved assays. A further tested BIR2 P-site within the JM is S286. A phosphorylation of this residue seems to stabilize the BIR2-BAK1 interaction. Summing up, BIR2 P-sites could have specificities to trigger release and to stabilize BIR2-BAK1 complex formation, depending on the incoming signal. In the case of the pseudokinase BIR2, P-sites could be mainly linkers for protein-protein interaction, and not switch on buttons for general protein activity as described for many active kinases, especially P-sites within in KD. For instance, BAK1 Y463 located in the KD is essential for the catalytic activity of BAK1 (Oh et al., 2010). Commonly, JM-located P-sites are more connected to a docking function (Pawson, 2004), and protein stability (Xu et al., 2006). However, there is a discussion about the different activation mechanism of RD and non-RD kinases. Autophosphorylation in the activation segment seem to be RD-kinase specific, whereby non-RD kinases are activated via P-sites in the JM domain (Dardick & Ronald, 2006; Johnson, Noble, & Owen, 1996; Liu et al., 2002). The high number of BIR2 P-sites in the KD could be evolutionary relics, and might be modified to additional docking sites. Mutations of single BIR2 P-sites have mostly led to weak phenotypes, hinting to phosphorylation events including multiple residues at once or a time depending mechanism. Nevertheless, BAK1 is involved in this action too, and therefore there might be independent phosphorylation events in both proteins to trigger effective cell responses for single signaling pathways. Furthermore, BIR2 and BAK1 act in many different signaling pathways (Chinchilla et al., 2009; Halter et al., 2014a), making them to essentially regulatory proteins in plant signaling, and thus P-sites could trigger specificity. The finding of BAK1-unspecific BIR2 P-sites S263 and S279 could support this idea, where a pathway-dependent kinase could transphosphorylate BIR2 in addition to BAK1. One prominent candidate could be the cytoplasmic kinase BIK1 (Lu et al., 2010; Veronese et al., 2006; Zhang et al., 2010b), being in complex with BAK1, and thus might be able to interact with BIR2, too. Ligand perception starts several phosphorylation events between BIK1, BAK1, FLS2, and possibly but not yet tested BIR2. Also PRRs could act as such additional BIR2 transphosphorylation kinases, even though a direct interaction of

PRRs and BIR2 could not be shown yet (Halter et al., 2014a). In contrast, BIR3, another member of the BIR family has the ability to interact with PRRs (Imkampe, 2015).

Surprisingly, the outcomes of functional complementation assays of BIR2 mutated P-sites, treated with *Alternaria* could not indicate a functional relevance of tested P-sites. This could show that phosphorylation is not that crucial for cell death control. As mentioned earlier in this thesis, there is no evidence that BIR2-BAK1 interaction has an effect or is generally involved in cell death control. More likely, the total protein amounts of each protein trigger responses in cell death control (Domínguez-Ferreras et al., 2015; Halter et al., 2014a; Imkampe, 2015; Kemmerling et al., 2007). However, phosphorylation is important for BIR2-BAK1 complex formation, and consequently the function of BIR2-BAK1 dependent signaling pathways in the plant cells. Phosphorylation itself is a reversible protein modification, controlled by kinases and the antagonistically acting phosphatases. The investigation of BIR2 and BAK1 related phosphatases is just as important to fulfill the whole process. For instance, the rice PRR XA21 can interact with the protein phosphatase 2C (PP2C) XA21-binding proteins 15 (XB15) through at least one JM-located serine, and *in vitro* autophosphorylation of XA21 is dephosphorylated by XB15 (Park et al., 2008), underlining the important function of phosphatases in plant signaling. Other regulatory functioning phosphatases are PP2C KAPP, interacting with FLS2 (Gómez-Gómez et al., 2001) and PP2A, linked to BAK1 (Segonzac et al., 2014)**.** A BIR2 pulldown assay, followed by MS analysis to identify novel BIR2 interactors (performed by Dr. Thierry Halter), is screened for potential BIR2-related phosphatases. At3g25800 and At1g25490 are two serine/threonine protein phosphatases of type 2A (PP2A). They belong to the same family as the above mentioned negative regulator of BAK1 (Segonzac et al., 2014) and might be good candidates to be studied in the future to get a better understanding of whole phosphorylation processes influencing BIR2 functionality.

# 5. Summary

Plants have to defend themselves against environmental threats, such as microbiological foes. Their immune system bases on a two-layered detection mechanism in each plant cell. The first layer, also called pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), takes place at the plasma membrane, where plant receptors are involved in recognition of plant-unrelated patterns derived from microbes or pathogens. To trigger an effective immune response, the small leucine-rich repeat-receptor-like kinase (LRR-RLK) BAK1 acts as a co-receptor, and is a central key player in diverse signaling pathways. This co-receptor positively supports PTI responses, growth and development, by interaction with the corresponding pattern recognition receptors (PRRs). It is also involved in cell death control. Cell signaling pathways have to be monitored, even in a negative manner to avoid unrestrained responses, harming the total plant performance. Thus, the discovery of BIR2, a negative regulator of BAK1 in PAMP signaling, brought new facts to light. This small LRR-RLK belongs to a small protein family with four members in *Arabidopsis*. BIR2 is an atypical kinase, unable to catalyze auto- or transphosphorylation events. Thus, a function as adaptor protein in signaling control and transduction could be its central role. BIR2 is constitutively bound to BAK1 in the unactivated state. PAMP perception by its receptor results in a partial release of BAK1 from the BIR2-BAK1 complex. Consequentially, BAK1 can fulfill its skills as a positive co-receptor of PRRs. Complex formation of kinases is often phosphorylation-dependent. The aim of this work was to show whether phosphorylation events are necessary for the association or dissociation of BIR2-BAK1 complexes, and for the function of BIR2. 13 *in vivo* phosphorylation sites (P-sites) have been identified, partially supported by former *in vitro* data (Blaum et al., 2014; Mazzotta, 2012). Selected P-sites have been mutated to alanine (A) or aspartate (D) to prevent or mimic phosphorylation. Characterization of *bir2* mutant lines, complemented with these constructs have revealed the potential action of six tested P-sites. Five P-sites (S263, T266, S271, T304 and S448) seem to positively influence the release of complex, whereby mutations of the P-site T304 has shown the strongest effect. Phospho-mimicking T304 lines (T304D) have revealed a reduced ability to interact with BAK1, an output with a direct influence on downstream responses, such as elf18-induced ROS burst, where no rescue of the *bir2* phenotype was detectable. Another P-site S286 might be important for a stabilization of BIR2-BAK1 complex. Phospho-preventing S286 lines (S286A) have interacted less strong with BAK1 in yeast-two-hybrid (Y2H) assays, and slightly over-complemented the ROS burst phenotype. This outcome could describe a dual function of BIR2 transphosphorylation; strengthen and weaken the protein-protein interaction to regulate specific signaling responses. In contrast to the *in vitro* identified BAK1-dependent P-sites, in the *in vivo* approach BAK1-unrelated BIR2 P-sites are detected, pointing to an involvement of other kinases in this complex action. These findings underline the importance of phosphorylation as a key protein modification for the regulation of complex dynamics.

# 6. Zusammenfassung

Pflanzen müssen sich gegen verschiedenste Umwelteinflüsse verteidigen, wie mikrobiologische Feinde. Ihr Immunsystem basiert auf einem zwei-Stufen Erkennungssystem in jeder einzelnen Pflanzenzelle. Die erste Stufe, auch Pathogen assoziierten molekularen Signaturen (PAMP, englisch, pathogen associated molecular pattern) - induzierten Immunität (PTI, englisch, PAMP-triggered immunity) genannt, findet auf dem Level der Plasmamembran statt, wo bestimmte Pflanzenrezeptoren in der Erkennung von pflanzenunspezifischen Strukturen, von Pathogenen und Mikroben, beteiligt sind. Das Auslösen von effektiven Immunantworten geschieht mit Hilfe von einer kleinen LRR-RLK, die als zentrale Schlüsselfigur in verschiedenen Signalwegen aktiv ist. Dieser Co-Rezeptor, genannt BAK1, kann positive PTI Antworten beeinflussen, so wie Wachstum und Entwicklung, und pflanzlichen Zelltod kontrollieren. Zellsignalwege müssen beaufsichtigt werden, auch auf eine negative Art und Weise. Dies dient dem Schutz vor unkontrollierten Reaktionen, die der Pflanzen sonst Schaden könnten. Folglich brachte die Entdeckung von BIR2, einem negativen Regulator von BAK1 im PAMP Signalweg, neue Tatsachen ans Licht. BIR2 ist eine kleine LRR-RLK und gehört zu einer *Arabidopsis* Familie mit vier Mitgliedern. Zudem ist BIR2 eine untypische Kinase, nicht in der Lage Auto- oder Transphosphorylierung zu katalysieren, und dürfte daher Aufgaben als Adapterprotein für Signalkontrolle und -weiterleitung übernehmen. Außerdem bindet BIR2 konstitutiv an BAK1, und PAMP Erkennung vom pflanzlichen Immunsystem führt zu einer Trennung von einigen BAK1 Proteinen aus dem BIR2-BAK1 Komplex. Folglich kann nun BAK1 seiner Aufgabe als positiver Co-Rezeptor nachgehen. Komplexbildung von Kinasen ist häufig abhängig von Phosphorylierung. Ziel der Arbeit war die Untersuchung von BIR2 Phosphorylierung (P) und ob diese notwendig für die Komplexbildung und somit für die BIR2 Funktionalität ist. Es wurden 13 *in vivo* P-Reste identifiziert, die teilweise alte *in vitro* Daten bestätigen (Blaum , 2014; Mazzotta, 2012). Ausgesuchte P-Reste wurden mutiert zu Alanin (A) oder Aspartat (D) um Phosphorylierung zu verhindern oder nachzuahmen. Charakterisierung von *bir2* Mutanten, die mit diesen Konstrukten komplementiert wurden, haben die potentiale Funktion von sechs getesteten P-Resten gezeigt. Fünft P-Reste (S263, T266, S271, T304 und S448) scheinen einen positiven Einfluss auf die Trennung des BIR2-BAK1 Komplexes zu haben. Hier zeigen mutierte T304 Linien den stärksten Effekt. Phosphorylierungs-nachahmende T304 Linien (T304D) können nicht mehr so stark mit BAK1 interagieren, ein Ergebnis, welches einen direkten Einfluss auf weitere Immunantworten hat. Der erhöhte PAMP-induzierte ROS Ausbruch (englisch, ROS burst) in *bir2* Pflanzen kann nicht komplementiert werden. Ein anderer P-Rest in der Juxtamembrandomäne S286 könnte wichtig für die Stabilisierung des Komplexes sein. Phosphorylierungs-verhindernde S286 Linien (S286A) zeigen eine schwächere Interaktion zu BAK1 im Hefedihybrid-System (englisch, Y2H), sowie über-komplementieren den ROS burst Phänotyp. Diese Ergebnisse könnten eine duale Funktion von BIR2 Transphosphorylierung beschreiben; zu stärken und zu schwächen die Protein-Protein Interaktion

um spezifische Signalantworten auszulösen und zu regulieren. Vielmehr wurden auch BAK1-unspezifische BIR2 P-Reste gefunden, die zu einem zusätzlichen Einfluss von anderen Kinasen in dieser Komplexdynamik hindeuten. Diese Beobachtungen unterstreichen die Wichtigkeit von Phosphorylierung als zentrale Proteinmodifikation für die Regulierung von Komplexformationen.

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# 8. Supplemental data

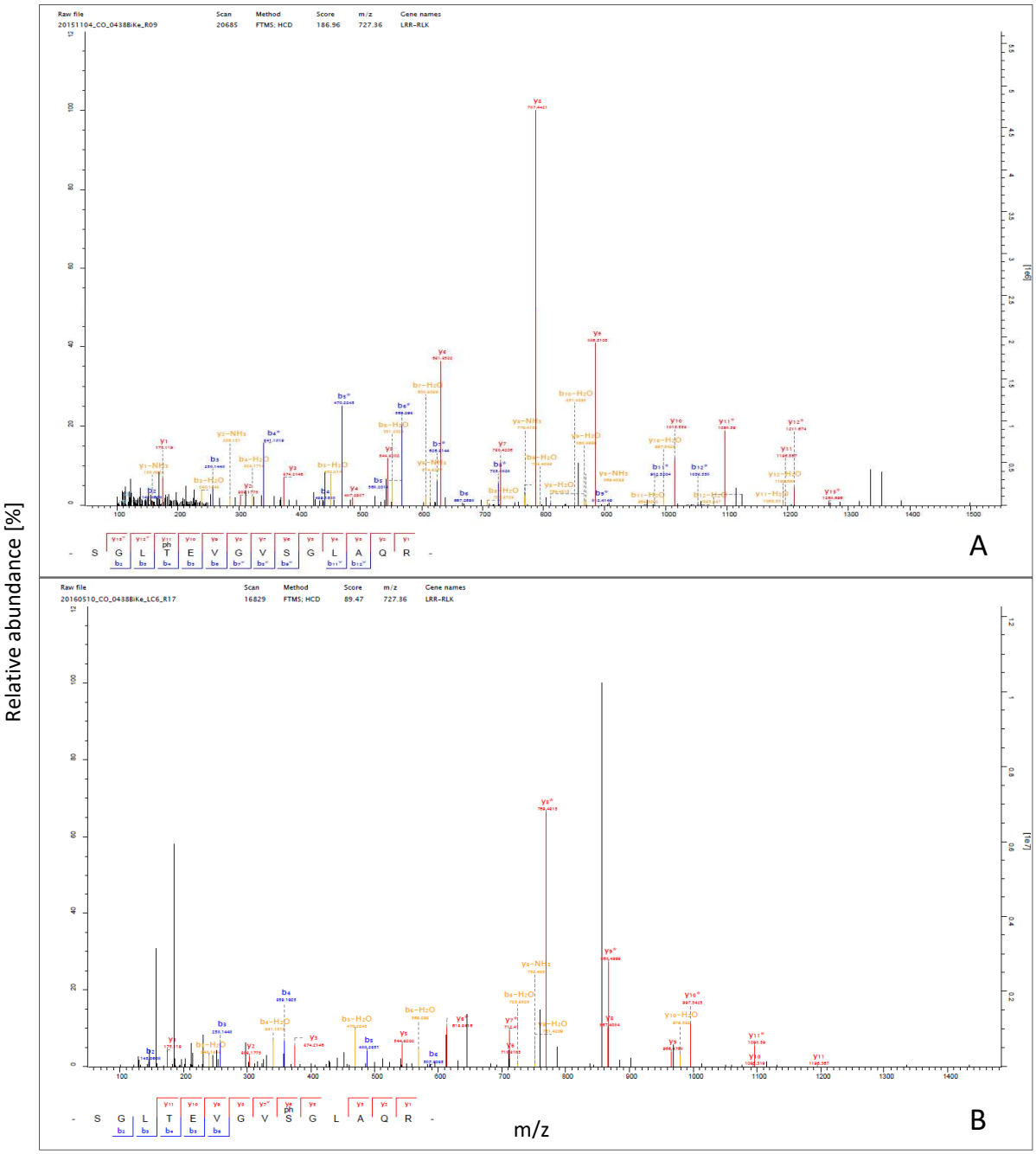


**Figure 8-1: MS-spectra of two identified phosphopeptides of BIR2 in the juxtamembrane domain.**
**A:** Spectra of BIR2 peptides containing phosphorylated T266 and in **B:** phosphorylated S271. Mass spectra are given in relative abundance (%) vs. mass to charge (m/z).

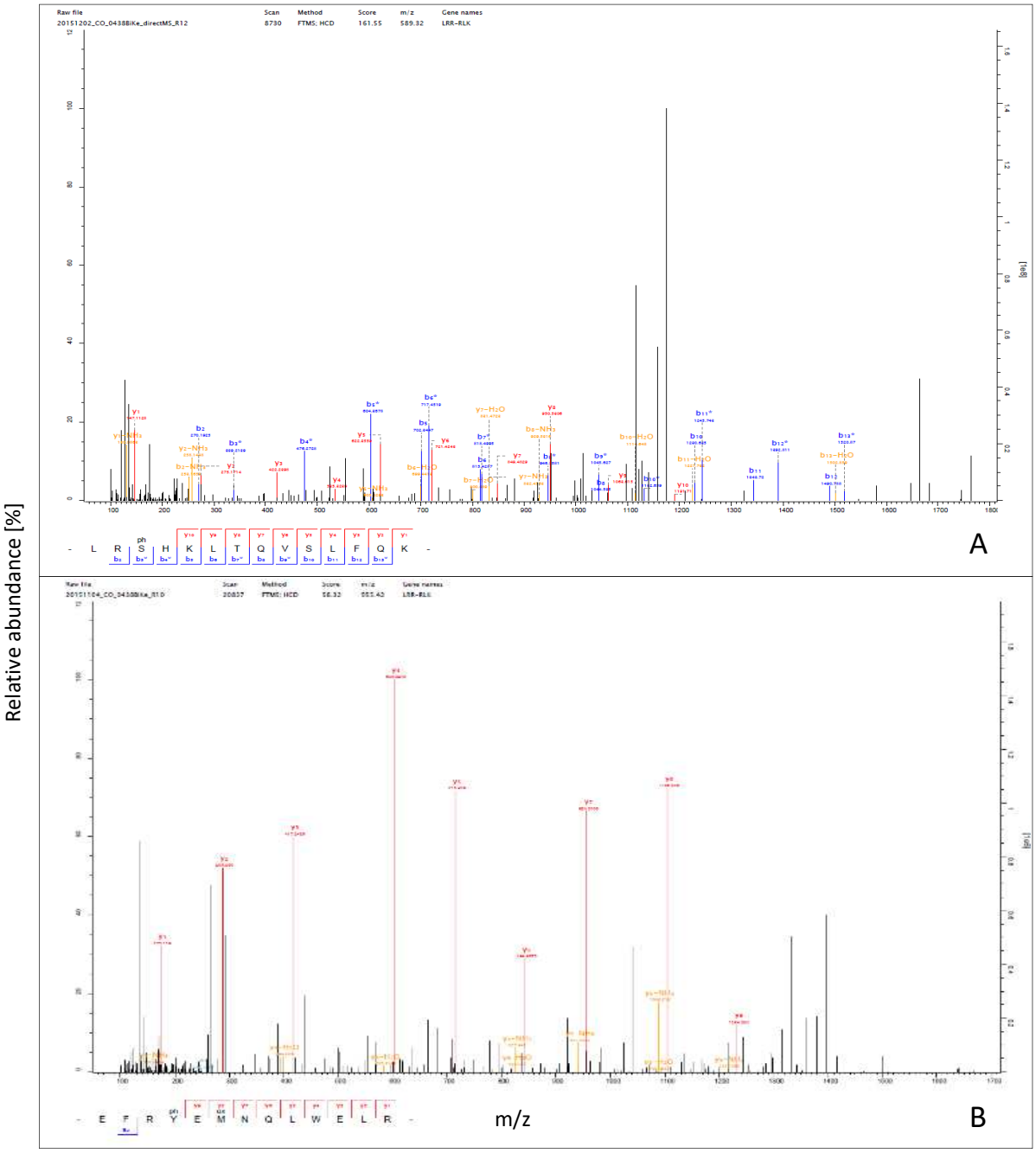


**Figure 8-2: MS-spectra of two identified phosphopeptides of BIR2 in the juxtamembrane and kinase domain.**
**A:** Spectra of BIR2 peptides containing phosphorylated S279 and in **B:** phosphorylated Y349. Mass spectra are given in abundance intensity (%) vs. mass to charge (m/z).

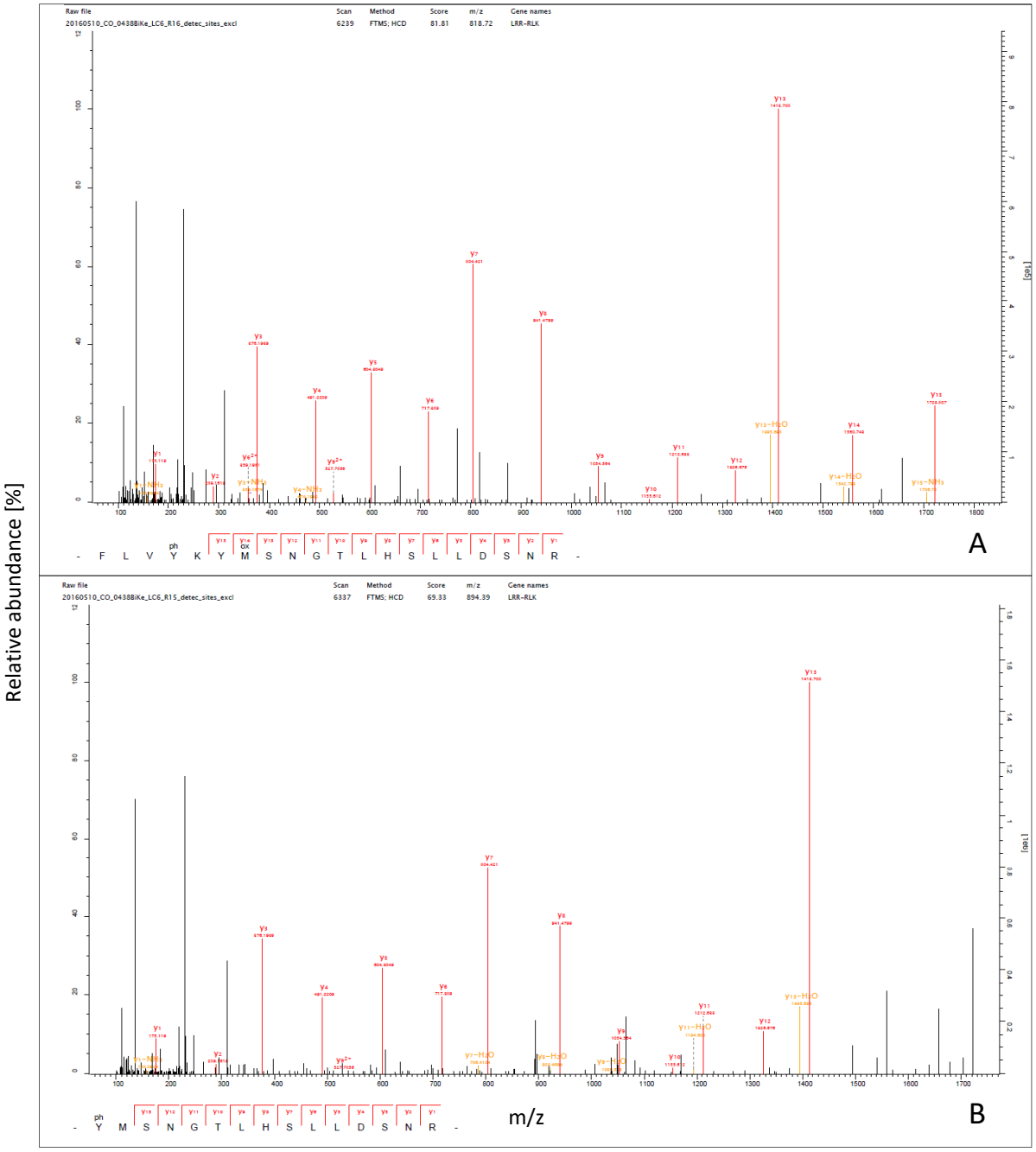


**Figure 8-3: MS-spectra of two identified phosphopeptides of BIR2 in the kinase domain.**
**A:** Spectra of BIR2 peptides containing phosphorylated Y379 and in **B:** phosphorylated Y381. Mass spectra are given in relative abundance (%) vs. mass to charge (m/z).

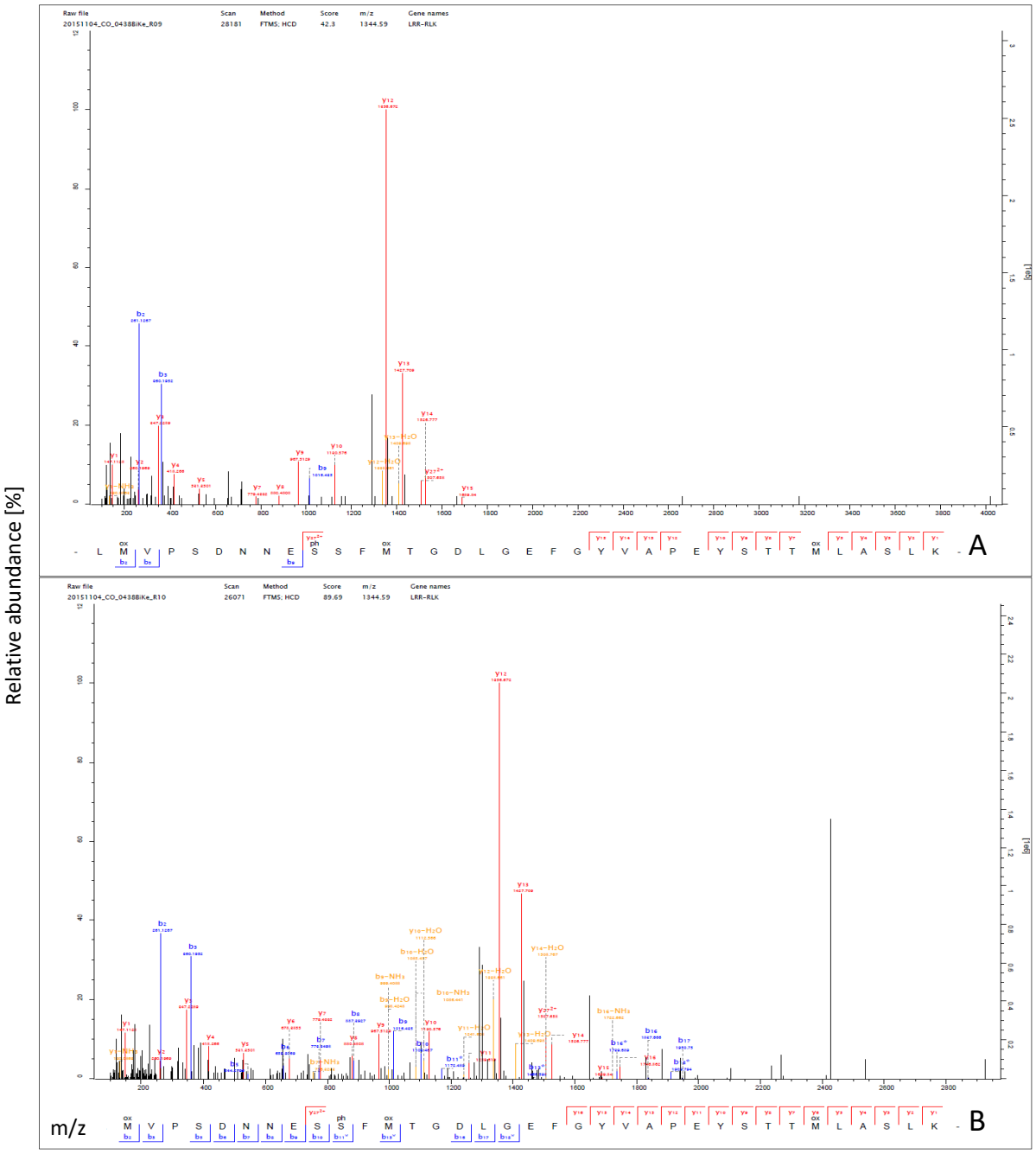


**Figure 8-4: MS-spectra of two identified phosphopeptides of BIR2 in the kinase domain**.
**A:** Spectra of BIR2 peptides containing phosphorylated S462 and in **B:** phosphorylated S463. Mass spectra are given in relative abundance (%) vs. mass to charge (m/z).

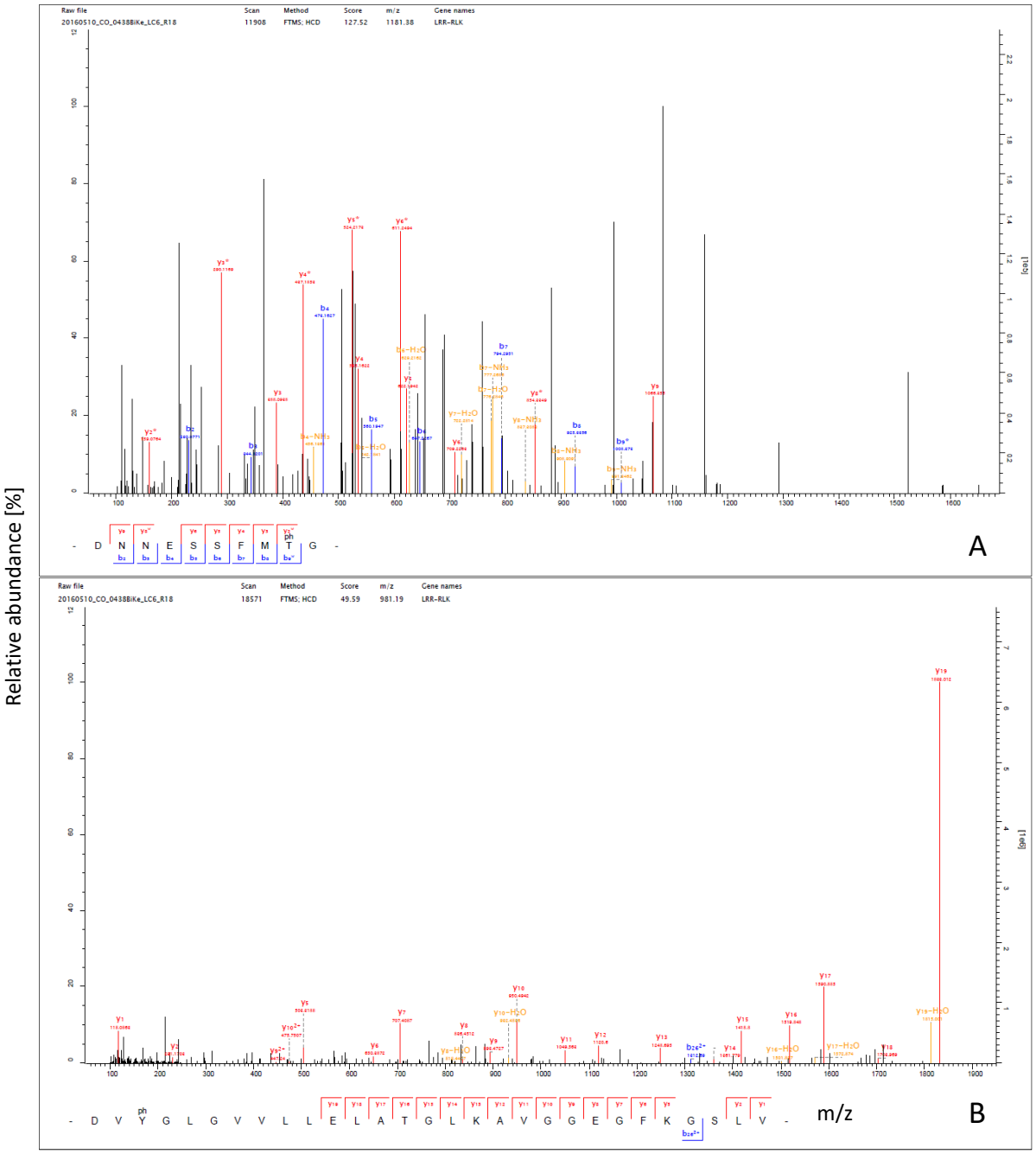


**Figure 8-5: MS-spectra of two identified phosphopeptides of BIR2 in the kinase domain.**
**A:** Spectra of BIR2 peptides containing phosphorylated T466 and in **B:** phosphorylated Y492. Mass spectra are given in relative abundance (%) vs. mass to charge (m/z).

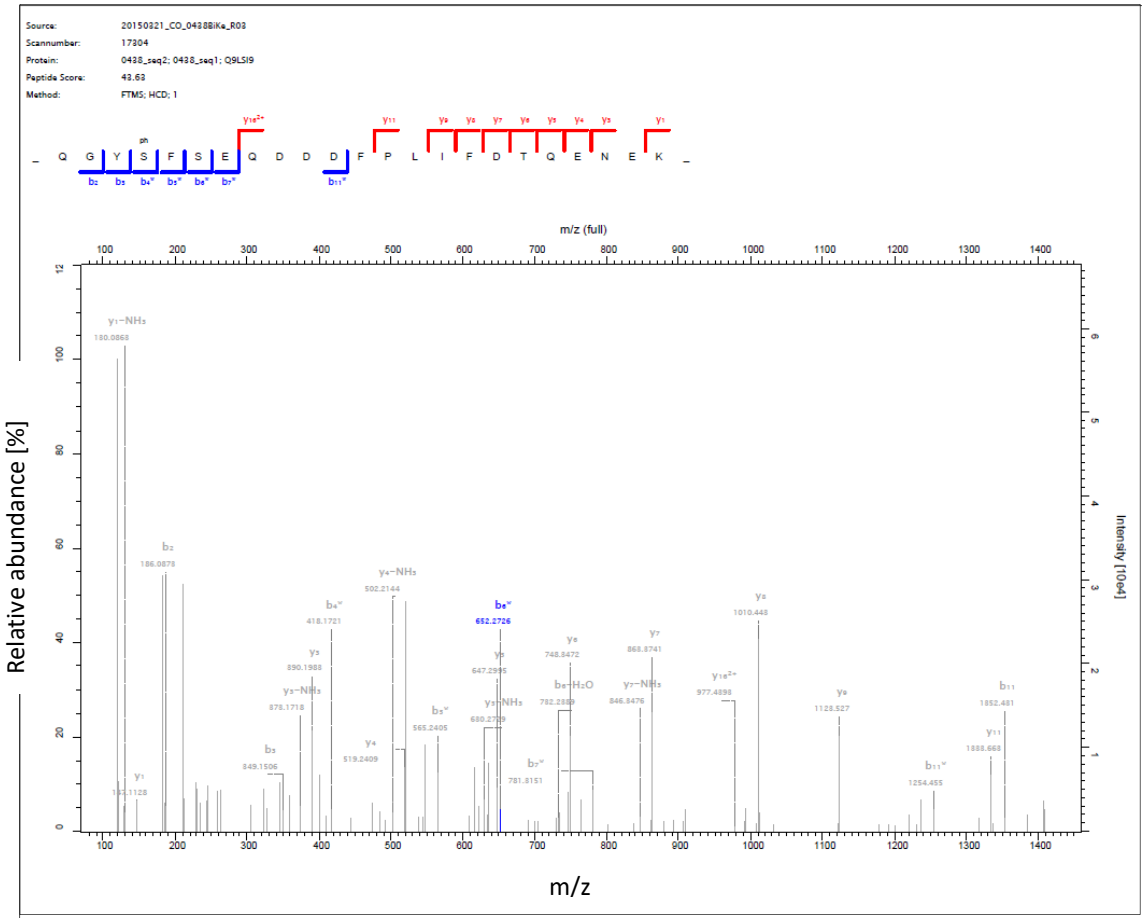


**Figure 8-6: MS-spectrum of one identified phosphopeptides of BIR2 in the C-terminus.**
**A:** Spectrum of BIR2 peptide containing phosphorylated S585. Mass spectra are given in relative abundance (%) vs. mass to charge (m/z).

**Table 8-1: Summary of identified *in vitro* and *in vivo* phosphorylation sites of BIR2.**
Total overview of P-sites out of untreated and treated experiments and literature screen for potential P-sites of BIR2. *In vitro* data are generated in the PhD work of Dr. Sara Mazzotta. Literature is screened for potential P-sites of BIR2. Numbers in brackets indicating no significant values (related to PEP and localization values, not shown). Juxtamembrane= JM, kinase domain= KD and C-terminus= CT.

| Domain | P-site | Number of identification | | Reference |
|---|---|---|---|---|
| | | *in vitro* (BIR2-KD + BAK1-KD in *E. coli*) | *in vivo* (35S::BIR2-YFP in *Arabidopsis*) | |
| JM | S263 | 2 | 20 | Benschop et al., 2007; Blaum et al., 2014; Nakagami et al., 2010; Roitinger et al., 2015; Sugiyama et al., 2008 |
| | T266 | 2 | 10 | Benschop et al., 2007; Blaum et al., 2014 |
| | S271 | 2 | 1 | Blaum et al., 2014 |
| | S279 | | 7 | |
| | T283 | 2 | | Blaum et al., 2014 |
| | S286 | 2 | 15 | Blaum et al., 2014 |
| | T304 | 2 | | Blaum et al., 2014 |
| KD | S330 | 2 | | Blaum et al., 2014 |
| | Y349 | | 2 | |
| | Y379 | | 2(1) | |
| | Y381 | | 1(2) | |
| | Y389 | 1 | | Blaum et al., 2014 |
| | S448 | 2 | | Blaum et al., 2014 |
| | S462 | 2 | (4) | Blaum et al., 2014 |
| | S463 | 1 | (7) | |
| | T466 | 2 | 1(3) | Blaum et al., 2014 |
| | Y492 | | 2 | |
| | T533 | 1 | | Blaum et al., 2014 |
| CT | S585 | | 3(1) | Nühse et al., 2004; Benschop et al., 2007; Dr. Andrea Gust (personal communication) |
| | S587 | | 2 | Nühse et al., 2004; Benschop et al. 2007 |

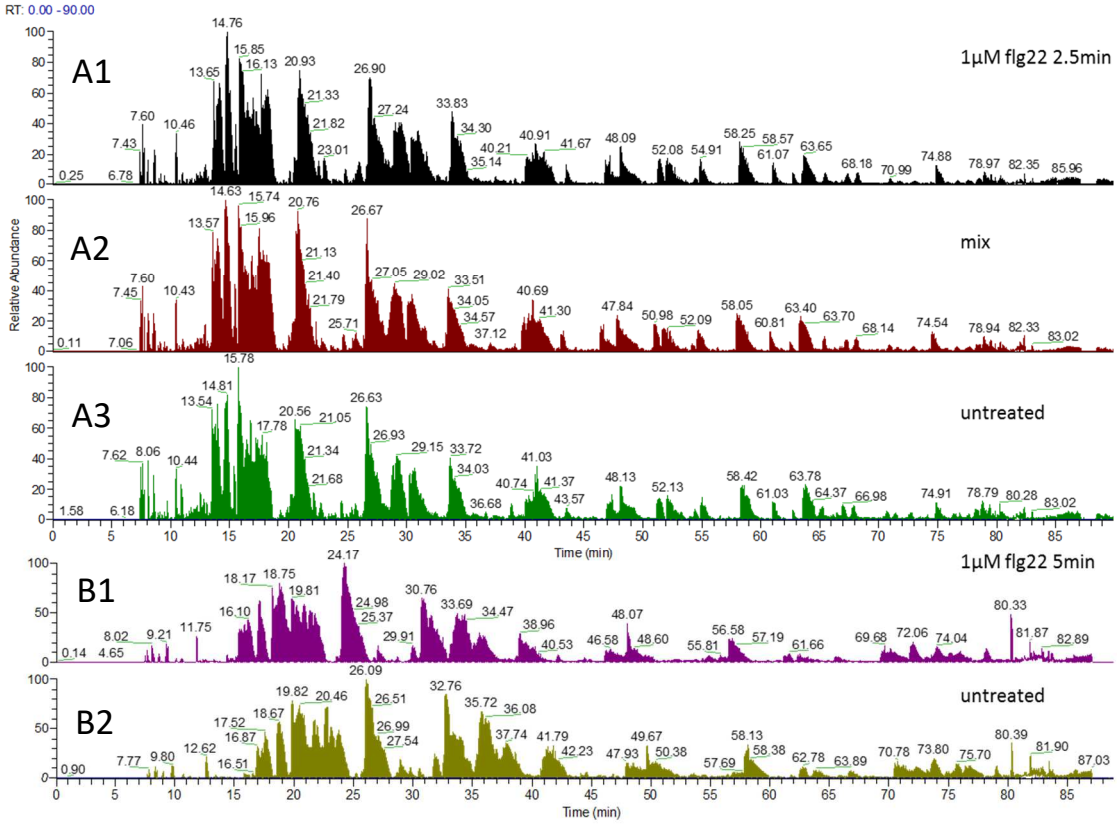


**Figure 8-7: Chromatograms of MS analyses representing the base peaks. Equal patterns between untreated and treated samples allow a comparison of intensities of detected phosphopeptides.**
**A1-3:** Chromatograms of untreated (**A3**) versus 2.5 min 1 µM flg22 (**A1**) and/or mix (1 µM flg22, elf18, pep1 (5 min) and 10 nM BL (90 min)) treated material (**A2**). **B1-2:** Chromatograms of untreated (**B1**) versus 5 min 1 µM flg22 (**B2**) treated samples. Shown chromatograms represent examples of each treatment. Graphs given in relative abundance (%) vs. time (min).

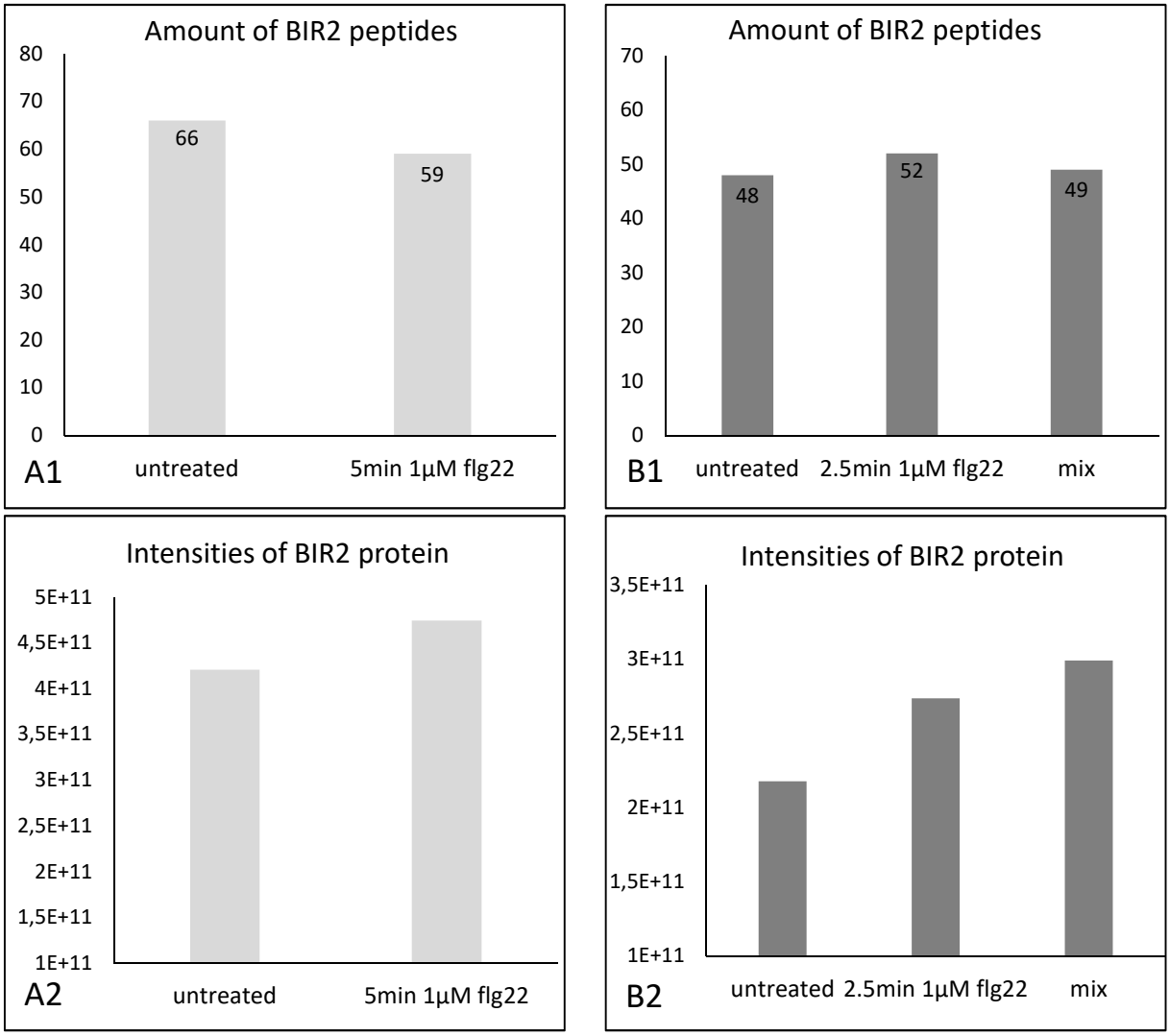


**Figure 8-8: Total amount of detected BIR2 peptides, and corresponding intensities of the protein. Similar values between untreated and treated samples allow a comparison of intensities of detected phosphopeptides.**

**A1-2:** Comparison untreated versus 5 min 1 µM flg22 treated samples in their total amount of BIR2 peptides (**A1**) and total BIR2 protein intensities (**A2**). **B1-2:** Comparison untreated versus 2.5 min 1 µM flg22 and/or mix (1 µM flg22, elf18, pep1 (5 min) and 10 nM BL (90 min)) treated material in their total amount of BIR2 peptides (**B1**) and total BIR2 protein intensities (**B2**). Shown values represent examples of each treatment.

**Table 8-2: Overview of functional and interaction analyses of potential BIR2 P-sites.**
Outcome of independent T2 plants of investigated P-sites which are summed up in Table 3-5. * Only mutated intracellular part of BIR2 is investigated in a Y2H. (+) indicating phenotypes comparable to Ws-0 levels and interaction ability to BAK1, respectively. (-) indicating phenotypes comparable to *bir2-2,* and no interaction to BAK1 detectable. (+/-) partial complementation, and weak interaction stability to BAK1. (++) over-complementation phenotypes. n.d. (not determined).

| | Functional analyses | | | Interaction assays | |
|---|---|---|---|---|---|
| Investigated P-sites in *bir2-2* background T2 generation | Growth phenotype | Ros burst | *Alternaria* symptoms | Y2H | Co-IP (IP@BAK1) |
| BIR2-myc #1 | + | + | + | + | + |
| BIR2-myc #2 | + | ++ | + | + | ++ |
| | | | | | |
| S263A-myc #1 | + | +/- | + | ++ | + |
| S263A-myc #3 | + | + | + | ++ | n.d. |
| S263A-myc #5 | + | + | + | ++ | n.d. |
| S263D-myc #1 | + (bigger) | + | ++ | ++ | + |
| S263D-myc #4 | + (bigger) | +/- | + | ++ | n.d. |
| | | | | | |
| T266A* | n.d. | n.d. | n.d. | ++ | n.d. |
| T266D* | n.d. | n.d. | n.d. | + | n.d. |
| | | | | | |
| S271A* | n.d. | n.d. | n.d. | + | n.d. |
| S271D* | n.d. | n.d. | n.d. | +/- | n.d. |
| | | | | | |
| S263A/T266A/S271A-myc #1 | + | + | + | ++ | + |
| S263D/T266D/S271D-myc #1 | + | + | + | + | + |
| | | | | | |
| S286A-myc #2 | + | ++ | + | +/- | + |
| S286A-myc #5 | + (bigger) | + | ++ | +/- | n.d. |
| S286A-myc #6 | + (bigger) | + | +/- | +/- | n.d. |
| S286D-myc #3 | + (bigger) | ++ | + | + | + |
| S286D-myc #4 | + (bigger) | + | n.d. | + | n.d. |
| S286D-myc #5 | + (bigger) | ++ | +/- | + | n.d. |
| | | | | | |
| T304A-myc #1 | ++ (OE-like) | ++ | + | ++ | + |
| T304A-myc #2 | + | n.d. | + | ++ | n.d. |
| T304D-myc #1 | + | - | + | - | +/- |
| T304D-myc #2 | + | - | + | - | n.d. |
| | | | | | |
| BIR2 #1 | ++ (OE-like) | + | + | + | + |
| | | | | | |
| S448A #1 | + | + | - | + | n.d. |
| S448A #2 | ++ (OE-like) | + | + | + | + |
| S448D #1 | + | + | + | - | + |
| S448D #2 | + | +/- | - | - | n.d. |

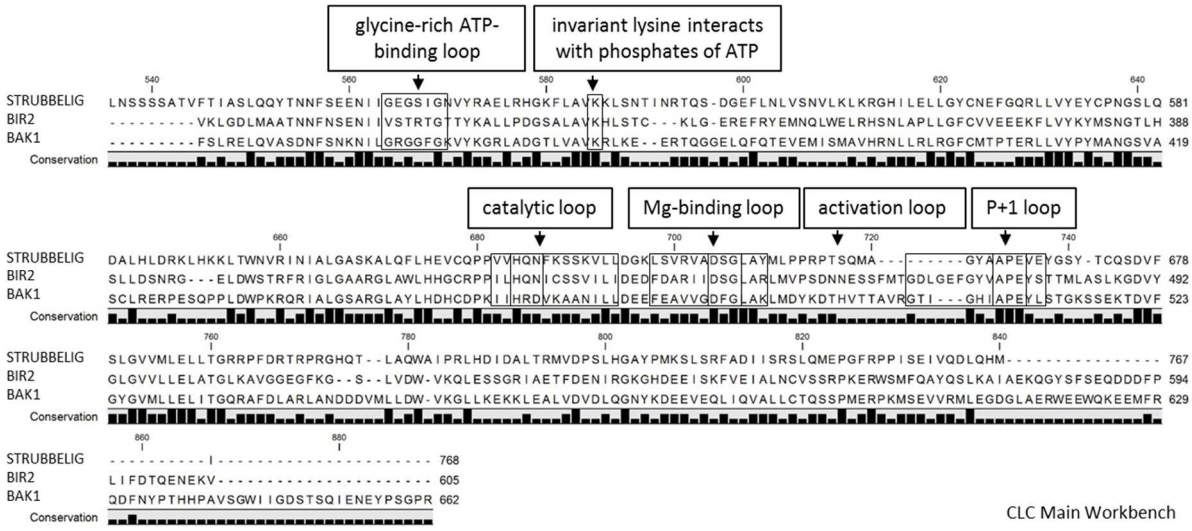


**Figure 8-9: Alignment of amino acid sequences of the intracellular parts of SUB, BIR2 and BAK1.**
BAK1 as an active RD-kinase, and BIR2 and SUB as inactive non-RD kinases. In boxes important features and motifs of kinase activity are labeled. Alignment created in CLC Main Workbench.

# 9. Appendix

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