



Identification and characterization of a novel PAMP  
from a widespread microbial virulence factor and its  
perception system in *Arabidopsis*

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Hannah Undine Böhm  
aus Friedrichshafen

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1. Berichterstatter:	Prof. Dr. Thorsten Nürnberger
2. Berichterstatter:	Prof. Dr. Georg Felix
3. Berichterstatter:	Prof. Dr. Ralph Hückelhoven

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# 1. Introduction

## 1.1 Concepts of Immunity

Immunity describes a state in which an organism holds enough defense mechanisms to protect itself from unwanted biological invasion, for example through harmful microorganisms. This principle is a quite ancient and concomitant phenomenon of life, even unicellular organisms like bacteria and archaea feature enzymatic systems protecting them against viruses and plasmids (Horvath and Barrangou 2010).

Plants, animals and other organisms continuously need to deal with invading pathogens as potential disease-causing agents. Fortunately, different barriers prevent pathogen intrusion most commonly. Constitutively, physical (body surface and mucosal epithelia, epidermis and cuticle) and chemical (antimicrobial secondary compounds) barriers constitute a first hindrance for the pathogen. However, most of the host defense mechanisms are inducible through specific recognition of the infectious microorganism (Medzhitov and Janeway 2000). Vertebrates rely on two main branches of immunity, termed innate and adaptive immunity. Innate immunity is the more ancient one, providing germline-encoded and preformed receptors for pattern perception of common microbial structures referred to as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) (Janeway and Medzhitov 2002, Dempsey *et al.* 2003). PAMPs are often highly conserved molecular structures found across whole classes of microbes which feature an essential function for the microorganism's lifestyle and cannot be found in the host. Thus, recognition of these patterns by pattern recognition receptors (PRRs) allows the host to distinguish between non-infectious self and infectious non-self (Medzhitov and Janeway 2002).

Besides, endogenous molecules passively released through cellular damage like injury or actively secreted by living cells under stress can be sensed by PRRs as well and hereby induce immune responses (Matzinger 1994, Bianchi 2007). Analogous to the nomenclature of PAMPs or MAMPs these molecules are designated as DAMPs,

or damage-associated molecular patterns. Exemplary DAMPs are the High Mobility Group Box 1 (HMGB1), uric acid, DNA or ATP (Venereau *et al.* 2015).

The adaptive immune system is evolutionarily younger and only a trait of the *Gnathostomata*. It provides specific recognition of foreign antigens and an immunological memory and relies on clonal expansion of antigen-specific effector cells selected for by gene rearrangement (Janeway and Medzhitov 2002, Dempsey, *et al.* 2003). This enables the production of an unlimited number of highly variable receptor molecules. However, in the event of a first infection effector cells get activated comparatively slowly, and defects during somatic selection might lead to allergies and autoimmune disorders.

Innate and adaptive immunity evolved independently, albeit adaptive immunity is only stimulated once a pathogen is recognized by the innate immune system (Medzhitov and Janeway 1998). Innate immunity is the more ancient and prevalent form of immunity in plants, fungi, insects and primitive multicellular organisms and can be mounted by each cell autonomously (Litman *et al.* 2005, Pancer and Cooper 2006). Animals and plants feature similar components of innate immunity, suggesting a convergent evolution of this type of immunity in the two lineages (Nürnbergger *et al.* 2004, Ausubel 2005, Zipfel and Felix 2005, Staal and Dixelius 2007).

PRRs of animals and plants can be either membrane-bound for extracellular MAMP-recognition or localized intracellularly. In the animal system, two cytosolic receptor classes can be distinguished. NOD (nucleotide-binding and oligomerization domain)-like receptors (NLRs) contain a central nucleotide-binding oligomerization domain (NOD), a C-terminal LRR domain and variable N-terminal interaction domains like the CARD (caspase recruitment)-domain, the PYR (pyrin)-domain or baculovirus inhibitor repeats (BIRs) and are necessary for the regulation of inflammatory and apoptotic responses (Franchi *et al.* 2009, Barbe *et al.* 2014, Motta *et al.* 2015). Another class, the RIG I (retinoic acid inducible gene I)-like receptors sense viral RNA and consist of an N-terminal CARD-domain followed by a RNA helicase domain (Creagh and O'Neill 2006, Meylan *et al.* 2006). In plants, NBS-LRRs (nucleotide-binding site leucine-rich repeat) are the cytoplasmic equivalents to animal NLRs but differ regarding the structure of the N-terminal domains. Instead of a CARD, PYR or BIR domain NBS-LRRs possess a TIR (Toll/interleukin-1 receptor) or a coiled-coil (CC) domain (DeYoung and Innes 2006, Jones and Dangl 2006, Rosenstiel *et al.* 2008).

C-type lectin receptors (CLRs) can be soluble or membrane-bound. CLRs can be classified into subtypes according to their function and structure and many recognize their ligands through the  $\text{Ca}^{2+}$ -dependent carbohydrate recognition domains (CRDs). In general these receptors serve two purposes; mediating cell-cell contact and acting as PRRs. Some CLRs induce signaling that directly activate nuclear factor-kappaB (NF- $\kappa$ B), others effect signaling mediated by Toll-like receptors (TLRs) (Cambi and Figdor 2003, Geijtenbeek and Gringhuis 2009). TLRs are a class of membrane-bound PRRs of animals, named after the first characterized member from *Drosophila melanogaster*, TOLL, which was shown to have a role in the fly's innate immunity (Lemaitre *et al.* 1996). TLRs consist of an extracellular leucine-rich domain (LRR) for ligand binding, a class 1 transmembrane domain and a cytoplasmic TIR-domain (Gay and Gangloff 2007). Upon ligand binding, dimerization with adaptor proteins like MyD88 (Myeloid differentiation primary response gene 88) and subsequent recruitment of kinases like IRAK (Interleukin-1 receptor-associated kinase) leads to signal transduction and activation of immunity (Akira and Takeda 2004). To date, 10 (human) and 12 (mouse) TLRs are identified, with high conservation of TLR1-TLR9 (Kawai and Akira 2009). Various MAMPs of different classes are recognized by TLRs like lipids, lipoproteins, proteins, cell wall fragments or nucleic acids (Jin and Lee 2008, Kawai and Akira 2009, O'Neill *et al.* 2013) thus covering a broad spectrum of pathogens like parasites, bacteria, fungi and viruses (Akira *et al.* 2006). TLR9 for example recognizes bacterial CpG-DNA and parasitic malaria hemozoin, TLR7 viral ssRNA, lipopolysaccharide (LPS) of gram-negative bacteria is perceived by TLR4 and TLR2 recognizes PGN (peptidoglycan) from gram-positive bacteria (Kawai and Akira 2009). TLR5 was identified as the receptor for flagellin, a highly conserved PAMP from bacterial flagella (Hayashi *et al.* 2001). Also plants are able to perceive flagellin by a receptor with an extracellular LRR domain, the receptor-like kinase (LRR-RK) FLS2 (flagellin-sensing 2) (Gomez-Gomez *et al.* 1999, Gomez-Gomez and Boller 2000). Instead of the TIR domain of TLRs, FLS2 and other plant LRR-RKs feature an intracellular kinase domain. Actually, the only characteristic TLR5 and FLS2 do have in common, is the extracellular LRR domain suggesting an independent evolution of the flagellin perception system in plants and animals. Supportive for this hypothesis is the fact that TLR5 and FLS2 recognize conserved epitopes of flagellin which are structurally distinct from each other (Felix *et al.* 1999, Smith *et al.* 2003).

## 1.2 Plant innate immunity

Plants rely on only one tier of immunity, the innate immune system. Plants need to deal with the biotic challenge of invading pathogens like viroids, viruses, bacteria, fungi and oomycetes. Physical barriers constitute a first line of defense like a rigid cell wall and a wax-containing hydrophobic cuticle. Chemical barriers like antimicrobial compounds or enzymes form another constitutive line of defense (VanEtten *et al.* 1994, Heath 2000, Dixon 2001, Reina-Pinto and Yephremov 2009, Piasecka *et al.* 2015). Once pathogens manage to overcome these constitutive barriers, they are confronted with the plants inducible innate immune system. In a first instance, invading pathogens are recognized through their PAMPs by membrane-bound plant PRRs leading to the activation of plant immunity called PTI (PAMP-triggered immunity). Similarly, PTI is activated through PRR-mediated recognition of DAMPs. Thus, PTI is a form of basal resistance effective against non-adapted pathogens called non-host resistance and limited in effectiveness against host-adapted microbes of susceptible cultivars (Dodds and Rathjen 2010, Böhm *et al.* 2014a). However, successful pathogens secrete effectors into host cells which promote virulence and suppress PTI, thereby establishing a state called ETS (effector-triggered susceptibility) (Jones and Dangl 2006, Win *et al.* 2012). As a counter measure, plants evolved mechanisms to detect these effectors or effector-mediated host-manipulation in a co-evolutionary arms race. NBS-LRRs are the main class of intracellular resistance proteins (R-proteins) sensing effectors and thereby activating immunity (Dodds and Rathjen 2010, Elmore *et al.* 2011). This kind of robust immune activation is called effector-triggered immunity or ETI and is proposed to be a faster, stronger and more prolonged version of PTI that often culminates in a hypersensitive response (HR) restricting pathogen growth (Tao *et al.* 2003, Jones and Dangl 2006, Tsuda *et al.* 2009, Tsuda and Katagiri 2010, Dou and Zhou 2012, Cui *et al.* 2015). However, this discrimination of ETI and PTI was recently challenged since several effectors and PAMPs do not match the common classification (Thomma *et al.* 2011, Shibuya and Desaki 2015). For example, some effectors are quite conserved and elicit defense responses, while some PAMPs are narrowly distributed or contribute to pathogen virulence. Also, some PAMPs elicit a quite strong defense response resulting in an HR, whereas some effectors are rather weak elicitors (Bailey *et al.* 1990, Wei *et al.* 1992, Khatib *et al.* 2004, Ron and Avni 2004, Wirthmueller *et al.* 2007, Thomma, *et al.* 2011, Schwessinger and Ronald 2012).

Classification by division into intracellular NBS-LRR proteins mediating ETI and extracellular PRRs mediating PTI is also blurred, since effector recognition not only occurs intracellularly but also at the plasma membrane. As such, there is rather a continuum between ETI and PTI than a precise distinction.

Membrane depolarization is the earliest measurable response upon PAMP perception. Increased influx of  $\text{Ca}^{2+}$  and  $\text{H}^+$  and simultaneous efflux of  $\text{K}^+$ ,  $\text{Cl}^-$  and  $\text{NO}_3^-$  within the first minutes lead to an extracellular alkalization (Jabs *et al.* 1997, Zimmermann *et al.* 1997, Wendehenne *et al.* 2002, Garcia-Brugger *et al.* 2006). Calcium-dependent protein kinases (CDPKs) sense rapid intracellular changes of  $\text{Ca}^{2+}$ -concentrations and mediate further signaling steps (Blume *et al.* 2000, Hrabak *et al.* 2003, Ludwig *et al.* 2004, Romeis and Herde 2014). The production of reactive oxygen species (ROS) constitutes another early response. ROS feature antimicrobial effects, act as signaling components and fortify the cell wall by mediating crosslinking events (Bradley *et al.* 1992, Apel and Hirt 2004, Torres *et al.* 2006). Activation of MAP kinases (mitogen-activated protein kinases) and phosphorylation cascades lead to regulation of transcription factors and defense gene expression (Zhang and Klessig 2001, Asai *et al.* 2002, Jonak *et al.* 2002, Colcombet and Hirt 2008). Thus, transcriptional reprogramming leads for example to regulation of the phytohormone cross-talk between jasmonic acid (JA), salicylic acid (SA) and ethylene production (Feys and Parker 2000, Kunkel and Brooks 2002). Hereby, SA-signaling mainly mediates responses against biotrophic pathogens and is necessary to establish systemic acquired resistance (SAR), whereas JA and ethylene mediate signaling to fight necrotrophic pathogens (Glazebrook 2005). Moreover, antimicrobial substances like phytoalexins are produced (Tsuji *et al.* 1992, Mao *et al.* 2011). Stomata constitute an entry point for pathogens, which need to reach the plant apoplast to proliferate. Upon PAMP perception, stomatal closure takes place in a manner dependent on abscisic acid, SA,  $\text{K}^+$ -fluxes and heterotrimeric G-proteins (Melotto *et al.* 2006, Zhang *et al.* 2008). Furthermore, enzymes like chitinases or glucanases directly inhibit fungal growth by destroying fungal cell wall components (Mauch *et al.* 1988, Joosten and De Wit 1989, van den Burg *et al.* 2006, van Loon *et al.* 2006). Deposition of callose, a  $\beta$ -1,3-glucan polymer, between the plasma membrane and the cell wall is a late response physically reinforcing the leaf and thereby insulating the plant from invading pathogens (Ellinger and Voigt 2014).

## 1.3 Molecular principles of plant-microbe interaction

Plants need to ward off a multitude of pathogens including bacteria, oomycetes and fungi. As sessile organisms, plants need to induce a prompt defense response upon threat by potential disease-causing pathogens and unlike animals, plants lack specialized immune cells. Therefore, plants evolved sophisticated perception systems and regulatory mechanisms to initiate appropriate defense responses.

### 1.3.1 Plant-Bacteria

Plant pathogenic bacteria cause many diseases in plants all over the world. Recently, the top 10 of pathogenic bacteria were nominated mainly based on economic but also on scientific importance (Mansfield *et al.* 2012). *Pseudomonas syringae* pathovars get to the first place, including pv. *tomato*, causing bacterial speck on tomato (Shenge *et al.* 2007) or pv. *aesculi* inducing bleeding-canker on horse-chestnut (Green *et al.* 2010). 28 different pathovars are described in the *European Handbook of Plant Diseases*, each causing disease on a different host. *Ralstonia solanacearum* was rated second, because it is probably the most destructive plant pathogenic bacterium worldwide. Many strains exist varying in their host range, geographical distribution and pathogenic behavior and these are responsible for potato brown rot, bacterial wilt of tomato, eggplant and tobacco as well as Moko disease of banana (Denny 2006, Genin 2010). In the third place, *Agrobacterium tumefaciens* was listed. This is mainly due to its scientific importance through identification of the Ti-plasmid as a vector for directed gene transfer into plants. However, *Agrobacterium* causes also disease on economically important crops as the agent for crown gall tumors. *Xanthomonas* species come to the fourth, fifth and sixth place constituting a severe threat for many crop plants. *Xanthomonas oryzae* pv. *oryzae* causes bacterial leaf blight (BLB) on rice, *Xanthomonas campestris* pathovars like pv. *campestris* cause black rot on crucifers affecting all cultivated brassicas, pv. *euvesicatoria* induces bacterial spot on pepper and tomato, pv. *malvacearum* now *X. axonopodis* pv. *malvacearum* angular leaf spot of cotton and *Xanthomonas axonopodis* pv. *manihotis* causes CBB (cassava bacterial blight) on cassava plants. Seventhly, *Erwinia amylovora* is nominated causing fire blight disease of apple, pear, quince, raspberry, blackberry and many cultivated and

wild ornamentals. At place eight comes *Xylella fastidiosa*, the causal agent of grapevine Pierce's disease, citrus variegated chlorosis and almond leaf scorch disease. Two *Dickeya* species make up position nine, *Dickeya dadantii* and *Dickeya solani* causing potato tuber rot. Last but not least, *Pectobacterium carotovorum* and *P. atrosepticum* come to place 10 of the most important plant pathogenic bacteria, causing soft rot disease in several crop plants and blackleg disease in potato respectively.

Most of the bacteria possess structures for locomotion called flagella, which are made up by the protein flagellin. In plants, recognition of flagellated bacterial invaders is mediated by the LRR-RK FLS2, which binds a conserved 22 amino acid stretch close to the N-terminus of flagellin, called flg22 (Felix, *et al.* 1999, Chinchilla *et al.* 2006). FLS2 was first identified in *Arabidopsis*, but most higher plants are able to sense flg22 and functional homologs have been identified in several other higher plants like in tomato (*Solanum lycopersicum*), rice (*Oryza sativa*), grapevine (*Vitis vinifera*) and *Nicotiana Benthamiana* (Hann and Rathjen 2007, Robatzek *et al.* 2007, Takai *et al.* 2008, Trda *et al.* 2014). Interestingly, different plant species differ in ligand specificities. For example, *Vitis vinifera* FLS2 does not recognize flg22 derived from *Burkholderia phytofirmans* which can be sensed by *Arabidopsis* FLS2 instead (Trda, *et al.* 2014). Likewise, plants seem to be able to perceive multiple epitopes within flagellin. Several pathovars of *Pseudomonas syringae* harbor another immunogenic epitope in this region, referred to as flgII-28. This epitope is not the agonist of FLS2 but several members of the Solanaceae family are able to sense it. Results from a genomic field study suggest that this immunogenic pattern might be under selective pressure to avoid recognition by tomato (Cai *et al.* 2011, Clarke *et al.* 2013).

Upon binding of flg22, the LRR-RK SERK3/BAK1 (somatic embryogenesis receptor 3/BRI1-associated kinase 1) is recruited into a complex with FLS2 (Chinchilla *et al.* 2007, Heese *et al.* 2007). BAK1 consists of a small extracellular LRR domain with 5 repeats followed by a serine and proline rich region (SPP motif), a transmembrane domain and a cytoplasmic kinase domain and is member of the SERK protein family together with its four closest homologs (Chinchilla *et al.* 2009). BAK1 acts as a co-receptor for multiple LRR-RLKs, such as the brassinosteroid (BR) receptor BRI1 (brassinosteroid insensitive 1). Also other SERK protein family members were found to interact with BRI1, like SERK1 and SERK4/BKK1 (BAK1-like kinase 1) (Kinoshita *et al.* 2005). BRI1 exists as homodimers at the plasma membrane and binding of BRs

results in transphosphorylation and dimer stabilization, hetero-oligomerization with BAK1 and activation of BR-signaling (Wang *et al.* 2005a, Wang *et al.* 2005b) Additionally, BAK1 functions in the containment of cell death and light signaling (Whippo and Hangarter 2005, Kemmerling *et al.* 2007).

FLS2 and most probably also BAK1 constitutively interact with soluble receptor-like cytoplasmic kinases (RLCKs) BIK1 (*Botrytis*-induced kinase 1) and the paralogous proteins PBS1, PBL1 (PBS1-like kinase 1) and PBL2 which have a positive regulatory function in PTI (Lu *et al.* 2010, Zhang *et al.* 2010, Monaghan and Zipfel 2012). BAK1 phosphorylates BIK1 upon flg22 binding, and BIK1 subsequently transphosphorylates FLS2 and BAK1. Flg22 binding induces association of BAK1 and FLS2 and the fully activated BAK1-FLS2 complex may further phosphorylate BIK1 and other substrates. BIK1 most likely dissociates from the complex to induce further downstream signaling (Lu, *et al.* 2010). Recently, the NADPH oxidase *At*RBOHD was identified as a direct phosphorylation target of BIK1 (Kadota *et al.* 2014). Another RLCK with positive regulatory function in PTI was lately identified as BSK1 (BR-signaling kinase 1), previously known for functioning as a positive regulator in BR-signaling (Tang *et al.* 2008). BSK1 is associated with FLS2, partially dissociates after binding of flg22 and is required for flg22-mediated signaling except for MAP kinase activation (Shi *et al.* 2013).

Phosphatases instead, can act as negative regulatory components in PTI signaling. The protein phosphatase 2C (PP2C) KAPP (kinase-associated protein-phosphatase) was found to physically interact with the kinase domain of FLS2 and to negatively regulate flg22-triggered responses (Gomez-Gomez *et al.* 2001). Further negative regulatory function in flg22-mediated signaling was assigned to the E3 ligases PUB12 (plant U-Box 12) and PUB13. These molecules are present in a constitutive complex with BAK1, get recruited into a complex with FLS2 upon flg22 treatment and are phosphorylated by BAK1 (Lu *et al.* 2011). FLS2 gets then polyubiquitinated by PUB12/13, endocytosed and degraded. Thus, the cells are desensitized and continuous signaling is prevented by ligand-induced receptor degradation (Smith *et al.* 2014). The BAK1-interacting receptor-like kinase 2, referred to as BIR2, is a LRR-RK constitutively interacting with BAK1 and functions as a negative regulator of PTI by preventing interaction of BAK1 with FLS2. Upon perception of flg22, BIR2 is released from BAK1 and enables FLS2 to recruit BAK1 into the signaling complex (Halter *et al.* 2014).



Crystallization of FLS2 and BAK1 ectodomains in complex with flg22 revealed that the ligand mainly binds on the concave surface of FLS LRRs 3-16 (Sun *et al.* 2013). FLS2 and BAK1 directly interact at FLS2 LRRs 18-20/23-26 and BAK1 LRRs 1-5. One glycine residue of C-terminal flg22 solely binds to BAK1 without contacting FLS2, hence acting as a 'molecular glue' between receptor and co-receptor. (Sun, *et al.* 2013). Heterodimerization of FLS2 and BAK1 is necessary for full signaling triggered by flg22 (Chinchilla, *et al.* 2007). Interestingly, a flg22-induced association of FLS2 with other SERK protein family members could be shown. FLS2 not only dimerizes with BAK1, but to the same extent also with SERK1 and SERK4/BKK1. In fact, BKK1 is necessary for flg22-induced signaling, suggesting that BAK1 and BKK1 complement each other to achieve full signaling strength (Roux *et al.* 2011).

The most abundant bacterial protein is the highly conserved Ef-Tu (elongation factor thermo unstable), one of the prokaryotic elongation factors functioning in the ribosomal translation machinery. In *Arabidopsis*, the N-acetylated epitope elf18 (the first 18 amino acids of Ef-Tu) is recognized by EFR (Ef-Tu receptor) (Kunze *et al.* 2004, Zipfel *et al.* 2006). EFR is a LRR-RK belonging to the same clade of LRR-RK genes (clade XII) as does FLS2 (Shiu and Bleecker 2001, Shiu *et al.* 2004) and both are considered to function similarly. Indeed, both recruit BAK1 and other members of the SERK family proteins upon ligand binding and induce a similar set of defense responses (Roux, *et al.* 2011, Greeff *et al.* 2012). Interestingly, Ef-Tu is perceived by *Arabidopsis* and various members of the *Brassicaceae* family but not by other plant families tested (Kunze, *et al.* 2004, Boller and Felix 2009). Similar to the observations made for flg22 perception systems of *Solanaceous* species, rice is able to perceive a different epitope within Ef-Tu than elf18. This pattern is located in the central region of Ef-Tu, is 50 amino acids long and is referred to as EFa50 (Furukawa *et al.* 2014).

The major constituent of cell walls of Gram-positive and also of Gram-negative bacteria is peptidoglycan (PGN), composed of alternating  $\beta(1-4)$ -linked N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) residues (Schleifer and Kandler 1972, Glauner *et al.* 1988). Binding of PGN in *Arabidopsis* is mediated by two LysM-RPs (lysin motif receptor proteins), LYM1 (lysin motif domain-containing glycosylphosphatidylinositol-anchored protein 1) and LYM3 (Willmann *et al.* 2011). Plant lysin-motif domain proteins consist of an ectopic LysM-domain, a transmembrane domain and a cytoplasmic kinase domain (LysM-RKs or LYKs) or in the case of LysM-RPs (or LYPs) which lack the kinase domain, a GPI-anchor attaching them to the

plasma membrane. These receptors are implicated in symbiosis and immunity by recognizing GlcNAc-containing ligands such as PGN, bacterial nodulation factors (NF) and fungal chitin (Gust *et al.* 2012). Likewise, LysM-RPs OsLYP4 and OsLYP6 mediate PGN perception in rice (Liu *et al.* 2012a). PGN-induced signaling in *Arabidopsis* is dependent on a third LysM protein, the LysM-RK CERK1 (chitin elicitor receptor kinase 1), albeit it does not directly bind PGN (Willmann, *et al.* 2011).

Lipopolysaccharides (LPSs) are glucoconjugates of the outer membrane of Gram-negative bacteria and composed of a hydrophobic lipid section referred to as lipid A, connecting to the core oligosaccharide by a sugar. The oligosaccharide contains some sugars and ends in oligosaccharide repeats called O-antigen (Raetz and Whitfield 2002). LPS is a potent inducer of PTI in various plant species (Silipo *et al.* 2010, Newman *et al.* 2013). Recently, the receptor for *Pseudomonas sp.*- and *Xanthomonas campestris*-derived LPS was identified in *Arabidopsis* as LORE (Lipooligosaccharide-specific reduced elicitation), a bulb-type (B-type) lectin S-domain (SD)-1 RLK, also known as SD1-29 (Ranf *et al.* 2015). Chemical degradation of *Pseudomonas species* LPS revealed that LORE mainly detects the lipid A moiety.

Sulfated RaxX (required for activation of Xa21 X), a protein recently identified in *Xanthomonas oryzae* pv. *oryzae*, is highly conserved in many plant pathogenic *Xanthomonas* species and required for Xa21-mediated immunity (Pruitt *et al.* 2015).

Flg22, Ef-Tu, PGN and LPS are generic examples for highly conserved molecular structures of bacteria, for which *Arabidopsis* and other higher plants evolved perception systems to ward off potential disease-causing agents. Orphan bacterial PAMPs for which the perception system still needs to be identified are for example cold-shock proteins (csp15) (Felix and Boller 2003) and bacterial DNA (Yakushiji *et al.* 2009). Also, some PRRs are identified for which the specific ligand remains enigmatic like for the rice LRR-RK Xa26 which confers resistance to *Xanthomonas oryzae* pv. *oryzae* (Sun *et al.* 2004). In *Arabidopsis*, the PAMP eMax (enigmatic MAMP of *Xanthomonas*) from *Xanthomonas axonopodis* pv. *citri* and other *Xanthomonas* species is recognized by RLP1/ReMAX (receptor of eMax) together with the LRR-RLK SOBIR1/EVR (suppressor of BIR1-1/evershed) (Jehle *et al.* 2013a, Jehle *et al.* 2013b).

### 1.3.2 Plant-Fungi, Plant-Oomycetes

Not only bacterial but also fungal and oomycete plant pathogens constitute a threat for many crops and are responsible for regular crop shortfalls. *Phytophthora infestans* for example is the causal agent of potato blight and accountable for the Great Irish Famine between 1845 and 1852 where about 1 million people died and a million more emigrated from Ireland. To date, *Phytophthora* is still a major constraint to potato production and was nominated for position one in the list of the 10 most important oomycete plant pathogens (Kamoun *et al.* 2015). Oomycetes superficially resemble filamentous fungi, but phylogenetically they are related to diatoms and brown algae in the stramenopiles (Thines 2014). They contain cellulose and glucans instead of chitin as the main cell wall polymer (Bartnicki-Garcia 1968). *Hyaloperonospora arabidopsidis* is a downy mildew within the *Peronosporaceae* and ranked for the second place. Downy mildews cause harmful diseases on many crops like on brassica crops, grape (*Plasmopara viticola*, place 6), cucurbits, lettuce (*Bremia lactucae*), sorghum and maize. On the third, fourth, fifth, seventh and eighth place five *Phytophthora* species are placed; *P. ramorum* (sudden oak/larch death, ramorum blight), *P. sojae* (stem and root rot disease on soy bean), *P. capsici* (blight, stem and fruit rot on pepper and tomato, lima and snap beans and cucurbits), *P. cinnamomi* (fruit rot, dieback, broad host spectrum), *P. parasitica* (root, crown and stem rot, citrus, herbaceous, solanaceous crops). *Pythium ultimum* is on position 9, causing damping off and root rot on more than 300 hosts including corn, wheat and soy bean. On place 10 comes *Albugo candida*, a white rust responsible for significant crop losses of Indian mustard in India, Canada and Australia.

The list for the 10 most important fungal plant pathogens (Dean *et al.* 2012) starts with *Magnaporthe oryzae*, due to its economic importance as the causal agent of rice blast disease, the most destructive rice disease worldwide. Grey mold, caused by the necrotroph *Botrytis cinerea* is listed next. It causes severe damage pre- and post-harvest and has a broad host range. Thirdly, *Puccinia spp.* are placed infecting wheat (*P. graminis* (stem/black rust), *P. striiformis f. sp. tritici* (stripe/yellow rust) and *P. triticina* (leaf/brown rust)). On the fourth and fifth place are two *Fusarium* species, *F. graminearum* being highly destructive on all cereal species, and *F. oxysporum* causing vascular wilt on a wide range of plants such as tomato, cotton and banana. *Blumeria graminis* (position 6) and *Mycosphaerella graminicola* (place 7) both cause severe

damage on cereals. Eighthly, *Colletotrichum spp.* is placed as one of the most important plant pathogenic fungi causing anthracnose spots, post-harvest rots and blight. Virtually every crop (fruit, vegetable) is susceptible to at least one *Colletotrichum* species. *Ustilago maydis* and *Melampsora lini* fill the last positions, rather for scientific than economic reasons.

The major component of fungal cell walls is chitin, a polysaccharide consisting of  $\beta(1-4)$ -linked N-acetylglucosamine (GlcNAc) residues. Chitin represents a highly conserved fungal molecular structure for which plants evolved perception systems to sense and to fend off invading fungi (Shibuya and Minami 2001, Wan *et al.* 2008a). In rice, chitin is perceived by the LysM-RP *OsCEBiP* (Chitin elicitor-binding protein) (Kaku *et al.* 2006). Upon chitin binding, *OsCEBiP* homodimerizes and forms a complex with *OsCERK1* (Chitin elicitor receptor kinase 1) (Shimizu *et al.* 2010, Hayafune *et al.* 2014). To form this sandwich-type receptor system and for optimum induction of PTI, seven to eight N-acetylated GlcNAc units are required. One ligand molecule is sandwiched between two *OsCEBiP* molecules, anchoring the GlcNAc units within central LysM motifs of *OsCEBiP*. Upon *OsCEBiP* homodimerization, *OsCERK1* dimers are proposed to be recruited forming a  $\alpha_2\beta_2$  tetramer (Hayafune, *et al.* 2014). In *Arabidopsis*, the LysM-RK *AtLYK5* was identified as the main chitin receptor quite recently, whereas *AtLYK4* shows some functional redundancy (Wan *et al.* 2012, Cao *et al.* 2014). *AtLYK1/AtCERK1* associates with *AtLYK5* in a chitin-dependent manner, *AtLYK5* binds chitin with a much higher affinity than *AtCERK1* and phosphorylation and homodimerization of *AtCERK1* is dependent on *AtLYK5* what refutes previous findings presenting *AtCERK1* as the main chitin receptor (Miya *et al.* 2007, Wan *et al.* 2008b, Iizasa *et al.* 2010, Petutschnig *et al.* 2010, Liu *et al.* 2012b, Cao, *et al.* 2014). The kinase domain of *AtLYK5* is not enzymatically active, but seems to function in protein-protein interaction with *AtCERK1*. As such, *AtLYK5* seems to play a similar role as *OsCEBiP* as chitin-binding receptor recruiting CERK1 proteins to mediate downstream signaling. *AtLYK5* exists as a homodimer independent of the presence of chitin and *AtCERK1*, but whether a heterotetramer of *AtLYK5* and *AtCERK1* proteins is formed comparable to the chitin  $\alpha_2\beta_2$  signaling complex in rice remains elusive (Cao, *et al.* 2014). *AtLYM2*, a homolog of *OsCEBiP*, is enriched in plasmodesmata, binds to chitin and mediates antifungal immunity. This happens independently of *AtCERK1*, suggesting two independent chitin-induced response ways in *Arabidopsis* (Miya, *et al.* 2007, Shinya *et al.* 2012, Faulkner *et al.* 2013).

Besides chitin, several other fungal PAMPs and their corresponding PRRs have been identified. Tomato evolved an array of Cf-genes, LRR-RPs conferring resistance to *Cladosporium fulvum*. Cf-proteins recognize fungal effectors by direct binding or in complex with host proteins (Wulff *et al.* 2009, Thomma, *et al.* 2011). All Cf-proteins interact with the LRR-RK SOBIR1 (Liebrand *et al.* 2013). Fungal  $\beta$ -1-4 endoxylanase (EIX, ethylene-inducing xylanase), a highly purified 22 kDa protein from *Trichoderma viride*, binds to tomato (*Solanum lycopersicum*) LRR-RPs *S/EIX1* and *S/EIX2* (Dean and Anderson 1991, Ron and Avni 2004). *S/EIX2* mediates signaling upon endocytosis and interacts with *S/SOBIR1* (Ron and Avni 2004, Liebrand, *et al.* 2013). BAK1 directly interacts with *S/EIX1*, attenuating plant responses mediated by *S/EIX2* (Bar *et al.* 2010). Another LRR-RP of tomato, *S/Me1* recognizes Ave1 (avirulence on Ve1 tomato) and governs resistance to *Verticillium* race 1 strains of *V. dahliae* and *V. albo-atrum* together with *S/BAK1* and *S/SOBIR1* (Fradin *et al.* 2009, de Jonge *et al.* 2012, Liebrand, *et al.* 2013). Ave1 is an effector protein consisting of 134 aa and a potent virulence factor of *Verticillium*. It exhibits homology to plant natriuretic peptides and to proteins from one bacterial and three other fungal pathogens, suggesting that Ave1 was horizontally acquired from plants (de Jonge, *et al.* 2012). In *Arabidopsis*, the LRR-RP RLP42/RBPG1 (Responsiveness to botrytis polygalacturonases 1) recognizes fungal endopolygalacturonases from the necrotroph *Botrytis cinerea* and interacts with SOBIR1 to mediate downstream signaling (Zhang *et al.* 2014).

Also, oomycete-derived PAMPs and their corresponding PRRs have been identified. The  $\beta$ -glucan binding protein GBP from soybean (*Glycine max*) binds *Phytophthora sojae*-derived 1,6- $\beta$ -linked and 1,3- $\beta$ -branched heptaglycoside (HG), but the final signaling process and possible interactors still need to be uncovered (Fliegmann *et al.* 2004). Recently, the LRR-RP ELR (Elicitin response) from the wild potato *Solanum microdontum* was identified to mediate recognition of elicitin, a conserved pattern from *Phytophthora* species (Du *et al.* 2015b). ELR associates with BAK1 and leads to enhanced resistance against *Phytophthora infestans* upon transfer into cultivated potato.

Moreover, some LRRs have been identified recognizing fungal patterns for which the specific immunogenic epitope remains to be characterized. The elicitor SCFE1 (*Sclerotinia* culture filtrate elicitor 1), partially purified from the necrotrophic fungus *Sclerotinia sclerotiorum*, triggers typical immune responses in *Arabidopsis*. Signaling is mediated through the LRR-RP RLP30 and the LRR-RKs SOBIR1 and BAK1 (Zhang

*et al.* 2013). The wheat (*Triticum aestivum*) LRR-RP *TaRLP1.1* mediates resistance to stripe rust disease *Puccinia striiformis* f. sp. *tritici* as well as the LRR-RKs *TaRLKs* R1-3 (Zhou *et al.* 2007, Jiang *et al.* 2013). Wheat resistance against *Puccinia recondita*, the causal agent of leaf rust disease, is mediated by *TaRLK10* (Feuillet *et al.* 1997) and in rice, the B-lectin-type LRR-RK *OsPi-d2* is involved in resistance against *Magnaporthe grisea* (Chen *et al.* 2006).

Ergosterol is a fungal-derived orphan PAMP for which the perception system still needs to be elucidated (Klempner *et al.* 2014). Also, oomycete PAMPs have been identified, for which the PRRs are not found yet like arachidonic acid (Bostock *et al.* 1982, Dedyukhina *et al.* 2014), Pep-13, a surface-exposed fragment within cell-wall transglutaminase (TGase) from *Phytophthora* species (Nürnberg *et al.* 1994, Brunner *et al.* 2002) and *Phytophthora* cellulose-binding elicitor lectin (CBEL) (Gaulin *et al.* 2006, Larroque *et al.* 2013).

### 1.3.3 Plant-Plant/ DAMPs

Besides the ability to sense signals of danger derived from non self-representing microbial PAMPs, plants can also sense self-derived molecules originated from pathogen recognition or tissue damage. *Arabidopsis* PEP1, a 23-aa peptide from the precursor PROPEP1 is an immune stimulating DAMP which is perceived by the LRR-RKs PEPR1 and PEPR2 thereby interacting with BAK1 and BIK1 (Huffaker *et al.* 2006, Yamaguchi *et al.* 2006, Postel *et al.* 2010, Yamaguchi *et al.* 2010, Liu *et al.* 2013). Wounding, pathogen challenge, PAMP treatment and the defense hormones jasmonate, ethylene and salicylate transcriptionally induce expression of PROPEP1 and its six paralogs, coupling local and systemic immunity (Ross *et al.* 2014). *ZmPEP1*, an *AtPEP1* ortholog has been characterized in maize and orthologs from several other species seem to have a regulatory function in antiherbivore defense (Huffaker *et al.* 2011, Huffaker *et al.* 2013). Another peptide, the 18-aa systemin, was first identified in the leaves of wounded tomato as the primary signal for systemic activation (Pearce *et al.* 1991) and is perceived by the tomato LRR-RK SR160 (Scheer and Ryan 2002). Cell wall integrity in plants is maintained by the pectic polysaccharide homogalacturonan (HGA). During fungal infection, fragments thereof named oligogalacturonides (OGs) are released, which induce immunity and act as DAMPs. In *Arabidopsis*, OGs are perceived by the EGF (epidermal growth factor)-motif containing

LRR-RK WAK1 (Brutus *et al.* 2010). ATP is another molecule released upon cell damage playing an essential role as DAMP in mammals, perceived by P2-type purinoceptors. AtDORN1 (does not respond to nucleotides 1), a lectin receptor kinase 1.9 was identified to bind extracellular ATP in *Arabidopsis*, mediating calcium-responses, activation of MAP kinases and gene expression (Choi *et al.* 2014). As such, DORN1 is the founding member of the new plant-specific purinoceptor subfamily P2K (P2 receptor kinase). Further plant DAMPs for which the perception system is not identified yet are NAD(P)H, DNA, sucrose, green leaf volatiles and various peptides (Tanaka *et al.* 2014).

#### **1.3.4 ETI and directed manipulation of PTI by bacteria, fungi and oomycetes**

Recognition of microbial PAMPs or plant-derived DAMPs leads to plant immune activation and PTI. Successful pathogens evolved effectors to suppress PTI and to establish ETS, thus enabling infection. Effectors are defined as secreted molecules altering host cell processes or structures which promote the pathogen's lifestyle. These molecules do not only feature immune suppressing function, but also might enhance access to vital nutrients (Win, *et al.* 2012). Effectors are either delivered at the interface of the host and the pathogen as apoplastic effectors or inside the cell as cytoplasmic effectors. Upon delivery, effectors traffic to different compartments and bind to their host targets. In susceptible plants, effector binding leads to host manipulation, suppression of immune responses and colonization by the pathogen. Resistant genotypes recognize effectors via surface PRRs or intracellular NBS-LRR proteins, trigger immunity and ward off the pathogen. Thus, effectors can have a positive or negative effect (as virulence or avirulence factor respectively) on the fitness of the pathogen and are as such among the most rapidly evolving genes of plant-associated microbes (Win, *et al.* 2012).

Bacterial type 3 effectors are delivered directly into to cytoplasm by a type III secretion system (T3SS) (Deslandes and Rivas 2012). AvrPto from *Pseudomonas syringae* pv.*tomato* JL1065 is a kinase inhibitor and interacts for example with the PRRs BAK1, FLS2 and EFR (Shan *et al.* 2008, Xiang *et al.* 2008), AvrPtoB from *Pseudomonas syringae* pv.*tomato* DC3000 mimics a U3 ubiquitin ligase and binds to

FLS2, BAK1 and CERK1 (Gohre *et al.* 2008, Shan, *et al.* 2008, Gimenez-Ibanez *et al.* 2009). HopF2, another effector from *Pst*DC3000 interacts with BAK1, BIK1 and some homologs and is able to attenuate multiple MAP kinases, like MKK5 through its mono-ADP-ribosyltransferase activity (Wang *et al.* 2010, Zhou *et al.* 2014). AvrPto, AvrPtoB and HopF2 are all effectors interacting with RIN4 (RPM1-interacting protein 4), a plasma membrane-anchored protein with negative regulatory function in PTI (Deslandes and Rivas 2012). RIN4 is exemplary for the 'guard'-hypothesis, the indirect perception of effectors through surveillance of their targets. AvrRPM1 and AvrB from *P. syringae* pv. *glycinea* phosphorylate RIN4 leading to activation of RPM1 (Mackey *et al.* 2002). TAL (transcription activator-like) effectors of *Xanthomonas* species and *Ralstonia solanacearum* are DNA-binding proteins (Scholze and Boch 2011). By mimicking eukaryotic transcription factors TAL effectors bind to promoter regions and activate transcription of certain host genes that are beneficial for the pathogen. PthXo1 for example induces the expression of *OsSWEET11*, a sugar transporter supposed to mediate sugar efflux to feed the bacteria (Chen *et al.* 2010).

Unlike bacteria, fungi and oomycetes do not feature a T3SS for effector delivery into the cell. Oomycetes and fungal germ tubes form appressoria, which directly pierce the cuticle and cell wall by enzymatic softening and mechanical force (Dou and Zhou 2012). Through invagination of the host plasma membrane, haustoria are formed which deliver effectors into the apoplast, some of which are further translocated inside the cell. Fungal LysM-type effectors like ECP6 from *Cladosporium fulvum* or Slp1 from *Magnaporthe oryzae* are apoplastic effectors, competing with chitin receptors for chitin binding (de Jonge *et al.* 2010, Mentlak *et al.* 2012). Effectors can also target hormone signaling, as does Cmu1, a cytoplasmic chorismate dismutase produced by *Ustilago maydis*. Cmu1 reduces chorismate levels, a precursor molecule for salicylic acid biosynthesis (Djamei *et al.* 2011).

Oomycete RXLR effectors are cytoplasmic effectors characterized by their highly invariant sequence RXLR, serving as translocation signal into the cell. Avr3a for example is a RXLR effector from *Phytophthora infestans* interacting with CMPG1, an E3 ubiquitin ligase which negatively regulates HR (Bos *et al.* 2010). Another class of oomycete effectors are the Crinklers or crinkling and necrosis (CRN) effectors, featuring a LXLFLAK motif for translocation into the host cell (Schornack *et al.* 2010). CRN proteins localize to and target nuclear compartments and some trigger cell death (Schornack, *et al.* 2010, Stam *et al.* 2013). Apoplastic oomycete effectors include



glucanase and protease inhibitors, which interfere with cell wall-degrading proteins produced by the host (Kamoun 2006). Next to hydrolytic proteins like proteases or glycanases, hemibiotrophic and necrotrophic oomycetes utilize toxins like PcF proteins to enhance invasion success (Wawra *et al.* 2012).

### 1.3.5 Phytotoxins

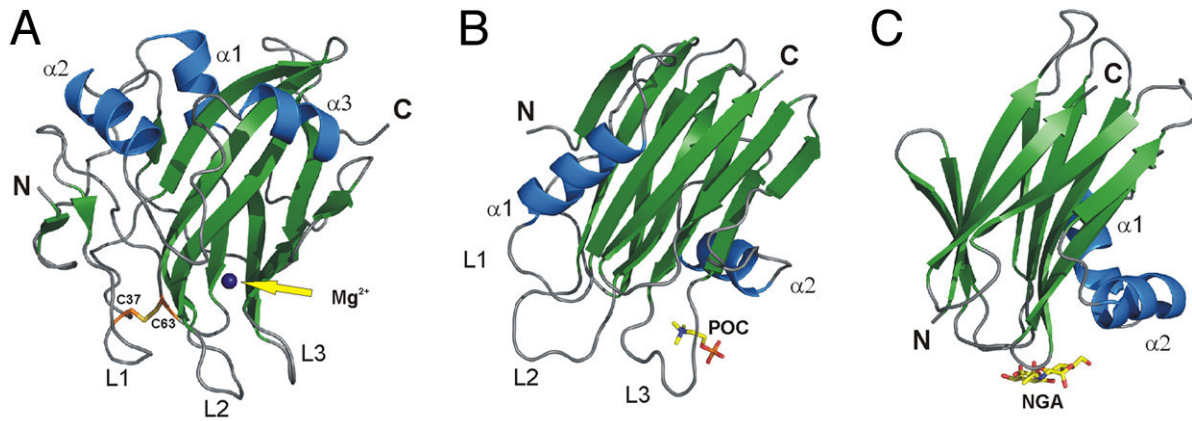
Phytotoxins are produced by pathogens to directly destroy plant cells or manipulate biochemical metabolic processes, leading to beneficial conditions for pathogen proliferation (Bender *et al.* 1999). Toxins can be classified into low-molecular toxins like terpenoids, polyketides and peptides and into high-molecular-weight molecules like proteins. Host-selective toxins (HSTs) produced by *Alternaria alternata* have diverse target sites in the cell such as the plasma membrane (AK-, AF-, ACT- toxin), the chloroplast (AM-toxin), mitochondria (AT-, ACR-toxin) and the endoplasmic reticulum (AAL-toxin) (Tsuge *et al.* 2013), suggesting selective binding of HSTs to host receptors. Receptors for HSTs could be identified including ToxABP1 (ToxA binding protein 1) from wheat which recognizes Ptr ToxA from *Pyrenophora tritici-repentis* (Manning *et al.* 2007), maize URF13 binding *Cochliobolus heterostrophus* T-toxin (Dewey *et al.* 1988) and oat VBP (victorin binding protein) the receptor for victorin produced by *Cochliobolus victoriae* (Wolpert and Macko 1989).

Coronatine is a non-HST chlorosis-inducing polyketide produced by *Pseudomonas syringae* with a molecular structure resembling methyl jasmonate and is involved in stomatal re-opening (Melotto *et al.* 2008). Oxalic acid is secreted by various phytopathogenic fungi and presumably promotes disease on various hosts by sequestering Ca<sup>2+</sup>, favouring polygalacturonase activity and occluding xylem vessels through calcium oxalate crystal formation leading to wilt (Ferrar and Walker 1993). Another non-HST protein family is the NEP1 (necrosis and ethylene-inducing peptide 1)-like or NLP protein family, that comprises conserved virulence factors which can be found in bacteria, oomycetes and fungi causing cytolysis by plasma membrane disruption (Ottmann *et al.* 2009).

## 1.4 NEP1-like proteins

The first characterized member of the NEP1-like protein family was isolated from *Fusarium oxysporum* culture filtrates and named after its ability to induce necrosis and ethylene in dicot plant species (Bailey 1995). NLPs are secreted proteins with an approximate size of 24-26 kDa exhibiting a high degree of sequence conservation. Several NLPs have been identified and characterized so far, secreted by various oomycetes like *Phytophthora* species (Fellbrich *et al.* 2002, Qutob *et al.* 2002, Feng *et al.* 2014), *Pythium aphanidermatum* (Veit *et al.* 2001), *Hyaloperonospora arabidopsidis* (Cabral *et al.* 2012), the bacterium *Pectobacterium carotovorum* (Mattinen *et al.* 2004) and the fungi *Botrytis cinerea* (Schouten *et al.* 2008), *Moniliophthora perniciosa* (Garcia *et al.* 2007), *Mycosphaerella graminicola* (Motteram *et al.* 2009), *Sclerotinia sclerotiorum* (Dallal Bashi *et al.* 2010) and *Verticillium dahliae* (Zhou *et al.* 2012).

Structural analysis of an NLP from *Pythium aphanidermatum* revealed similarities to pore-forming toxins from marine invertebrates called actinoporins, such as equinatoxin II from *Actinia equina* and sticholysin produced by *Stichodactyla helianthus* (Küfner *et al.* 2009, Ottmann, *et al.* 2009). Also, structural similarities to fungal lectins like XCL from *Xerocomus chrysenteron* and ABL from *Agaricus bisporus* could be found (Fig. 1-1). The crystal structure of *Pya*NLP revealed a single-domain molecule with a central  $\beta$ -sandwich, one sheet with three strands and an antiparallel second sheet with 5 strands (Figure 1-1 A). At the top of the sandwich are three helices, and three broad loops form the base (L1-L3). L1 is anchored to the central sheet core by two cysteine residues building a disulfide bridge. Above L2/L3 a negatively charged cavity binds a divalent cation involving residues D93, D104, E106, H159 and H101 stabilizing the water network (Ottmann, *et al.* 2009). A groove at the bottom of lectins and actinoporins targets carbohydrates and sphingomyelin respectively (Mancheño *et al.* 2003, Birck *et al.* 2004, Bakrač *et al.* 2008), suggesting that also the groove of *Pya*NLP might target specific components of the plasma membrane. The negatively charged cavity of *Pya*NLP contains the hepta-peptide motif GHRHDWE, which is highly conserved among NLPs. Mutational analysis of *Pya*NLP revealed that residues H101, D104 and E106 within the hepta-peptide motif and residue D93 are crucial for cytotoxic activity and for the coordination of the ion inside the cavity (Ottmann, *et al.* 2009).



**Figure 1-1: 3D structures of PyaNLP, the actinoporin sticholysin and the fungal lectin ABL.**

Ribbon plot of *Pya*NLP (A), the actinoporin sticholysin (B) and the fungal lectin ABL (C). Helices are shown in blue,  $\beta$ -strands in green. The bound magnesium atom is shown as a blue sphere (A). Adopted from Ottmann, *et al.* (2009).

Previously, the presence of either two or four conserved cysteine residues classified NLPs as type 1 or type 2 NLPs respectively (Gijzen and Nurnberger 2006). More recently, phylogenetic analysis revealed a third type which only occurs in fungi (Oome and Van den Ackerveken 2014). Moreover, a non-toxic subgroup of type 1 NLPs was identified (type 1a), which lack the cation-binding pocket required for cytotoxicity. Also the classification by the number of disulfide bridges seems to be blurred, since members of type 1 and type 2 NLPs harbor additional disulfide bridges, most probably serving stabilization purposes upon secretion (Oome and Van den Ackerveken 2014).

NLPs are virulence-promoting toxins, as overexpression of *Fo*NLP in a hypovirulent strain of *Colletotrichum coccodes* resulted in increased virulence towards *Abutilon theophrasti* (Amsellem *et al.* 2002). Virulence-promoting function could also be assigned to bacterial *Pcc*NLP, and oomycete-derived *Pya*NLP and *Pp*NLP, restoring aggressiveness of a *Pcc* *nlp*-deficient *Pectobacterium carotovorum* strain (Ottmann, *et al.* 2009). In contrast, no virulence-promoting function could be shown for *Myg*NLP, a NLP from the monocot-specific pathogen *Mycosphaerella graminicola*. A *Myg* *nlp*-deficient strain neither lost aggressiveness in infection analysis on susceptible wheat cultivars, nor gained virulence under overexpression conditions (Motteram, *et al.* 2009). Purified *Myg*NLP is able to exert toxin activity on the dicot *Arabidopsis* but not on its host wheat, although it is highly expressed during onset of the necrotrophic stage (Motteram, *et al.* 2009). Similarly, *Be*NLP1 and *Be*NLP2 from *Botrytis elliptica* do neither induce necrosis on lily nor are these NLPs essential virulence factors during lily

infection (Staats *et al.* 2007). Moreover, disrupted expression of FoNLP in *Fusarium oxysporum* f. sp. *erythroxyli* did not result in reduced aggressiveness on *Erythroxylum coca* (Bailey *et al.* 2002).

Interestingly, a growing number of secreted NLPs can be classified as non-toxic, giving rise to new questions concerning the functionality of this protein family. Especially oomycetes encode a large number of NLPs like *Phytophthora infestans* encoding for almost 70 NLPs, including non-toxic ones. In *Phytophthora sojae*, only eight out of 19 representative NLPs were found to be toxic whereas the rest lacked necrosis-inducing activity (Dong *et al.* 2012). Similarly, only two out of seven *Verticillium dahliae* NLPs exhibited necrotic activity (Santhanam *et al.* 2013). More importantly, the obligate biotroph *Hyaloperonospora arabidopsidis* encodes 12 NLP genes, 10 of which were tested for necrosis-inducing activity and shown to be non-toxic (Cabral, *et al.* 2012). NLP expression profiling of hemibiotrophic pathogens revealed an upregulation of non-toxic NLPs in early infection stages like in germinating cysts, whereas toxic NLPs are expressed during transition from biotrophic to necrotrophic infection stages (Qutob, *et al.* 2002, Kanneganti *et al.* 2006, Santhanam, *et al.* 2013). This suggests an additional role for NLPs apart from their function as virulence factors in hemibiotrophic and necrotrophic pathogens operating in early infection stages. Expansion, pseudogenization, positive selection, varying expression patterns and existence of non-toxic versions suggests that NLPs have diversified in function most possibly due to different lifestyles of the pathogens, and these alternative roles remain to be elucidated (Dong, *et al.* 2012, Santhanam, *et al.* 2013).

Immune stimulation by NLPs reflects typical responses observed during PTI, such as induction of ion fluxes, activation of MAP kinases, production of reactive oxygen species and phytoalexins, defense gene induction and deposition of callose (Küfner, *et al.* 2009). However, elicitor active epitopes could not be identified so far (Fellbrich, *et al.* 2002, Schouten, *et al.* 2008). Instead, immunogenic activity exerted by bacterial PccNLP (*Pectobacterium carotovorum* subsp. *carotovorum*) could be linked to the membrane disintegrating activity suggesting that plants sense the toxin activity but not an immunogenic epitope within NLPs (Ottmann, *et al.* 2009).

## **1.5 Aims of the thesis**

NEP1-like proteins exert cytotoxic activity on dicot plant species, fulfill a role as virulence factors and are, concurrently, potent inducers of plant immunity. Aim of this work was to gain deeper insight into how NLPs induce plant immune signaling and to hereby attain better understanding of the biological significance of such diverse features within one protein. Identification and characterization of immunogenic molecules directly connected to NLP activity should shed light on how this protein family triggers immune responses in plants.

## **2. Materials and Methods**

### **2.1 Materials**

#### **2.1.1 Chemicals**

Chemicals were purchased in standard purity from Sigma-Aldrich (Taufkirchen), Carl Roth (Karlsruhe), Merck (Darmstadt), Qiagen (Hilden), Invitrogen (Karlsruhe), Duchefa (Haarlem, The Netherlands), Molecular Probes (Leiden, The Netherlands), Fluka (Buchs, Switzerland) and BD (Sparks, USA). Restriction enzymes, ligase and DNA modification enzymes were purchased from Thermo Fisher Scientific (St. Leon-Rot) and New England Biolabs (Beverly, USA). Oligonucleotides were received from Eurofins MWG Operon (Ebersberg). Primary antibodies were purchased from Cell Signaling Technology (Phospho p44/42 MAPK (Erk1/2)), Sigma-Aldrich ( $\alpha$ -Myc,  $\alpha$ -HA), Sicgen ( $\alpha$ -GFP) and Agrisera ( $\alpha$ -BAK). Alkaline phosphatase conjugated secondary antibodies  $\alpha$ -rabbit IgG,  $\alpha$ -goat IgG and  $\alpha$ -mouse IgG were purchased from Sigma-Aldrich. Synthetic peptides were purchased from Genscript Inc., dissolved in 100 % DMSO as 10mM stock solutions and working dilutions thereof were prepared with water prior to use.

## 2.1.2 Media and Antibiotics

Media used in this study are listed in table 2-1.

Medium	Ingredients per 1 liter	Species
LB	10 g Bacto-tryptone, 5 g Yeast extract, 5 g NaCl, pH 7,0	<i>Escherichia coli</i>
YPD	20 g Peptone, 20 g Glucose, 10 g Yeast extract	<i>Pichia pastoris</i>
MD	1.34 % (w/v) YNB, $4 \times 10^{-5}$ % (w/v) Biotin, 2 % (w/v) Glucose	<i>Pichia pastoris</i>
BMGY /BMMY	1 % (w/v) Yeast extract, 2 % (w/v) Peptone, 1,34 % (w/v) YNB, 100 mM potassium phosphate pH 6,0, $4 \times 10^{-5}$ % (w/v) Biotin, 1 % (v/v) Glycerol for BMGY or 2 % (v/v) Methanol for BMMY	<i>Pichia pastoris</i>
$\frac{1}{2}$ MS	2,2 g MS (Duchefa), pH 5.7 (KOH)	<i>Arabidopsis thaliana</i>
$\frac{1}{2}$ PDB	12 g PDB (Potato Dextrose Broth, Duchefa), pH 5.8 (NaOH)	<i>Botrytis cinerea</i>
King's B	20 g glycerol, 40 g Proteose Pepton No. 3, addition of sterile 0,1 % (v/v) MgSO <sub>4</sub> and KH <sub>2</sub> PO <sub>4</sub> after autoclaving	<i>Pseudomonas syringae</i>
Minimal medium	1 g NaOH, 3 g DL-malic acid, 2 g NH <sub>4</sub> NO <sub>3</sub> , 0,1 g MgSO <sub>4</sub>	<i>Sclerotinia sclerotiorum</i>
Rye sucrose	200 ml rye extract (60 g rye in 200 ml H <sub>2</sub> O, soaked 36-48 h, 50 °C 3h, filtered), 20 g saccharose, 15 g agar	<i>Phytophthora infestans</i>

**Table 2-1: Media used**

Antibiotics used in this study are listed in table 2-2.

Antibiotics	Concentration µg/µl	Solvent
Carbenicillin	100	Water
Cycloheximid	50	Water
Kanamycin	50	Water
Rifampicin	50	Methanol
Spectinomycin	100	Water
Tetracyclin	50	Ethanol

**Table 2-2. Antibiotics used**

### 2.1.3 Vectors and constructs

Vectors used in this study are listed in table 2-3.

Vectors	Characteristics	Reference
pCR8/GW/TOPO	Ori Puc, rrnB, T2, rrnB,T1, attP1, attP2, ccdB, Sm/Sp <sup>r</sup>	Thermo Fisher Scientific
pB7FWG2.0	p35S, t35S, attR1, attR2, ccdB eGFP	Karimi <i>et al.</i> (2002)
pGWB14	p35S, t35S, attR1, attR2, ccdB, Kan <sup>r</sup> , Hyg <sup>r</sup> , 3x-HA	Nakagawa <i>et al.</i> (2007)
pGWB17	p35S, t35S, attR1, attR2, ccdB, Kan <sup>r</sup> , Hyg <sup>r</sup> , 4x-Myc	Nakagawa, <i>et al.</i> (2007)
pUBC-nYFP-DEST	Sm <sup>r</sup> , Hyg <sup>r</sup> , Cm <sup>r</sup> , ccdB, pUBQ10, pBR322, pVS1, T35S, nYFP, attR1, attR2	Grefen <i>et al.</i> (2010)
pUBC-cYFP-DEST	Cm <sup>r</sup> , ccdB, Bar <sup>r</sup> , Sm <sup>r</sup> , pUBQ10, pBR322, pVS1, T35S, cYFP, attR1, attR2	Grefen, <i>et al.</i> (2010)
pPIC9K	5'AOX1, $\alpha$ -factor, MCS, TT, HIS4, 3'AOX1 Amp <sup>r</sup> , Kan <sup>r</sup>	Thermo Fisher Scientific
pBluescript KS(-)	f1(-) origin, lacZ, MCS, T7, T3, lac, pUC, Amp <sup>r</sup>	Invitrogen

**Table 2-3: Vectors used**

SERK constructs for Co-IP experiments were kindly provided by Dr. Eric Melzer (SERK1, SERK2, SERK4 and SERK5 in pGWB17). *PpNLP\_mut* (H121A D124A) in pPIC9K was kindly provided by Dr. Isabell Albert.

## 2.2 Organisms and cultivation conditions

### 2.2.1 Plants

*Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used for these studies, 135 ecotype accessions (ordered at NASC (The European Arabidopsis Stock Centre) and kindly provided by the lab of Dr. Detlef Weigel, MPI Tübingen) RLP and RLK T-DNA



insertion mutant collections (Wang *et al.* 2008, Postel, *et al.* 2010), T-DNA insertion mutant lines *rlp23-1* (SALK\_034225), *rlp23-2* (GK-738D01), *sobir1-12* (SALK\_050715), *sobir1-13* (SALK\_009453), *bak1-3* (SALK\_034523), *bak1-4* (SALK\_116202), *bak1-5*, *bak1-5/bkk1-1* (Schwessinger *et al.* 2011), *bkk1-1* (SALK\_057955), *efr/fls2*, *PR1::GUS* (Shapiro and Zhang 2001). Close relatives of *Arabidopsis thaliana* (ordered at NASC): *Arabis alpina*, *Thlaspi arvense*, *Draba rigida*, *Arabidopsis lyrata*, *Capsella rubella*. The monocot *Triticum aestivum*, *Nicotiana benthamiana*, *Solanum lycopersicum* ‘MoneyMaker’, *Solanum tuberosum* ‘Desirée’ (provided by the greenhouse), *Lactuca sativa*, *Petroselinum crispum*.

All plants were grown on soil under standard conditions (8h light, 150µmol/cm<sup>2</sup>s light, 40-60 % humidity, 22°C) and used for the experiments at an age of 5-6 weeks. Plants used for infection assays with *Pseudomonas syringae* and *Botrytis cinerea* were grown under translucent cover. *Arabidopsis* plants used for seedling growth inhibition were surface sterilized and grown under long-day conditions (16 h light) in ½ MS liquid medium supplemented with the respective peptides.

*N. benthamiana*, *S. lycopersicum* and *N. tuberosum* plants were grown in the greenhouse (16 h light, 22°C).

## 2.2.2 Bacteria, fungi and oomycetes

*Escherichia coli* One Shot TOP10 (mcrA, delta (mrr-hsdRMS-mcrBC), phi, 80delta lac delta M15, delta lacX74, deoR, recA1, araD139 delta (ara, leu), 7697, galU, galK, lambda-, rpsL, endA1, mupG) or XL1 blue (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F' proAB, lacIqZΔM15, Tn10 (Tet<sup>r</sup>)] were cultivated on LB-plates or at 37°C in liquid LB-medium overnight at 230 rpm with the corresponding antibiotics coded on the plasmids.

*Agrobacterium tumefaciens* GV3103::pmP90 (T-DNA<sup>-</sup> vir<sup>+</sup> rif<sup>r</sup>, pMP90 gen<sup>r</sup>) was grown on LB-agar or in liquid LB-medium at 28 °C with the corresponding antibiotics for 48 h at 180 rpm.

*Pseudomonas syringae* pv. *tomato* strain DC3000 (rif<sup>r</sup>) were grown for 24-48 h at 28 °C either on King’s plates or in liquid King’s B medium at 180 rpm. For the determination of bacterial growth the *Pseudomonas* strains were re-isolated from plant material (see 2.5.2) and dilution series thereof plated on LB-plates containing cycloheximide and rifampicin.

*Pectobacterium carotovorum* subsp. *carotovorum* SCC3200 (Cm<sup>r</sup>) *PccNLP*-deficient strain (Mattinen, *et al.* 2004) was grown at 28 °C in LB-medium, 230 rpm.

*Pichia pastoris* GS115 was grown in YPD-medium (30 °C, 230 rpm) to prepare electrocompetent cells. Transformed yeast cells were selected on MD-plates and grown in BMGY and BMMY for protein expression.

*Botrytis cinerea* BO-10 stock solutions with  $2 \times 10^7$  spores/ml in 25 % glycerol were prepared by Dagmar Kolb (induction of spore release with H<sub>2</sub>O from mycelium grown on malt tomato agar (10 g/l malt extract, 250 g/l tomato leaf extract) and kept at – 80°C.  $5 \times 10^6$  spores/ml in PDB were used for infection assays on *Arabidopsis*.

*Sclerotinia sclerotiorum* 1980 (provided by H. Stotz, University of Würzburg) was grown at 18 °C on minimal medium to reduce aggressiveness (Guo and Stotz 2007) and used for infection assays on potato.

*Phytophthora infestans* 88069 was grown on rye sucrose medium at 18 °C and used for infection assays on potato.

## 2.3 Methods

### 2.3.1 General molecular biology methods

PCR was performed with Taq Polymerase (Genaxxon) or Pfu Polymerase (Thermo Fisher Scientific) using the given primers and cycling conditions. Agarose gel electrophoresis to separate DNA fragments was performed with a 1 % agarose gel containing 0,5 µg/ml ethidium bromide in 1 x TAE buffer (4 mM Tris/acetate, 1 mM EDTA pH 8,0). Samples were mixed with loading dye (3 x loading dye: 87 % (v/v) glycerine, 30 mM Tris-HCl, 3 mM EDTA pH 8,0, 0,4 % bromphenol blue (w/v)) and loaded next to PstI-digested λ-DNA (100 ng/µl) used as a size marker. Electrophoresis was performed at an electric field strength of 5 V/cm. DNA fragments were visualized in a UV-transilluminator (Infinity-3026 WL/26 Mx, Peqlab) with the software InfinityCapt 14.2 (Peqlab). DNA purification from agarose gels was performed with the GeneJet Gel Extraction Kit (Thermo Fisher Scientific). Nucleic acid concentrations were determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) at 220-340 nm and evaluated with the NanDrop Software. Sequencing of plasmid DNA was performed by GATC (Konstanz) and prepared according to the company's

instructions. Sequences were analyzed using the CLC Main Workbench (Qiagen). The enzymes were used according to the manufacturer's protocol (Thermo Fisher Scientific). Plasmid isolation was performed using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific).

### 2.3.2 Cloning

Cloning of RLP23, SOBIR1 and BAK1 constructs was performed by Dr. Isabell Albert. Coding sequences with or without the native promoter were cloned into the pCR8/GW/TOPO vector (Thermo Fisher Scientific) using the primers PromRLP23 fwd (ATACATGTTCACTCATCTTTCC), RLP23 fwd (ATGTCAAAGGCGCTTTTGCATTTGC), RLP23revW/oStop (ACGCTTTCTGCGTTTATTGAGACC), SOBIR1 fwd (ATGGCTGTTCCCACGGGAAGC), SOBIR1revW/oStop (GTGCTTGATCTGGGACAACATGG), BAK1 fwd (ATGGAACGAAGATTAATGATCC), BAK1revW/oStop (TCTTGGACCCGAGGGGTATTCG) and recombined to pB7FWG2.0 (Karimi, *et al.* 2002) for C-terminal GFP fusion, to pGWB14 for C-terminal HA fusion to pGWB17 for C-terminal Myc fusion (Nakagawa, *et al.* 2007), to pUBC-nYFP-DEST and pUBC-cYFP-DEST for C-terminal n- or cYFP fusions (Grefen, *et al.* 2010).

### 2.3.3 Site-directed mutagenesis

For site-directed mutagenesis of *PccNLP* mut (mutations in H121A and D124A) *PccNLP* D124A single mutant in pBluescript KS(-) vector was taken as template. In a total volume of 50  $\mu$ l, 20 ng of DNA was mixed in 1 x Pfu buffer containing MgCl<sub>2</sub>, 1  $\mu$ l Pfu Polymerase (Thermo Fisher Scientific), 0,2 mM dNTPs and 0,2  $\mu$ M of the fwd and rev mutagenesis primer respectively (*PccNLP* mut fwd 5'-GGAGTAAATTCAGGCGCCCGCCATGCCTGGGAA-3' and *PccNLP* mut rev 5'-TTCCCAGGCATGGCGGGCGCCTGAATTTACTCC-3'). Initial denaturation was done at 95 °C for 1 min, followed by 18 cycles of 50 s at 95 °C, annealing at 60 °C for 50 s

and elongation at 68 °C for 10 min. A final elongation was done for 7 min at 68 °C. Methylated template DNA was digested with DpnI (Thermo Fisher Scientific).

### 2.3.4 Transformation of bacteria

Chemical competent *Escherichia coli* XL1-blue were prepared according to Sambrook and Russel (2001). *Escherichia coli* One Shot TOP10 were acquired from Thermo Fisher Scientific. Plasmid DNA was added to 200 µl of competent cells, incubated for 30 min on ice and heat-shocked for 30 sec at 42 °C. 800 µl LB-medium were added to the cells, shaken at 37 °C for 1 h and plated on LB plates with the corresponding antibiotics for selection.

250 ml of a *Pectobacterium carotovorum* SCC3200 culture were grown to an OD<sub>600</sub> of 0,5, centrifuged at 4 °C for 10 min at 4500 g and resuspended in 250 ml transformation buffer (272 mM saccharose, 1 mM MgCl<sub>2</sub>, 7 mM sodium phosphate buffer pH 7,4). The cells were centrifuged, washed and resuspended in 2,5 ml transformation buffer. 200 µl aliquots were mixed with 100 ng plasmid DNA in electroporation cuvettes, incubated on ice for 5 min and pulsed at 2500 volts, 5 ms. Cells were mixed with 800 µl LB-medium, incubated for 1 h at 37 °C at 750 rpm and plated on LB plates with the corresponding antibiotics for selection.

### 2.3.5 Transformation of *Pichia pastoris*

Transformation of *Pichia pastoris* was performed by electroporation according to the protocol of the Multi-Copy Pichia Expression Kit (Thermo Fisher Scientific).

### 2.3.6 Stable transformation of plants

Stable transformation of *N. benthamiana*, *S. lycopersicum* and *S. tuberosum* was performed by Caterina Brancato. *N. benthamiana* leaf pieces were incubated in *Agrobacterium* cell suspensions (grown as described in 2.2.2 and resuspended in LB-medium without antibiotics) for 3 minutes, transferred to MS medium with 2 % sucrose and incubated for 48 hrs in the dark. Transgenic calli were selected on MS medium

containing BASTA. For transformation of *S. lycopersicum*, cotyledons were incubated in *A. tumefaciens* suspension for 2 days at room temperature in the dark and transferred to selection medium containing BASTA. Leaf pieces of *S. tuberosum* plants were floated in liquid 2 x MS medium containing *A. tumefaciens* for 3 days in the dark at room temperature. Subsequently, they were transferred to callus induction medium for 1 week and then to selection medium with BASTA. Transgenic plants selected in sterile culture were transferred to soil and grown in the greenhouse under long day conditions. Stable transformation of *Arabidopsis thaliana* plants was performed by Dr. Isabell Albert. Agrobacteria were harvested and resuspended in 5 % (w/v) sucrose, 10 mM MgSO<sub>4</sub> and 0.01 % (v/v) Silwet and sprayed on buds of 6 to 8-week-old *rlp23-1* and *rlp23-2* mutant plants or accessions Bor-4 and Kyoto. The T1 generation was selected with 0.2 % BASTA.

### **2.3.7 Transient transformation of *Nicotiana Benthamiana***

*Agrobacterium tumefaciens*-mediated transient transformation of *N. benthamiana* was used for transient protein expression. The bacterial strains carrying the appropriate expression constructs were cultured as described in 2.2.2. After harvesting the cells by centrifugation for 10 minutes at 2000 g, the pellet was washed for two times with 10 mM MgCl<sub>2</sub>. The density of the culture was adjusted to an OD<sub>600</sub> of 1 in 10 mM MgCl<sub>2</sub> and 150 µM acetosyringone. The bacterial suspension was then incubated at RT for 2 hours. Afterwards, the bacteria were mixed 1:1 with a suspension of bacteria carrying an p19 expression construct (Voinnet *et al.* 2003) and adjusted to an OD<sub>600</sub> of 0,2. The mixture was infiltrated into the leaves of 3-week-old tobacco plants and the leaf tissue was analyzed 2-3 days post infection for the presence of the protein.

### **2.3.8 Isolation and transformation of protoplasts**

Isolation and transformation of protoplasts was performed by Caterina Brancato using the standard protocol from Yoo *et al.* (2007) optimized by Dr. Kenneth W. Berendzen, ZMBP Central Facilities (unpublished).

### 2.3.9 DNA isolation

Isolation of *Phytophthora infestans* DNA of infected potato leaves was performed by homogenization of the samples in lysis buffer containing 200 mM Tris-HCl pH 8,0, 100 mM NaCl, 25 mM Na<sub>2</sub>EDTA, 3 % (w/v) SDS and 125 µg/ml proteinase K (freshly added). After incubation for 1 h at 37 °C, the samples were mixed with 1 volume of extraction buffer (100 mM Tris-HCl pH 8,0, 2,5 M NaCl, 20 mM Na<sub>2</sub>EDTA, 2 % (w/v) CTAB and 2 % (v/v) β-mercaptoethanol) and incubated for 15 min at 65 °C. 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added and samples were centrifuged at 10.000 g for 10 min. The aqueous phase was transferred into new tubes and mixed with 100 µg/ml RNase A. After incubation at 37 °C for 30 min, 0,25 volumes of 2 M NaCl-4 % (w/v) PEG<sub>6000</sub> and 0,7 volumes of isopropyl alcohol were added. After centrifugation at 15.000 g for 20 min, the pellets were washed with 1 ml cold 70 % (v/v) ethanol, air-dried and resuspended in 40 µl of TE buffer pH 8,0.

### 2.3.10 RNA isolation

RNA from *Arabidopsis* plants was isolated using the RNeasy Plant MiniKit (Qiagen) according to the manufacturer's recommended protocol and stored at -20 °C.

### 2.3.11 qRT-PCR

cDNA was synthesized with the RevertAid MuLV reverse transcriptase (Thermo Scientific) according to the manufacturer's protocol. PCR amplification was carried out in the presence of SYBR Green (Thermo Scientific) with an iQ5 iCycler (BioRad). Amplification of EF1-α (EF1-α fwd 5'-TCACATCAACATTGTGGTCATTGG-3' EF1-α rev 5'-TTGATCTGGTCAAGAGCCTACAG-3') served as internal standard. Primers used for amplification of PAD3 were PAD3 fwd 5'-CAGGGAAGATACGGATATAAAC-3' and PAD3 rev 5'-AGTGGCATTTAAGTAAAGGCC-3'. Samples were pipetted in triplicates. The data was analyzed according to the  $2^{-\Delta\Delta C(T)}$  method (Livak and Schmittgen 2001). PAD3 gene induction by NLPs was calculated as fold change and presented as relative to the expression level induced by H<sub>2</sub>O infiltration. Cycling conditions were 10 min at 95 °C, 40 cycles 10 s 95 °C, 15 s 57°C and 72 °C for 1 min.

### 2.3.12 qPCR

Quantification of *Phytophthora infestans* DNA was performed by real-time quantitative PCR in an iQ5 iCycler (BioRad) using 1 µl of extracted DNA (2.3.9) in 20 µl of buffer with SYBR Green (Thermo Scientific). DNA extracts of pure *Phytophthora infestans* mycelium were used to prepare a calibration curve in a range of 100 ng to 0,001 ng of DNA (spectrophotometric determination, NanoDrop 2000, Thermo Scientific) used to calculate the DNA content of the samples. Samples were pipetted in triplicates. Primers for amplification were chosen based on highly repetitive sequences from the *P. infestans* genome (PiO8-3-3 fwd 5'-CAATTCGCCACCTTCTTCTCGA-3', PiO8-3-3 rev 5'-GCCTTCCTGCCCTCAAGAAC-3') (Llorente *et al.* 2010). Cycling conditions were 10 min 95 °C, 40 cycles 10 s 95 °C, 15 s 59 °C, 20 s 72 °C.

## 2.4 Biochemical methods

### 2.4.1 Protein extraction from plant tissue

Total protein was extracted from 250 mg of frozen leaf material and solubilized with 1, 7 ml of solubilization buffer (25 mM Tris-HCl pH 8,0, 150 mM NaCl, 1 % (v/v) NP40, 0,5 % (w/v) DOC, 2 mM DTT, 8 µl plant protease inhibitor cocktail (PPI, Sigma-Aldrich) per 250 mg leaf material) after grinding in liquid nitrogen. The samples were solubilized for 1 h at 4 °C by overhead shaking (5-7 rpm). Centrifugation for 1 h at 4 °C and 20.000 g separated the soluble proteins from the cell debris and could be used for further analysis.

### 2.4.2 Immunoprecipitation

Leaves of transiently transformed *N. benthamiana* or *Arabidopsis rlp23-1/p35S::RLP23-GFP* plants were either harvested directly or 3 min after infiltration of 1 µM nlp20 (*N. benthamiana*) or 10 µM nlp20 (*Arabidopsis*). For immunoprecipitations, 250 mg ground leaf material from *N. benthamiana* or 500 mg from *Arabidopsis* was

used per sample. Membrane proteins were solubilized as described (2.4.1) and immuno-adsorbed with prewashed and equilibrated (in solubilization buffer) GFP-tag to GFP-Trap beads (ChromoTek). The protein extracts were incubated with the beads for 1-2 h at 4 °C in an overhead rotator (5-7 rpm). By carefully sedimenting the beads (1000 g, 4 °C, 1 min) they were washed two times with solubilization buffer and two times with washing buffer (25 mM Tris-HCl pH 8,0, 150 mM NaCl, 2 mM DTT, 8 µl PPI/sample) and boiled with SDS loading buffer for 10 min at 95 °C. After sedimenting the beads at 1000 g, the samples were subjected to SDS-PAGE and western blot analysis. For pull-down experiments of BAK1 from *Arabidopsis* tissue, NaCl concentrations of the solubilization and the washing buffer were reduced to 20 mM.

### **2.4.3 Heterologous protein expression in *Pichia pastoris***

*P. pastoris* was precultured for 24 h in 10-20 ml BMGY-medium. Cells were centrifuged (5 min, 1500 g, at RT), resuspended in 100-500 ml BMMY-medium, and cultured for 3-4 days. After 10 min of centrifugation at 10.000 g, the supernatant was precipitated with 60 % (w/v) ammonium sulfate. After 15 min of centrifugation at 20.000 g at 4 °C, the pellet was resuspended in water and dialysed in the buffer for ion exchange chromatography.

### **2.4.4 Heterologous protein expression in *Pectobacterium carotovorum***

*P. carotovorum* was cultured for 24 h in LB-medium containing ampicillin. To isolate periplasmic proteins, the bacteria were pelleted for 3 min at 6000 g (RT) and resuspended in 1 ml of a solution containing 10 mM Tris-HCl (pH 7,5) and 30 mM NaCl (4 °C). After another centrifugation step for 3 min at 6000 g (4 °C), the cells were resuspended in 200 µl 30 mM Tris-HCl pH 7,5 (RT) and 200 µl of a solution containing 30 mM Tris-HCl pH 7,5, 40 % (w/v) saccharose and 0,2 mM EDTA were added and incubated for 10 min at RT. After 5 min of centrifugation, the pellet was resuspended in 200 µl of a 20 mM MgCl<sub>2</sub> solution and incubated on ice for 10 min. After a 10 min centrifugation at 16.000 g and 4 °C, periplasmic proteins within the supernatant were desalted with a PD-10 column (GE Healthcare) and dialysed in the respective buffer for ion exchange chromatography.



### 2.4.5 Purification of recombinant proteins

The proteins were purified by ion exchange chromatography and subsequent gel filtration. The protein solution was dialysed in buffer A and the column equilibrated with the threefold column volume. The dialysed sample was applied to the column (1 ml/min) and washed with the tenfold column volume. Proteins were eluted with a gradient of 0-100 % (10 ml) of buffer B and the collected fractions were checked for protein quantity via SDS-PAGE. The fractions containing most protein were united and purified by gel filtration (1ml/min, HiLoad™ 16/60 Superdex 75, GE Healthcare) using the buffer GF. Protein containing fractions were pooled and dialysed in water. UV-spectroscopy (wavelength  $\lambda_{280}$ ) was used to determine protein concentrations using the protparam tool (<http://web.expasy.org/protparam>) to determine protein-specific extinction coefficients  $\epsilon_{280}$  for each protein. Protein concentrations were verified by SDS-PAGE using BSA as a standard protein.

Protein	Buffer A	Buffer B	Column	Buffer GF
<i>PpNLP</i> , <i>PpNLP</i> mut	20 mM Tris pH 8,5	20 mM Tris pH 8,5 500 mM KCl	HiTrap Q FF	20 mM Tris pH 8,5 150 mM KCl
<i>PccNLP</i> , <i>PccNLP</i> mut	50 mM MES pH 5,7	50 mM MES pH 5,7 500 mM KCl	HiTrap SP FF	50 mM MES pH 5,7 150 mM KCl

**Table 2-4: Buffer and columns used for protein purification**

### 2.4.6 Determination of protein concentration

The protein concentration was determined after the Bradford method (Bradford 1976) using Roti-Quant solution (Carl Roth). A standard curve was calculated with bovine serum albumin (BSA).

### 2.4.7 SDS-Page

SDS polyacrylamide gel electrophoresis was performed using the protocol of Laemmli (1970) by the method of Sambrook and Russel (2001). The acrylamide-

bisacrylamid mixture (37,5:1) was purchased as Rotiphorese Gel 30 (Carl Roth). Separating gels of 8 %, 10 % or 12 % were used with 5 % stacking gels in the gel chamber system of BioRad. Protein separation was performed at 20 mA and the prestained PageRuler™ protein ladder mix (Fermentas) was used as a protein marker.

#### **2.4.8 Coomassie blue stain**

Non-specific staining of proteins after SDS-PAGE was done using a Coomassie staining solution containing 0,125 % (w/v) Coomassie brilliant blue R-250, 50 % (v/v) MeOH and 10 % (v/v) acetic acid. The solution was heated up and shaken with the gel for 30 min. For destaining of the gel the solution was exchanged by 10 % (v/v) acetic acid, heated up and shaken until excess stain was removed.

#### **2.4.9 Westen blot analysis**

Western blotting was performed in a PerfectBlue semi-dry-blotting gadget (PeqLab, Erlangen). The SDS polyacrylamide gel was sandwiched together with a nitrocellulose Hybond ECL- Membrane (GE Healthcare) between three layers of whatman paper after all components were equilibrated in blotting buffer (25 mM Tris-HCl pH 8,3, 192 mM glycine, 20 % (v/v) MeOH). Blotting was performed for 1 h at 1 mA/cm<sup>2</sup> and stained with Ponceau-S (0,1 % (w/v) Ponceau-S, 5 % (v/v) acetic acid) to control blotting efficiency and evenness. The membrane was blocked for 1-2 h in 5 % (w/v) BSA in TBS-T (20 mM Tris-HCl pH 7,5, 150 mM NaCl, 0,1 % (v/v) Tween-20). The membrane was then incubated with the first antibody in 5 % BSA in TBS-T overnight at 4 °C, washed with TBS-T (3 x 5 min) and incubated in the second antibody in 5 % BSA in TBS-T for 2 h. After three washing steps the membrane was equilibrated in alkaline phosphatase buffer (AP-buffer, 150 mM Tris-HCl pH 9,5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>) and detected using either the substrates NBT (100 µg/ml) and BCIP (50 µg/ml) or by chemiluminescence using nitroblock solution (1:20 in AP, Thermo Fisher Scientific) to enhance the alkaline phosphatase signal and CDP Star (0,25 mM in AP). The chemiluminescent signal was detected with a CCD camera (Viber Louromat, PeqLAB).

### **2.4.10 Activation of MAP kinases**

Leaves of 5 to 6-week-old *Arabidopsis* plants were frozen in liquid nitrogen 15 min after infiltration of the respective peptide solution with a needle-free syringae. The samples were homogenized in 100 µl extraction buffer (50 mM Tris-HCl pH 7,5, Complete Protease Inhibitor Cocktail (Roche), PhosStop Phosphatase Inhibitor Cocktail (Roche)) and centrifuged at 10.000 g and 4 °C for 20 min. The protein concentration was determined (2.4.6) and 30 µg of protein were subjected to a 10 % SDS-PAGE (2.4.7). After western blotting, activated MAP kinases 6, 3 and 4 were detected using the phospho p44/42 MAPK (Erk1/2) primary antibody (Cell Signaling Technology).

### **2.4.11 Protein modeling**

The *PpNLP* sequence without the N-terminal signal peptide (SignalP 4.1 server, Petersen *et al.* (2011), cleavage site between position A19 and D20) was used to create a protein model applying the Phyre2 web portal for protein modeling, prediction and analysis (Kelley *et al.* 2015). For digital imaging, the software PyMOL (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC) and Microsoft Office 2013 was applied.

## **2.5 Bioassays**

### **2.5.1 Priming of *Arabidopsis* plants**

5 to 6-week-old *Arabidopsis* plants were primed by leaf infiltration with 1 µM nlp20 (*PpNLP*), flg22 or the chitin hexamer C6 (1 mM) or with 1 µM of the inactive peptides nlp20 (*PccNLP*) and nlp20\_W6A or mock-treated with H<sub>2</sub>O (0,01 % DMSO) 24 hours prior infection with *Pseudomonas syringae* or *Botrytis cinerea*.

### **2.5.2 Infection with *Pseudomonas syringae***

*Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000) was grown overnight in King's B medium, centrifuged, washed and diluted in 10 mM MgCl<sub>2</sub> to a density of 10<sup>4</sup> cfu/ml. Bacteria were pressure-infiltrated into primed *Arabidopsis* leaves and the plants were kept under translucent cover and high humidity. Leaves were harvested at day 0 and 3, surface sterilized in 70 % EtOH and washed in ddH<sub>2</sub>O for 1 min each. Two leaf discs (5mm diameter) per leaf were stamped out, ground in 200 µl of a 10 mM MgCl<sub>2</sub> solution, diluted serially 1:10 and plated on LB plates containing rifampicin and cycloheximide. After 2 days of incubation at 28 °C, colony-forming units were counted.

### **2.5.3 Infection with *Botrytis cinerea***

Spores of *Botrytis cinerea* isolate B0-10 were diluted to a final concentration of 5x10<sup>6</sup> spores/ml in PDB medium. Primed *Arabidopsis* leaves were droplet inoculated with 5 µl of the spore solution and kept under translucent cover and high humidity for 2 days. Lesion sizes were determined using the Photoshop CS6 Lasso tool. Selected pixels were counted and the lesion size in cm<sup>2</sup> was calculated using a 0,5 cm<sup>2</sup> standard.

### **2.5.4 Infection with *Sclerotinia sclerotiorum***

*Sclerotinia sclerotiorum* strain 1980 was grown on minimal medium to reduce aggressiveness of the fungus as previously described (Guo and Stotz 2007). An agar plug (5 mm diameter) with actively growing mycelium was placed on the adaxial surface of potato leaves. Leaves were kept in closed boxes under high humidity and disease development was scored at 3-4 days after infection. Lesion sizes were determined using the Photoshop CS6 Lasso tool. Selected pixels were counted and the lesion size in cm<sup>2</sup> was calculated using a 0,5 cm<sup>2</sup> standard.

### **2.5.5 Infection with *Phytophthora infestans***

*Phytophthora infestans* strain 88069 was maintained on plates with rye sucrose medium and release of spores was induced by covering 10 to 15-day-old mycelium with cold sterile water (4 °C) for 4 hours in the cold room. The concentration of spores was adjusted to  $5 \times 10^4$  zoospores/ml and potato leaves were droplet inoculated with 10  $\mu$ l drops and kept in boxes under high humidity. Pictures were taken 4-6 days after infection and lesion sizes were determined using the Photoshop CS6 Lasso tool. Selected pixels were counted and the lesion size in  $\text{cm}^2$  was calculated using a 0,5  $\text{cm}^2$  standard. For quantification of the DNA, infection sites were stamped out with a cork borer (12 mm diameter) and DNA was extracted as described in 2.3.9 (Llorente, *et al.* 2010) and subjected to quantitative real-time PCR (2.3.12).

### **2.5.6 Detection of reactive oxygen species**

For measurement of reactive oxygen species (ROS) production, small leaf pieces (~ 0,4 cm x 0,2 cm) of 5-week-old *Arabidopsis* plants were cut and floated overnight on ddH<sub>2</sub>O. The next day, two thereof were placed in one well of a 96-well plate containing 100  $\mu$ l of a 20 mM L-012 and 0,5  $\mu$ g/ml peroxidase solution. The background was measured before treatment with the respective peptide solution and luminol-chemoluminescence was quantified using a 96-well Luminometer (Mithras LB 940, Berthold Technologies) and the software MicroWin.

### **2.5.7 Biosynthesis of ethylene**

6-week-old *Arabidopsis* leaves were cut into ~ 0,4 x 0,4 cm pieces and floated overnight in ddH<sub>2</sub>O. Three thereof were transferred into glass vials (6 ml volum) with 400  $\mu$ l 20 mM MES buffer pH 5,7, treated with the corresponding protein or peptide and closed with rubber plugs. The samples were shaken for four hours at 170 rpm and 1 ml of air was taken of the glass vial and injected into a gas chromatograph (Shimadzu GC-14A).

### **2.5.8 Extracellular medium alkalization**

Alkalinization of the medium was measured as described (Felix, *et al.* 1999). *Arabidopsis* Col-0 cell suspension aliquots of 2,5 ml were placed in vials on a shaker at 120 rpm and treated with the respective peptide. The extracellular pH was measured continuously over a time period of 30 min using a glass electrode and a pen recorder.

### **2.5.9 Seedling growth inhibition**

*Arabidopsis* Col-0 seeds were surface-sterilized (1 min in 50 % EtOH, 10 min in 50 % sodium hypochlorite 0,1 % Triton-X 100, 5 x washing in ddH<sub>2</sub>O) and grown under long-day conditions (16 h light) in liquid ½ MS medium containing the respective peptide solution. After two weeks, the seedlings were transferred onto agar plates to determine the root length.

### **2.5.10 Analysis of cell death**

Leaves of 6-week-old *Arabidopsis* plants were infiltrated abaxially with 0,5 µM of the respective protein solution or given peptide concentrations with a needle-free syringe. Necrotic symptoms were analyzed 2 days post treatment.

### **2.5.11 Calcein release**

Isolation of plant plasma membranes from 6-week-old *Arabidopsis* Col-0 plants was done by phase partitioning of microsomal fractions using Dextran and PEG in a concentration of 6.4% (w/w) and 3 mM KCl performed by Dr. Isabell Albert (Larsson *et al.* 1994). For preparation of calcein-loaded vesicles, plasma membranes (100-500 µg protein) were sonified (30 min on ice, 20 sec pulse on, amplitude 25%, 20 sec pulse off) in the presence of calcein (60 mM) in calcein buffer (20 mM Tris-HCl, pH 8,5, 140 mM NaCl, 1 mM EDTA). After incubation at RT for 30 min and centrifugation (15 min, 16.000 g, RT) the excess calcein was removed by gel filtration using a Sephadex G-75 medium column (Sigma) equilibrated in calcein buffer. Fractions of about 120 µl

were collected of which 5  $\mu\text{l}$  were mixed with 195  $\mu\text{l}$  of vesicle buffer (20 mM MES pH 5,8, 140 mM NaCl) and measured with and without 0,5 % Triton X-100 in a microplate reader (Mithras LB 940, Berthold Technologies). The signal was excited at 485 nm collecting emission at 520 nm. Fractions with at least double a difference in the fluorescence signal between treated and untreated samples were united and protein concentration was determined. Measurements were performed in a total volume of 200  $\mu\text{l}$  with 1 ng/ $\mu\text{l}$  of plasma membrane vesicles in vesicle buffer. Vesicles were treated with the respective protein solution and calcein release was measured over 20 min. The percentage of calcein release was calculated according to the equation  $(F_{\text{meas}} - F_{\text{init}}) / (F_{\text{max}} - F_{\text{init}}) * 100$ , where  $F_{\text{meas}}$ ,  $F_{\text{init}}$ , and  $F_{\text{max}}$  are the measured, initial, and maximal fluorescence, respectively.  $F_{\text{max}}$  was obtained by the addition of Triton X-100 to a final concentration of 0.5% (v/v) at the end of each measurement.

## 2.6 Microscopy and histochemistry

### 2.6.1 Bimolecular fluorescence complementation

Bimolecular fluorescence complementation (BiFC) was investigated in transformed protoplasts (2.3.8) of *Arabidopsis* Col-0 plants on a Leica SP2 confocal microscope with a 20x/0.75 water-immersion objective. The YFP signal was excited using a 514 nm laser collecting emission between 520–560 nm.

### 2.6.2 Aniline blue stain

The induction of callose deposition was analyzed by aniline blue staining (Gomez-Gomez, *et al.* 1999). Leaves were harvested 24 hours after infiltration with the respective peptide solution, flooded with fixing solution (1% (v/v) glutaraldehyde; 5 mM citric acid; 90 mM  $\text{Na}_2\text{HPO}_4$  pH 7,4) in a 6-well plate and incubated for 24 hours. After fixation, chlorophyll was removed with 100% (v/v) EtOH for 1-2 days. The leaves were transferred to 50% (v/v) EtOH, subsequently equilibrated in 67 mM  $\text{K}_2\text{HPO}_4$  (pH 12,0) and finally stained for 1 h in 0,1 % (w/v) aniline blue dissolved in 67 mM  $\text{K}_2\text{HPO}_4$  (pH 12,0). The stained leaves were transferred to a microscopic slide and covered with 70%

(v/v) glycerol and 30% (v/v) staining solution and examined under UV epifluorescence. Quantification of callose was performed by counting selected pixels and calculated in % relative to the respective image section of the leaf surface. Pictures were analyzed using the Photoshop CS6 Magic tool, hereby removing background and leaf-veins within a certain color range. (Use: white, Mode: normal, Opacity: 100%).

### **2.6.3 GUS stain**

GUS-activity in *PR1::GUS* transgenic *Arabidopsis* plants (Shapiro and Zhang 2001) was histochemically determined 24 hours after leaf infiltration of the respective protein or peptide solution. Leaves were transferred into 2 ml Eppendorf tubes, flooded with X-Gluc buffer (50 mM Na<sub>3</sub>PO<sub>4</sub> pH 7,0, 0,5 mM K<sub>4</sub>[Fe(CN)<sub>6</sub>], 0,5 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>], 10 mM EDTA pH 8,0, 0,1 % (v/v) Triton X-100, 0,05 % (w/v) 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc, Carl Roth), two times vacuum infiltrated and incubated overnight at 37 °C. Chlorophyll was removed by repeated washing with 70 % EtOH.

## **2.7 Statistical analysis**

Statistical analysis was performed using Microsoft Office Excel or GraphPad QuickCalcs. The data represent the average of replicates with ± SD of the mean. The significance of the differences was calculated using the t-test.



### 3. Results

#### 3.1 Identification and characterization of the novel *Pp*NLP-derived PAMP nlp20

NLPs are widespread microbial virulence factors, some of which exert toxin activity on dicot plant species and strongly induce plant immunity (Qutob *et al.* 2006, Ottmann, *et al.* 2009).

Objective of this chapter was to identify and characterize this immunogenic activity. Comparative analysis on recombinant *Pcc*NLP (NLP derived from *Pectobacterium carotovorum* pv. *carotovorum*) and *Pp*NLP (*Phytophthora parasitica*-derived NLP) should reveal the presence of a novel immunogenic pattern. Deletion mapping and mutational analysis were applied to gain more insight into motif length and residues which are essential for elicitor activity. Moreover, studies on pattern distribution among microbes of different kingdoms of life and identification of sensitive plants should enlighten the spectrum of the novel immunogenic pattern.

##### 3.1.1 WT, mutagenized and heat-treated recombinant *Pcc*NLP and *Pp*NLP show distinct behaviors regarding cytotoxicity and immunogenic activity in *Arabidopsis*

Studies using recombinant *Pcc*NLP and *Pp*NLP cytotoxins should specify the toxic and immunogenic activity of NLPs in more detail. Therefore, wild-type and modified non-toxic versions of both recombinant proteins were applied to define cytolytic and immunogenic activities in *Arabidopsis*.

Mutational analysis within highly conserved amino acids of *Pectobacterium carotovorum* NLP (*Pcc*NLP) revealed that immune-associated defense responses and cytotoxic activity require same structural scaffolding (Ottmann, *et al.* 2009). Consistent with these findings, an exchange of two of the highly conserved amino acids within the hepta-peptide motif (H121A; D124A, analogous positions of *Pp*NLP) of recombinant *Pcc*NLP (*Pcc*NLP\_mut) led to a complete loss of cytolytic activity upon infiltration of

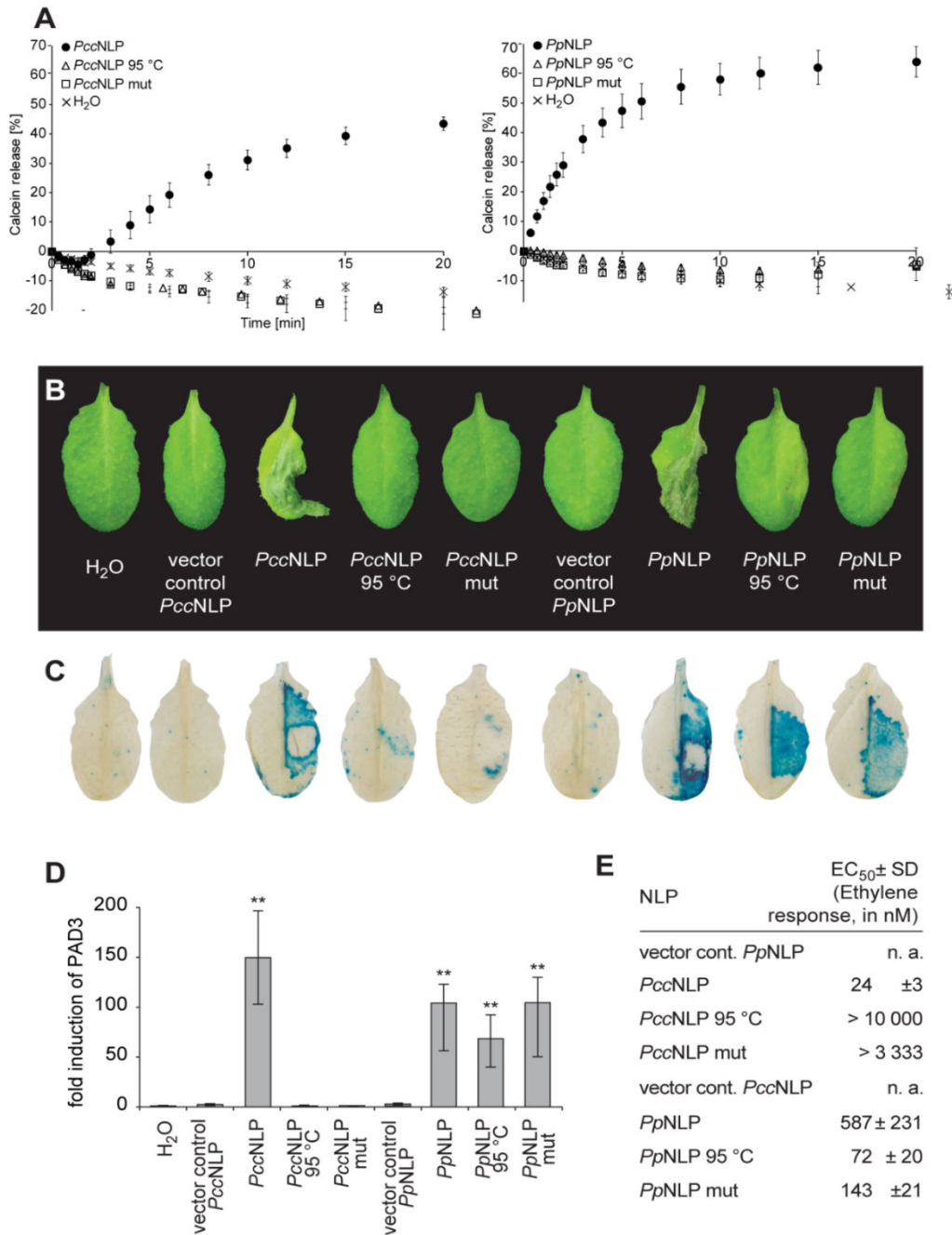
*Arabidopsis* leaves in comparison to the wild-type protein which triggered strong leaf necrosis (Fig. 3-1 B). In addition, treatment of purified calcein-filled plasma membrane vesicles from *Arabidopsis* leaves with *PccNLP\_mut* did not cause any measurable release of the fluorescent dye, whereas the wild-type protein triggered a strong calcein release within a few minutes (Fig. 3-1 A). Likewise, heat-inactivated *PccNLP* (*PccNLP* 95 °C) could neither provoke leaf necrosis nor any quantifiable calcein release (Fig. 3-1 A, B). In the same way, recombinant *PpNLP* from the oomycete *Phytophthora parasitica* triggered a fast and strong release of calcein as well as leaf necrosis upon infiltration though none of these effects could be observed for the mutated (*PpNLP\_mut*) or heat-denatured (*PpNLP* 95 °C) version of this protein (Fig. 3-1 A, B).

In agreement with the findings of Ottmann et. al., 2009, who showed that cytotoxic and immunogenic activities of *PccNLP* are based on identical structures, *PccNLP\_mut* and denatured *PccNLP* 95 °C did not only lose their cytotoxicity, but also their capability to trigger immunity-associated defense responses in *Arabidopsis*. Gene induction in transgenic reporter plants carrying a fusion construct consisting of the promoter of the defense gene PR1 (pathogenesis-related protein1) and the reporter enzyme GUS ( $\beta$ -glucuronidase) was abrogated (Fig. 3-1 C) upon leaf infiltration of both protein variants. Gene expression of PAD3 (phytoalexin deficient 3), a gene necessary for the biosynthesis of the phytoalexin camalexin, was also not induced by *PccNLP\_mut* and *PccNLP* 95 °C, whereas the wild-type protein was able to trigger PAD3 gene expression in a 150-fold induction compared to the control treatment (Fig. 3-1 D). Moreover, the mutated and heat-inactivated *PccNLP* variants failed to trigger ethylene formation, although a high  $EC_{50}$  in the range of  $24 \pm 3$  nM could be determined for the native protein (Fig. 3-1 E).

Intriguingly, the oomycete-derived *PpNLP* variants *PpNLP\_mut* and *PpNLP* 95 °C showed a strong induction of PR1::GUS and PAD3 gene expression comparable to the responses elicited by wild-type *PpNLP* (Fig. 3-1 C, D). Likewise, ethylene production was triggered not only by the native *PpNLP* ( $587 \pm 231$  nM), but also by the mutated ( $143 \pm 21$  nM) and the heat-denatured protein ( $72 \pm 20$  nM) (Fig. 3-1 E).

These findings suggest the presence of a yet unknown immunogenic feature within *PpNLP* which can be clearly distinguished from its toxin activity.

## Results



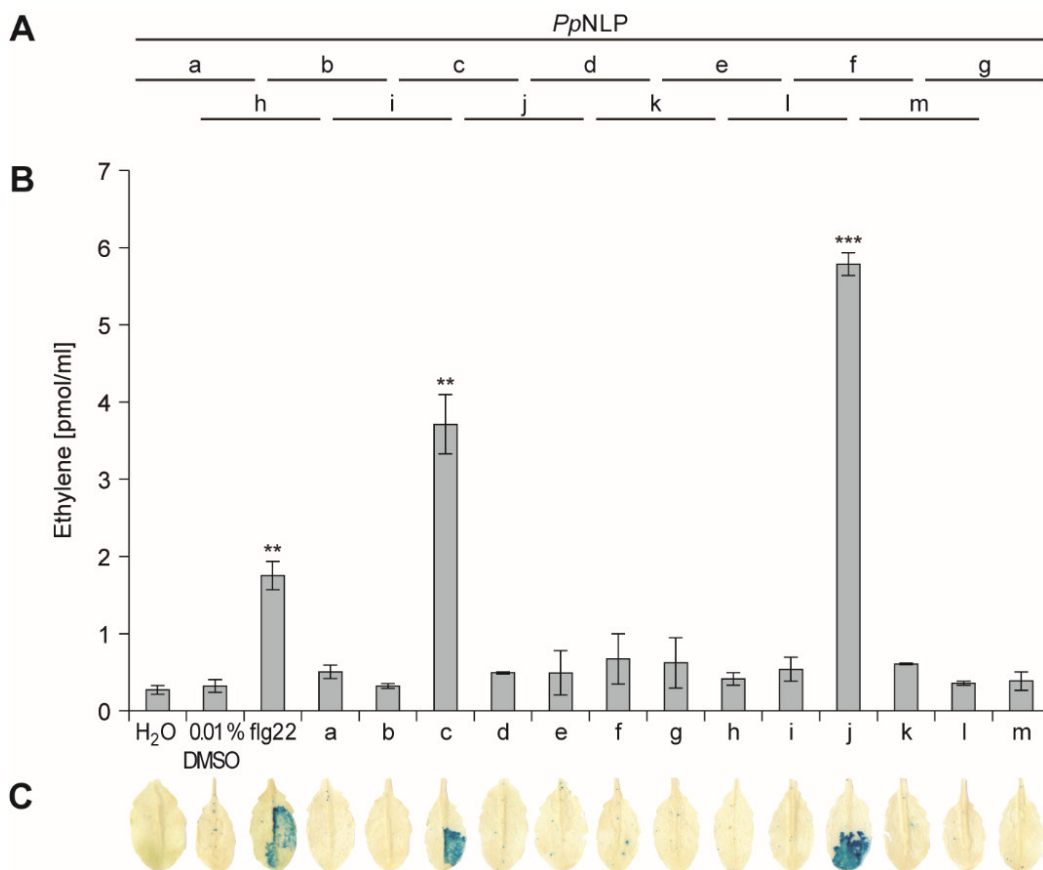
**Figure 3-1: Cytotoxic and immunogenic potential of *Pectobacterium carotovorum* (*PccNLP*) and *Phytophthora parasitica* (*PpNLP*) NLPs in *Arabidopsis*.**

Relative calcein release from purified *Arabidopsis* membrane vesicles given in % of maximum release after addition of Triton-X (0,5 %). Vesicles were treated with water or 333 nM of recombinant wild-type, heat-treated (95°C) or mutated (mut) protein (H121A D124A). Data points represent means of three replicates ± SD (A). Development of necrotic lesions (B) or blue stains indicating PR1::GUS activity in transgenic *Arabidopsis* plants (C) upon leaf infiltration of 0,5 µM recombinant *PccNLP* and *PpNLP* variants. Water and empty vector infiltration served as controls. Pictures were taken 2 dpi (B), GUS activity was histochemically visualized 1dpi (C). *PAD3* gene expression determined by qRT-PCR 4 hrs after infiltration of 0,3 µM NLP variants. Transcript levels shown as fold induction compared to water control treatment and normalized to EF1-α transcript. Bars represent means ± SD of three replicates, significant differences over control treatment were determined by Student's t test \*\*P≤0.01 (D). EC<sub>50</sub> value of ethylene production induced by the NLP variants after 4 hrs of incubation with leaf discs. n.a. = not applicable, numbers represent three replicates ± SD (E). All experiments were performed in triplicates with similar results.

### 3.1.2 Identification of an immunogenic core within *PpNLP*

Immunogenic activities of microbial patterns can characteristically be traced back to small immunogenic epitopes (Boller and Felix 2009, Monaghan and Zipfel 2012, Böhm, *et al.* 2014a). To scan the *PpNLP* protein for such a pattern, 13 nested synthetic peptides were synthesized spanning the whole protein sequence of *PpNLP* (30mer peptides a-m, Fig. 3-2 A). Most interestingly, peptides c and j were able to trigger ethylene formation as well as PR1::GUS gene induction in *Arabidopsis*, whereas the other peptides did not evoke immunogenic responses (Fig. 3-2 B, C).

Peptides c and j span residues G<sub>84</sub> to W<sub>130</sub> with an overlapping region from G<sub>100</sub> to D<sub>113</sub>, indicating that the immunogenic centerpiece is located within this peptide sequence (GVYAIMYSWYFPKD, Table 3-1, peptide 1).



**Figure 3-2 Immunogenic activity of *PpNLP* sequence-derived synthetic peptides in *Arabidopsis*.**

Schematic presentation of 13 overlapping peptides (a-m, 30mer peptides), synthesized based on the *PpNLP* sequence (A). Ethylene formation 4 hours upon induction with 1  $\mu$ M peptide. Water, 0,01 % DMSO (peptide dilution) and flg22 served as controls. Bars represent means  $\pm$  SD of three replicates, asterisks mark significant differences over DMSO control as determined by Student's t test, \*\* $P \leq 0,01$ , \*\*\* $P \leq 0,001$  (B). PR1::GUS gene activation 24 hours after infiltration of 1  $\mu$ M peptide solution (C). All experiments were performed three times with similar results.

### 3.1.3 Characterization of the minimal elicitor motif

To further specify the minimal elicitor motif required for the immunogenic activity detected within *PpNLP*, N- and C-terminal extension and deletion peptide derivatives based on peptide 1 were synthesized and  $EC_{50}$  values (ethylene formation) were determined (Table 3-1, peptides 2-14). C-terminal deletion of the two amino acids K<sub>112</sub> and D<sub>113</sub> (peptide 2) led to a drastic increase of the  $EC_{50}$  value ( $16667 \pm 3055$  nM), whereas the N-terminal deletion variant, in which amino acids G<sub>100</sub> and V<sub>101</sub> were lacking (peptide 3), did not exert such an effect on the immunogenic potential ( $EC_{50} = 217 \pm 65$ ) when compared to peptide 1 ( $322 \pm 165$  nM). Therefore, further stepwise N-terminal deletions were conducted (removal of 5mers, peptides 4-6), revealing a complete loss of the immunogenic potential ( $EC_{50} > 100000$ ) as early as amino acids Y<sub>102</sub>-Y<sub>106</sub> (peptide 4) were missing. So far, these findings indicate an essential role of amino acids Y<sub>102</sub> to D<sub>113</sub>, as the key part establishing an elicitor active peptide pattern within *PpNLP*. Further analysis on the function of adjacent amino acids by C-terminal extensions and N-terminal single deletions respectively, revealed two peptides with a high immunogenic potential (peptide 9,  $EC_{50} = 14 \pm 12$  nM, and peptide 13,  $EC_{50} = 1,5 \pm 0,7$  nM). Individual step by step deletion of the N-terminal part ( $\Delta$  A<sub>103</sub>,  $\Delta$  I<sub>104</sub>,  $\Delta$  M<sub>105</sub>, peptides 10-12 and  $\Delta$  Y<sub>104</sub>, peptide 14) led to a gradual increase of the  $EC_{50}$  ( $133 \pm 10$  nM,  $1550 \pm 328$  nM,  $>50000$  nM,  $>100000$  nM) underlining the importance of this motif for elicitor activity.

To obtain deeper insights into the significance of individual amino acids within the two most active peptides (peptide 9 and 13), an alanine-scanning mutagenesis was conducted exchanging single amino acids by alanine (peptide 16-38) or, in the case of peptide 15, by tryptophan. Four amino acids could be identified, whose mutagenesis led to a distinct loss of immunogenic activity: I<sub>104</sub>A (peptide 16,  $EC_{50} = 1567 \pm 252$  nM), Y<sub>106</sub>A (peptide 18,  $EC_{50} = 1500 \pm 436$  nM), W<sub>108</sub>A (peptide 20,  $EC_{50} = 2833 \pm 777$  nM) and Y<sub>109</sub>A (peptide 21,  $EC_{50} = 2633 \pm 208$  nM) whereas all other amino acid exchanges had no or significantly less impact on peptide activity. Since mutations in the C-terminal part of peptide 13 (peptide 35-38) revealed a rather moderate effect on the  $EC_{50}$  values, all further studies were conducted with the 20mer peptide 9 (A<sub>103</sub>IMYSWYFPKDSPVTGLGHR<sub>122</sub>).

Thus, peptide 9 was assigned as the minimal motif of immunogenic activity in *Arabidopsis* and named nlp20 (*PpNLP*), based on its origin and length.

## Results

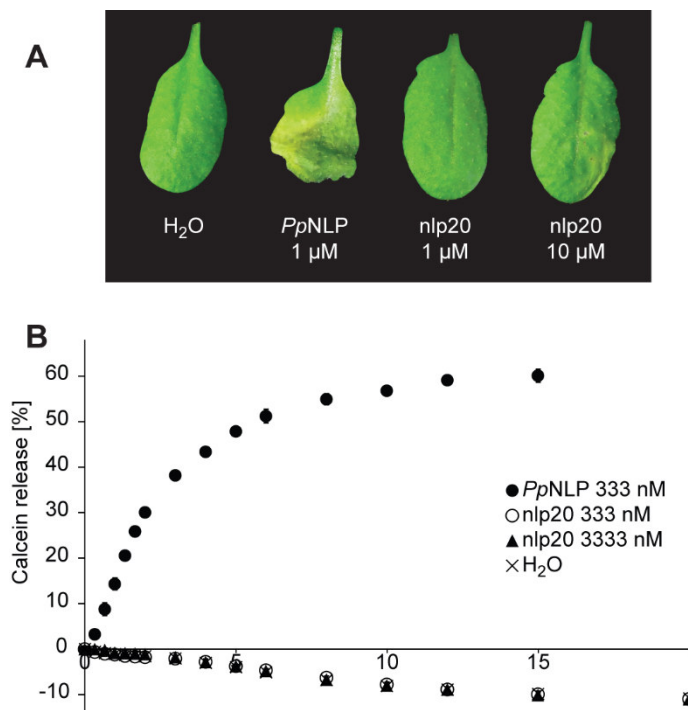
Peptide No.	<i>Pp</i> NLP consensus sequence (amino acids 84 - 131)	EC <sub>50</sub> ± SD [nM]
c	GSGYGSQVYGRVATYNGVYAIMYSWYFPKD	101 ± 34
j	GVYAIMYSWYFPKDSPVTGLGHRHDWEHV	34 ± 10
1	GVYAIMYSWYFPKD	322 ± 165
2	TYNGVYAIMYSWYFP	16667 ± 3055
3	YAIMYSWYFPKDSPV	217 ± 65
4	SWYFPKDSPVTGLGH	>100000
5	KDSPVTGLGHRHDWE	>100000
6	TGLGHRHDWEHV	>100000
7	RVATYNGVYAIMYSWYFPKD	84 ± 11
8	GVYAIMYSWYFPKDSPVTGL	482 ± 45
9	AIMYSWYFPKDSPVTGLGHR	14 ± 12
10	IMYSWYFPKDSPVTGLGHR	133 ± 10
11	MYSWYFPKDSPVTGLGHR	1550 ± 328
12	YSWYFPKDSPVTGLGHR	>50000
13	AIMYSWYFPKDSPVTGLGHRHDWE	1.5 ± 0.7
14	SWYFPKDSPVTGLGHRHDWE	>100000
15	WIMYSWYFPKDSPVTGLGHR	55 ± 24
16	AAMYSWYFPKDSPVTGLGHR	1567 ± 252
17	AIAYSWYFPKDSPVTGLGHR	42 ± 9
18	AIMASWYFPKDSPVTGLGHR	1500 ± 436
19	AIMYAWYFPKDSPVTGLGHR	18 ± 10
20	AIMYSAWYFPKDSPVTGLGHR	2833 ± 777
21	AIMYSWAWYFPKDSPVTGLGHR	2633 ± 208
22	AIMYSWYAPKDSPVTGLGHR	5.4 ± 3
23	AIMYSWYFAKDSPVTGLGHR	307 ± 153
24	AIMYSWYFPADSPVTGLGHR	89 ± 31
25	AIMYSWYFPKASPVVTGLGHR	97 ± 36
26	AIMYSWYFPKDAVVTGLGHR	57 ± 28
27	AIMYSWYFPKDSAVTGLGHR	76 ± 45
28	AIMYSWYFPKDSPATGLGHR	30 ± 11
29	AIMYSWYFPKDSPVAGLGH	25 ± 15
30	AIMYSWYFPKDSPVTALGH	23 ± 7.6
31	AIMYSWYFPKDSPVTGAGHR	24 ± 7
32	AIMYSWYFPKDSPVTGLAHR	220 ± 78
33	AIMYSWYFPKDSPVTGLGAR	48 ± 34
34	AIMYSWYFPKDSPVTGLGAA	283 ± 40
35	AIMYSWYFPKDSPVTGLGHRADWE	7.7 ± 1.7
36	AIMYSWYFPKDSPVTGLGHRHAW	6.2 ± 5.6
37	AIMYSWYFPKDSPVTGLGHRHDAE	7.3 ± 1.8
38	AIMYSWYFPKDSPVTGLGHRHDWA	7.3 ± 1.7

**Table 3-1: Ethylene-based identification of a minimum immunogenic motif within *Pp*NLP.**

### 3.1.4 Nlp20 (*Pp*NLP) does not exert any cytotoxic activity on *Arabidopsis*

To rule out a potential toxin activity within the nlp20 peptide pattern derived from the cytotoxin *Pp*NLP, leaf infiltration of *Arabidopsis* plants with nlp20 (*Pp*NLP) peptide was conducted. As shown in Figure 3-3 A, strong leaf necrosis developed upon infiltration of full-length recombinant *Pp*NLP, whereas equimolar (1  $\mu$ M) infiltration of synthetic nlp20 (*Pp*NLP) let the leaf completely intact as did the control infiltration with water. Even peptide infiltration of a 10  $\mu$ M solution did not exert any visible symptoms of necrosis. To quantify these observations, a calcein release assay with purified plasma membrane vesicles from *Arabidopsis* treated with native *Pp*NLP and nlp20 (*Pp*NLP) peptide was conducted. While a fast and strong release of calcein could be measured for the vesicles treated with 333 nM *Pp*NLP, no calcein release was observed for the peptide-treated vesicles, even not for those treated with a 10-fold higher peptide concentration (Fig. 3-3 B).

Consequently, nlp20 (*Pp*NLP) is not able to exert a toxic effect on *Arabidopsis* and can thus be clearly separated from toxin activity found for full-length *Pp*NLP (see also 3.1.1).

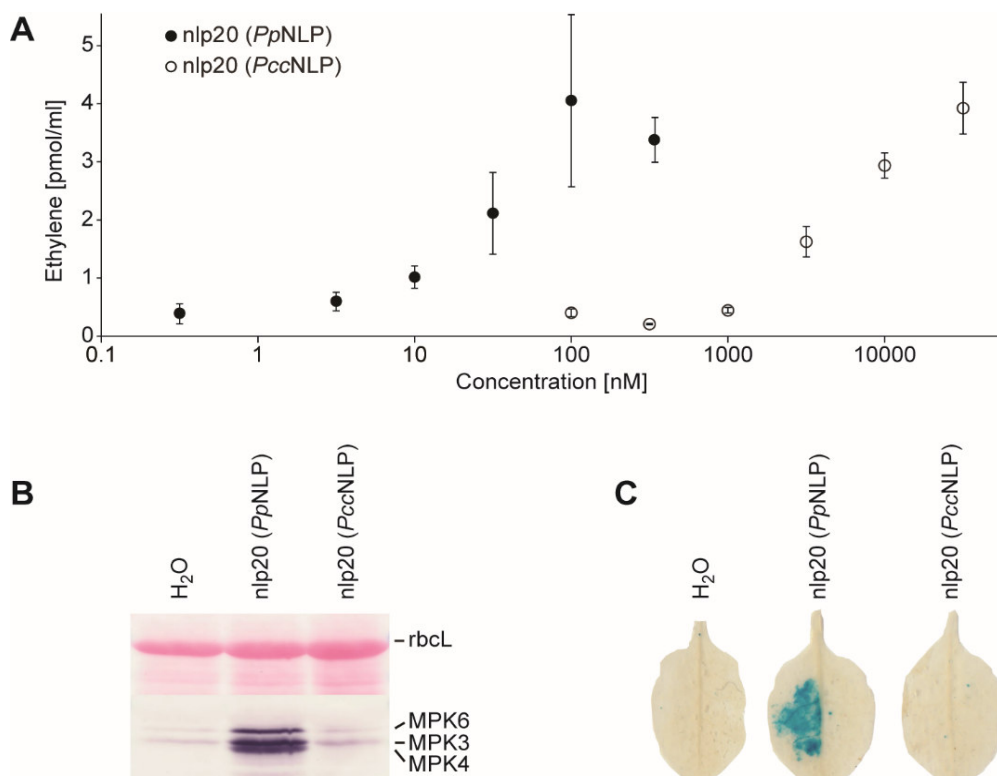


**Figure 3-3: Classification of nlp20 (*Pp*NLP) as non-toxic on *Arabidopsis*.**

Leaf necrosis 2 dpi of 1  $\mu$ M recombinant *Pp*NLP or 1  $\mu$ M and 10  $\mu$ M nlp20 (*Pp*NLP) peptide (A). Calcein release from purified plasma membrane vesicles upon incubation with 333 nM *Pp*NLP protein or 333 nM and 3333 nM peptide calculated in % of maximum release after Triton-X addition (B).

### 3.1.5 *Pcc*NLP does not contain an immunogenic core orthologous to nlp20 (*Pp*NLP)

Based on the present findings, the immunogenic potential of *Pp*NLP on *Arabidopsis* can not only be traced back to its cytotoxicity but also to the novel immunogenic peptide pattern nlp20. To complete the findings made in 3.1.1, a *Pcc*NLP peptide sequence orthologous to the immunogenic centerpiece within *Pp*NLP was synthesized (GSFYALYFLKDQILSGVNSGHR). This peptide, called nlp20 (*Pcc*NLP), was not capable to activate various immune responses tested in *Arabidopsis* like formation of ethylene, induction of MAP kinases or PR1::GUS gene expression whereas nlp20 (*Pp*NLP) elicited all responses tested (Fig. 3-4, A-C). These results show, that *Pcc*NLP does not harbor an elicitor-active motif orthologous to nlp20 (*Pp*NLP), whereby its immunogenic potential (Fig. 3-1) can be attributed to its cytotoxic activity.



**Figure 3-4: NLP 20 (*Pcc*NLP) does not induce immunity-associated defense responses in *Arabidopsis*.**

Ethylene formation 4 hours upon induction with different concentrations of nlp20 peptides derived from *Pp*NLP and *Pcc*NLP. Data points represent means of three samples  $\pm$  SD (A). Activation of MAP kinases 15 minutes after infiltration of 100 nM nlp20 (*Pp*NLP) or nlp20 (*Pcc*NLP) detected by p44/p42 anti-phospho antibody. Ponceau S staining of rbcL (Ribulose bisphosphate carboxylase large chain) is shown as loading control (B). PR1::GUS gene activation upon infiltration of 100 nM peptide. Histochemical staining was performed 1 dpi (C). One representative experiment out of three is shown.

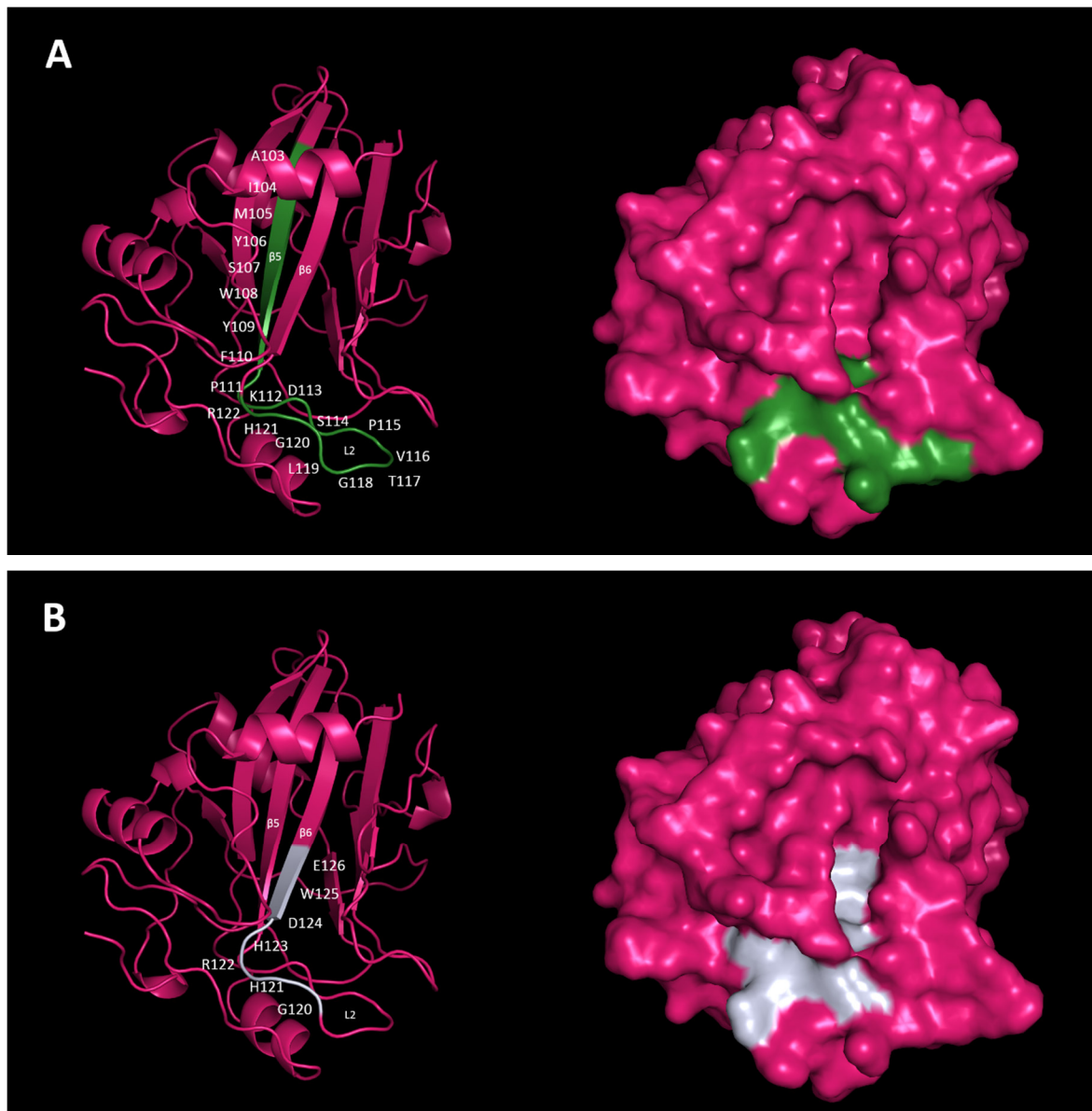


### 3.1.6 Position of the nlp20 motif within *PpNLP*

*PpNLP* exhibits a high primary sequence identity of 63,6 % to the crystallized NLP from *Pythium aphanidermatum* (*PyaNLP*), and displays almost identical structural fold conservation (Küfner 2009, Ottmann, *et al.* 2009). The *PyaNLP* crystal revealed a single-domain molecule consisting of a central  $\beta$ -sandwich (3+5 strands), three  $\alpha$ -helices at the top and three broad loops (L1, L2 and L3) at the base of the molecule. A negatively charged cavity above L2 and L3 at the surface of the protein coordinates a divalent cation, comprising the highly conserved hepta-peptide motif with amino acids assigned to be crucial for toxin activity (Ottmann, *et al.* 2009).

To gain insight into the localization of the nlp20 motif within the *PpNLP* protein, a protein model of *PpNLP* lacking the N-terminal signal peptide was created applying the Phyre2 web portal for protein modeling, prediction and analysis (Kelley, *et al.* 2015) and the graphics software PyMOL for digital imaging. The left panel of Figure 3-5 A shows the cartoon model of *PpNLP* with the nlp20 motif spanning amino acids A103-R122 highlighted in green, building  $\beta$ 5 (A103-Y109) and L2 (E110-R122). The right panel depicts the surface model, whereby  $\beta$ 5 is located inside the protein and residues of L2 can be found on the surface of the molecule only. These superficial residues are not elicitor-active and would require remote interior residues A103-Y106 in  $\beta$ 5 for immune stimulating activity (table 3-1, peptides 4, 5 and 6). For comparison, localization of the hepta-peptide motif is shown in Figure 3-5 B. Residues of the motif G120-E126 are surface-exposed, mainly span  $\beta$ 6 and are highlighted in grey. Thus, nlp20 and the hepta-peptide motif share three residues G120, H121 and R122 at the end of L2 and the beginning of  $\beta$ 6.

The position of the nlp20 motif within *PpNLP* might indicate that structural reorganization of the protein takes place upon the event of plant plasma membrane contact, leading to subsequent pattern recognition through the unfolded peptide pattern. This hypothesis is supported by findings made in 3.1.1 (Fig 3-1 C, D, E), where it is shown that non-toxic mutagenized and denatured *PpNLP* versions are capable of inducing immunity in *Arabidopsis*.



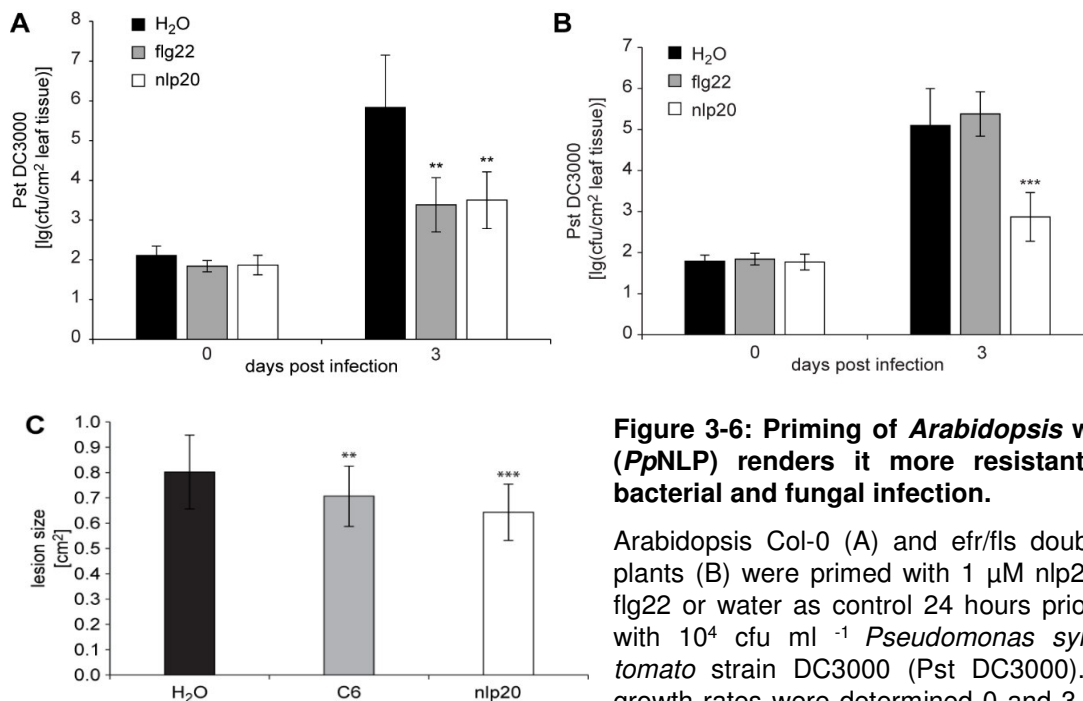
**Figure 3-5: Localization of nlp20 and the hepta-peptide motif within *PpNLP*.**

Cartoon (left panel) and surface view (right panel) of *PpNLP* showing the position of nlp20 (green, A) and the hepta-peptide motif (grey, B). Modeling was done with Phyre2 web portal for protein modeling, prediction and analysis and PyMOL software for graphical editing.

### 3.1.7 Pre-treatment with nlp20 (*PpNLP*) renders *Arabidopsis* less susceptible to bacterial and fungal infection

Priming is a state of induced resistance, associated with an enhanced immune reaction of plants to microbial attack at the advent of infection (Conrath *et al.* 2015). To test whether nlp20 (*PpNLP*) primes a defense response in *Arabidopsis*, leaves were infiltrated with 1  $\mu\text{M}$  peptide solution and infected with *Pseudomonas syringae* pv. *tomato* DC3000 24 hours later. After three days, significantly reduced bacterial growth rates could be observed for nlp20 (*PpNLP*)-treated plants when compared to mock-treated samples, just as for flg22-treated plants (Fig 3-6 A). This priming effect could be shown on *fls2/efr* double mutant plants as well, thereby ruling out a contamination with flg22 within the nlp20 (*PpNLP*) peptide preparation (Fig. 3-6 B). Likewise, *Arabidopsis* plants infected with the fungus *Botrytis cinerea* showed significantly smaller lesion size development when primed with nlp20 (*PpNLP*) or the chitin hexamer C6, when compared to mock-treated leaves (Fig. 3-6 C).

Thus, nlp20 (*PpNLP*) is capable to establish a primed state in *Arabidopsis* and thereby provides enhanced protection against bacterial and fungal infection.



**Figure 3-6: Priming of *Arabidopsis* with nlp20 (*PpNLP*) renders it more resistant towards bacterial and fungal infection.**

*Arabidopsis* Col-0 (A) and *efr/fls* double mutant plants (B) were primed with 1  $\mu\text{M}$  nlp20 peptide, flg22 or water as control 24 hours prior infection with  $10^4$  cfu ml<sup>-1</sup> *Pseudomonas syringae* pv. *tomato* strain DC3000 (Pst DC3000). Bacterial growth rates were determined 0 and 3 days after infection. Each bar represents mean value of 6 replicates  $\pm$  SD, asterisks indicate significant differences over control treatment determined by Student's t test, \*\* $P \leq 0,01$  (A, B). Spot inoculation with 5  $\mu\text{l}$  of a  $5 \times 10^6$  ml<sup>-1</sup> solution of *Botrytis cinerea* spores was performed 24 hours after priming with 1  $\mu\text{M}$  nlp20, chitin hexamer C6 or water respectively. Data represents means of  $n=28$  samples per treatment. Asterisks show significant differences over control treatment, \*\* $P \leq 0,01$ , \*\*\* $P \leq 0,001$  (C). All experiments were done in triplicate with similar results.

### 3.1.8 Immunogenic nlp20 (*Pp*NLP) orthologous sequences can be found in bacteria, fungi and oomycetes

NLPs are widely distributed microbial virulence factors that can be found in bacteria, fungi and oomycetes (Gijzen and Nurnberger 2006). To examine if an immunogenic nlp20 pattern is present in all three kingdoms of life, peptides derived from species of all kingdoms with sequences orthologous to the nlp20 (*Pp*NLP) motif were synthesized and tested for their ability to induce ethylene formation in *Arabidopsis*. Table 3-2 shows EC<sub>50</sub> values  $\pm$  SD determined for nlp20 (*Pp*NLP) and five orthologous peptides; one derived from another oomycete, *Pythium aphanidermatum* (nlp20 (*Pya*NLP)), two fungus-derived sequences from *Fusarium oxysporum* (nlp20 (*Fo*NLP)) and *Botrytis cinerea* (nlp20 (*Bc*NLP)) and two of bacterial origin from *Bacillus halodurans* (nlp20 (*Bh*NLP)) and *Bacillus subtilis* (nlp20 (*Bs*NLP)). All peptides were capable of inducing ethylene production with EC<sub>50</sub> values comparable to the EC<sub>50</sub> of nlp20 (*Pp*NLP) ( $14 \pm 12$  nM), ranging from  $5,7 \pm 2,4$  nM to  $43 \pm 13$  nM (Table 2, Fig. 3-7 A).

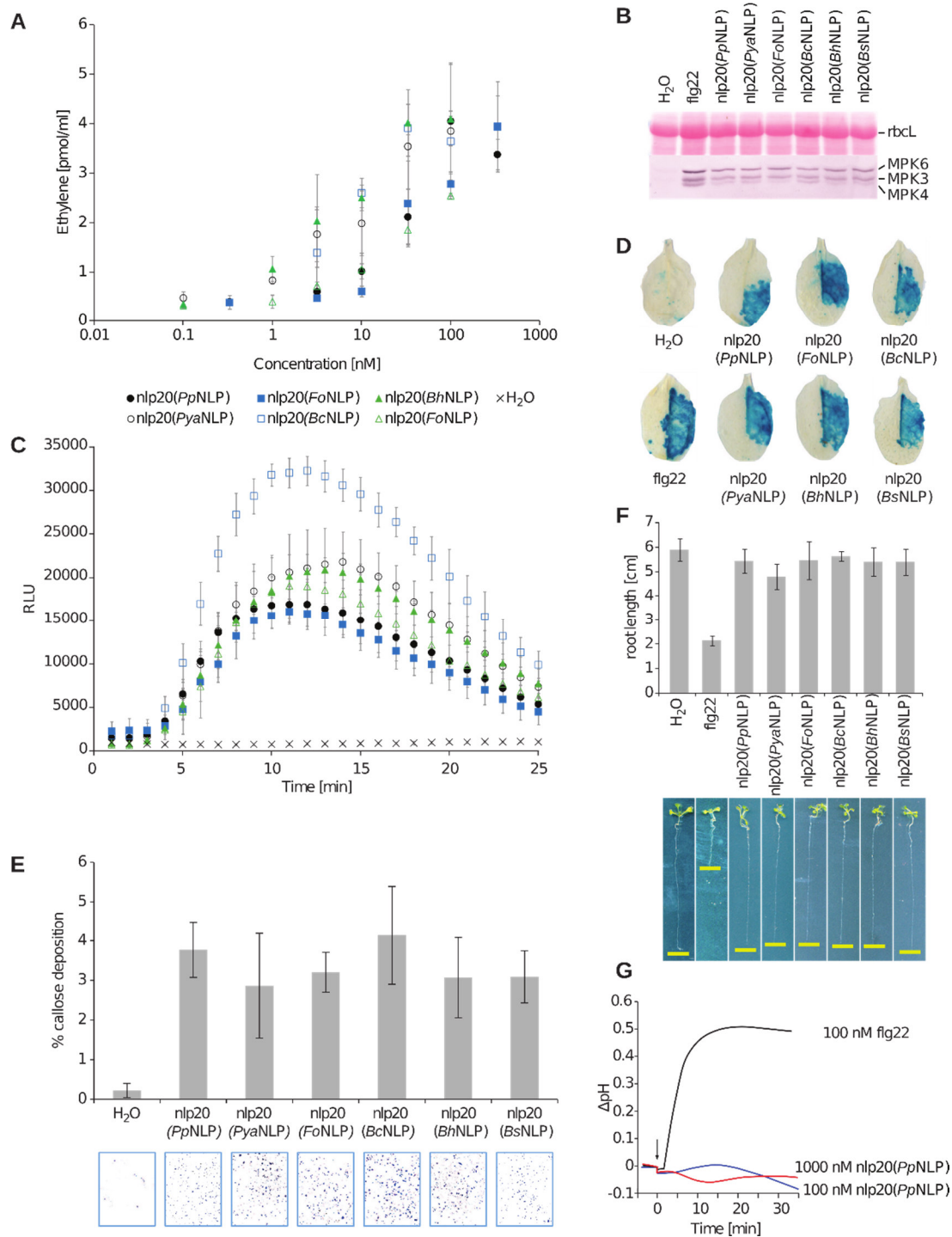
This immunogenic potential could not only be observed for ethylene formation, but also in additional common read outs of immune stimulating activities like the activation of MAP kinases (Fig. 3-7 B), initiation of an oxidative burst (Fig. 3-7 C), the induction of PR1::GUS gene expression (Fig. 3-7 D) and the deposition of callose (Fig. 3-7 E) all elicited by 100 nM peptide concentration. Interestingly, none of the peptides provoked seedling growth inhibition on *Arabidopsis* seedlings, which were grown on media supplemented with 1  $\mu$ M of the respective peptide. Flg22-treated roots instead showed a significant growth reduction of about 65% when compared to the water control (Fig. 3-7 F). Similarly, 100 nM of flg22 peptide triggered an extremely fast and strong extracellular shift of the pH in a medium alkalization assay ( $\Delta$ pH  $\approx$  0,5), whereas nlp20 (*Pp*NLP) did not show any effect on the  $\Delta$ pH, not even with a 10-fold higher final concentration of 1000 nM (Fig. 3-7 G).

In sum, oomycete-derived nlp20 (*Pp*NLP) and orthologs from bacteria and fungi are able to induce a comprehensive set of immune responses in *Arabidopsis*, but not seedling growth inhibition and extracellular pH-shift. This might point to differential downstream signaling upon ligand perception by RLPs or RLKs.

Organism	peptide	peptide sequence	ethylene induction (EC <sub>50</sub> in nM ± SD)
<i>Phytophthora parasitica</i>	nlp20 ( <i>Pp</i> NLP)	A I M Y S W Y F P K D S P V T G L G H R	14 ± 12
<i>Pythium aphanidermatum</i>	nlp20 ( <i>Pya</i> NLP)	A I M Y S W Y M P K D S P S T G I G H R	8.9 ± 2.7
<i>Fusarium oxysporum</i>	nlp20 ( <i>Fo</i> NLP)	A I M Y A W Y W P K D Q P A D G N L V S G H R	43 ± 13
<i>Botrytis cinerea</i>	nlp20 ( <i>Bc</i> NLP)	A I M Y S W Y M P K D E P S T G I G H R	7.7 ± 3.2
<i>Bacillus halodurans</i>	nlp20 ( <i>Bh</i> NLP)	A I M Y A W Y F P K D S P S P G L G H R	5.7 ± 2.4
<i>Bacillus subtilis</i>	nlp20 ( <i>Bs</i> NLP)	A I M Y S W Y F P K D E P S P G L G H R	11 ± 0.3

**Table 3-2: Nlp20 (*Pp*NLP) orthologous peptides of oomycete, fungal and bacterial origin show similar elicitor activity in *Arabidopsis*.**

## Results



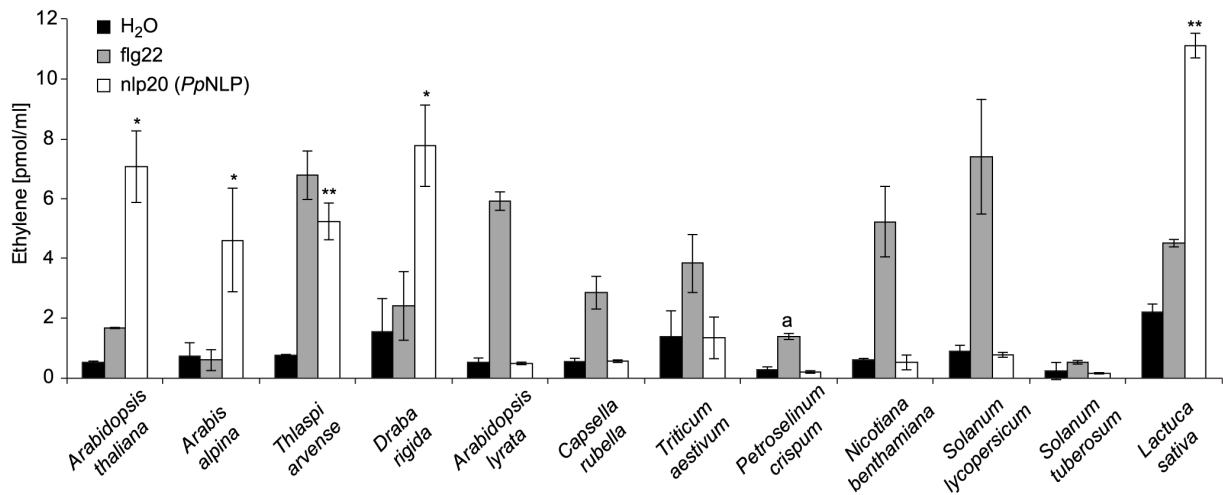
**Figure 3-7: Nlp20 (*PpNLP*) and its orthologs elicit immunity-associated defense responses in *Arabidopsis*.**

100nM of orthologous peptides (table 3-2) were leaf infiltrated to determine ethylene formation (A), MAP kinase activation (B), production of reactive oxygen species (C), PR1::GUS gene expression (D) and apposition of callose (E). Callose deposition is given in %  $\pm$  SD of three image sections counted as pixels after clearing of background and leaf-veins (E). *Arabidopsis* seedlings were grown in liquid  $\frac{1}{2}$  MS medium supplemented with 1  $\mu$ M nlp20 (*PpNLP*) peptide, its orthologs or flg22 respectively and grown under short day conditions for 2 weeks. For root length determination, seedlings were transferred onto agar plates. Bars in the upper panel represent root length in cm  $\pm$  SD of three representative seedlings depicted in the lower panel. Yellow marks highlight the position of the root tips (F). Extracellular pH shift of *Arabidopsis* cell suspensions after application of nlp20 (*PpNLP*) in given concentrations or 100 nM flg22 (G). Data points represent means of three samples  $\pm$  SD. All experiments were performed three times with similar results.

### 3.1.9 Screening of various plant families for their ability to recognize nlp20 (PpNLP)

To investigate whether the ability to perceive nlp20 (PpNLP) is restricted to *Arabidopsis thaliana* or can be found in other plants as well, an ethylene screen of different plant species was conducted. As depicted in Fig. 3-8, close relatives of *Arabidopsis thaliana*, thus members of the *Brassicaceae* family namely *Arabis alpina*, *Thlaspi arvense* and *Draba rigida* exhibited a distinct ethylene formation when treated with 1  $\mu$ M nlp20 (PpNLP). However, two other closely related plant species, *Arabidopsis lyrata* and *Capsella rubella*, did not produce any ethylene upon nlp20 (PpNLP) treatment (Fig. 3-8). Moreover, the monocot species *Triticum aestivum* (*Poaceae*) failed to trigger ethylene production as did so a member of the *Apiaceae* family, *Petroselinum crispum*, what is in accordance with findings made by Fellbrich, *et al.* (2002). Here, Pep-13 from *P.sojae* transglutaminase, was used as a positive control instead of flg22 peptide. Three solanaceous species (*Nicotiana benthamiana*, *Solanum lycopersicum* and *Solanum tuberosum*) were also not capable to produce ethylene, whereas lettuce (*Lactuca sativa*, an *Asteraceae*) exerted a strong ethylene formation upon peptide treatment (Fig. 3-8). 13 further members of the *Asteraceae* were tested for their capacity to induce ethylene formation (data not shown), but lettuce remained the only responder within this family. Also, among five vegetable cultivars from *Brassica oleracea* could none be identified as nlp20-sensitive (*Brassica oleracea* var. *gongylodes*, *Brassica oleracea* var. *italica*, *Brassica oleracea* var. *botrytis*, *Brassica oleracea* convar. *capitata* var. *alba*, *Brassica oleracea* convar. *capitata* var. *rubra*) as could none be identified out of 14 plants from 11 different plant families (*Adoxaceae*, *Araceae*, *Betulaceae*, *Convolvulaceae*, *Fabaceae*, *Malvaceae*, *Oleaceae*, *Plantaginaceae*, *Rosaceae*, *Rubiaceae* and *Urticaceae*, data not shown).

With this, three close *Arabidopsis* relatives and lettuce could be assigned receptive towards nlp20 (PpNLP).



**Figure 3-8: Identification of nlp20 (*PpNLP*)-sensitive plants.**

Ethylene formation of different plant species 4 hours after induction with 1  $\mu$ M nlp20 (*PpNLP*). Control treatments were performed with water and 1  $\mu$ M flg22. Pep-13 from *P.sojae* transglutaminase was used as positive control for *P.crispum* (a). Bars represent means  $\pm$  SD of three samples and asterisks show significant differences over water control as determined by Student's t test, \* $P \leq 0,05$ , \*\* $P \leq 0,01$ . One out of three experiments with similar results is shown.

### 3.2 An RLP23-SOBIR1-BAK1 receptor complex mediates nlp20 (*PpNLP*) recognition in *Arabidopsis*

Plants are able to sense an ample spectrum of PAMPs which might be of carbohydrate, proteinaceous or lipophilic origin (Boller and Felix 2009). Various receptor types exist which are implicated in sensing microbial patterns, either alone or jointly with other receptors by building complexes (Böhm, *et al.* 2014a, Zipfel 2014). LRR-RKs (leucine-rich repeat receptor-like kinases) like FLS2, the receptor for flg22, consist of an extracellular LRR domain, a transmembrane domain adjacent to juxta domain regions and a cytoplasmic kinase domain, whereas LRR-RPs (leucine-rich repeat receptor-like proteins) lack a cytoplasmic signaling domain.

Aim of this chapter was to identify and characterize the perception system of nlp20 in *Arabidopsis*. Reverse genetics were applied to identify the components of the signaling entity. Utilizing an interfamily receptor transfer, the potential of the nlp20 receptor for conferring resistance against NLP-expressing pathogens to crop plants should be examined. Furthermore, interaction studies should provide insight into nlp20-dependent and independent receptor interactions. If not stated otherwise in this section, nlp20 always refers to the *Phytophthora parasitica*-derived nlp20 (*PpNLP*).



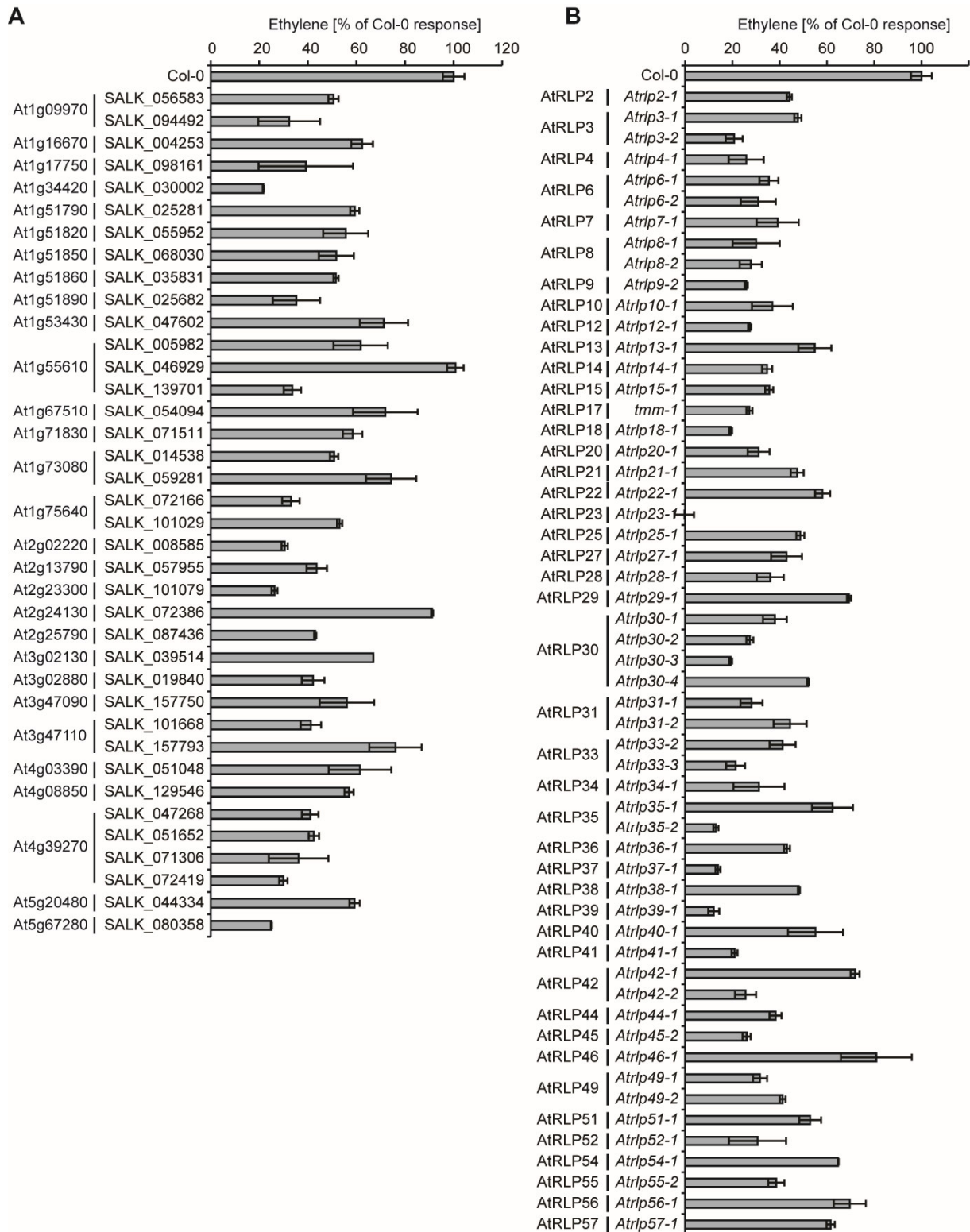
### **3.2.1 Identification of the leucine-rich repeat receptor protein RLP23 as the receptor for nlp20**

#### **3.2.1.1 A reverse genetic screen identifies the LRR-RP RLP23 as a prerequisite for nlp20-mediated immune signaling in *Arabidopsis***

In *Arabidopsis*, LRR-type PRR mediated immune signaling is often initiated by proteinaceous ligands (Böhm, *et al.* 2014a). To identify PRRs which are involved in recognition of nlp20, LRR-RK and LRR-RP T-DNA insertion mutant collections (Wang, *et al.* 2008, Postel, *et al.* 2010) were screened for ethylene formation upon treatment with nlp20 (*PpNLP*). In all of the tested 38 mutant genotypes representing 29 LRR-RKs an ethylene response was triggered upon treatment with 1  $\mu$ M of the ligand (Fig. 3-9 A, ethylene response shown in % of Col-0 response). Also, 55 mutant genotypes representing 44 LRR-RPs were tested whereby the mutant allele *Atrlp23-1* (SALK\_034225) proved to be insensitive to nlp20 (*PpNLP*) treatment (Fig. 3-9 B).

This suggests that the LRR-RP RLP23 (At2g32680) is required for nlp20-induced immune signaling in *Arabidopsis*.

## Results

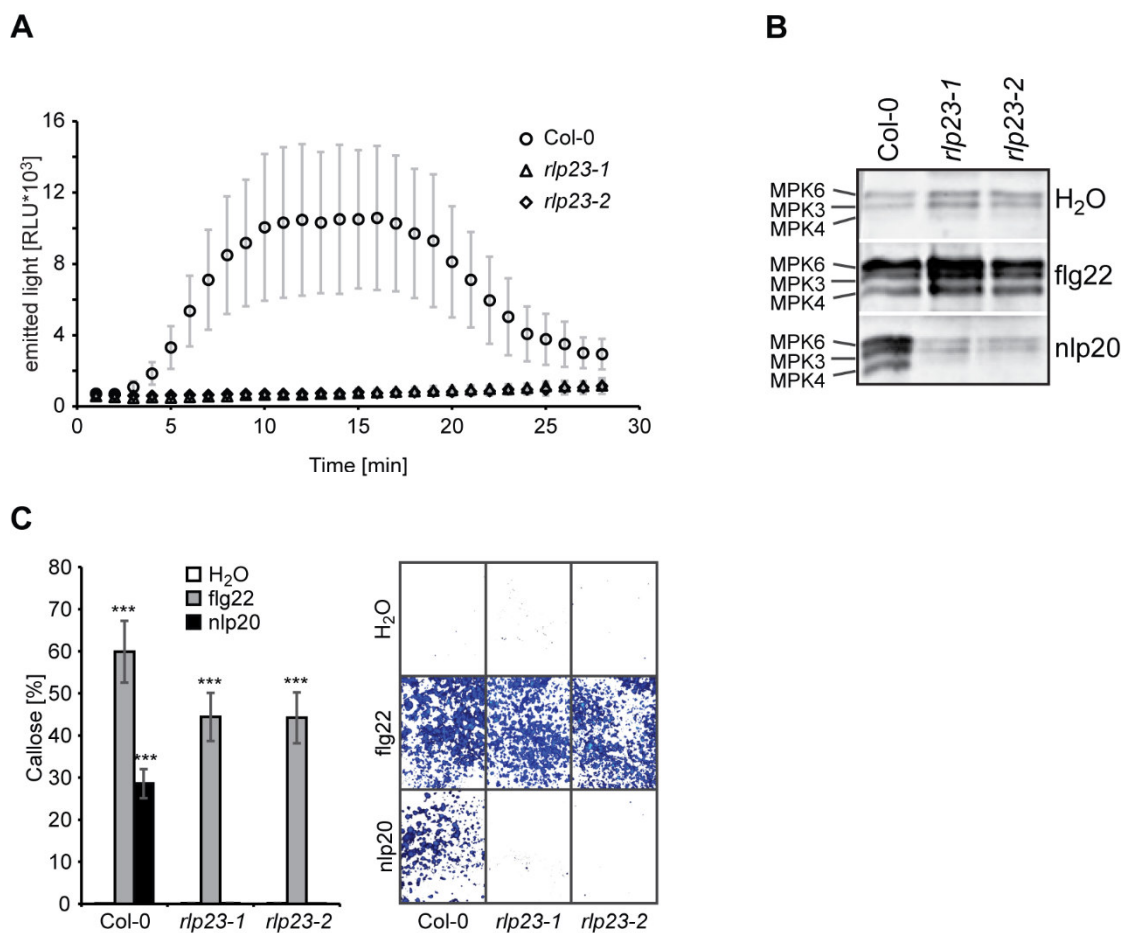


**Figure 3-9: Screen for nlp20-insensitive RLP and RLK T-DNA insertion mutants.**

38 T-DNA insertion mutants for 29 RLKs (A) and 55 T-DNA mutants for 44 RLPs (B) were tested for ethylene formation upon induction with 1  $\mu$ M nlp20 after 4 hours. Bars represent means of two replicates  $\pm$  SD as percentage of the response determined for Col-0.

To further substantiate this finding, *Atrlp23-1* mutant plants were tested together with another RLP23 T-DNA insertion mutant, *Atrlp23-2* (GK-738D01), for additional immunity-associated defense responses like production of reactive oxygen species, activation of MAP kinases and deposition of callose. Upon treatment with 1  $\mu$ M nlp20, both mutant lines failed to generate all responses tested but responded normally to flg22 treatment (Fig. 3-10 A-C).

This indicates specificity of nlp20 recognition by RLP23 due to the lack of functional RLP23.



**Figure 3-10: Nlp20-mediated defense responses are abrogated in *rlp23* mutant plants.**

Production of reactive oxygen species (A), activation of MAP kinases (B) and callose deposition (C) were determined for T-DNA insertion mutant lines *rlp23-1* and *rlp23-2* upon induction with 1  $\mu$ M nlp20 peptide. Water and flg22 were used as control treatments. Callose deposition is determined as percentage of the image section (one out of three representative pictures shown in the right panel) calculated in pixels. Bars and data points represent three replicates  $\pm$  SD, asterisks mark significant differences over control treatment determined by Student's t test, \*\*\* $P \leq 0,001$  (C).

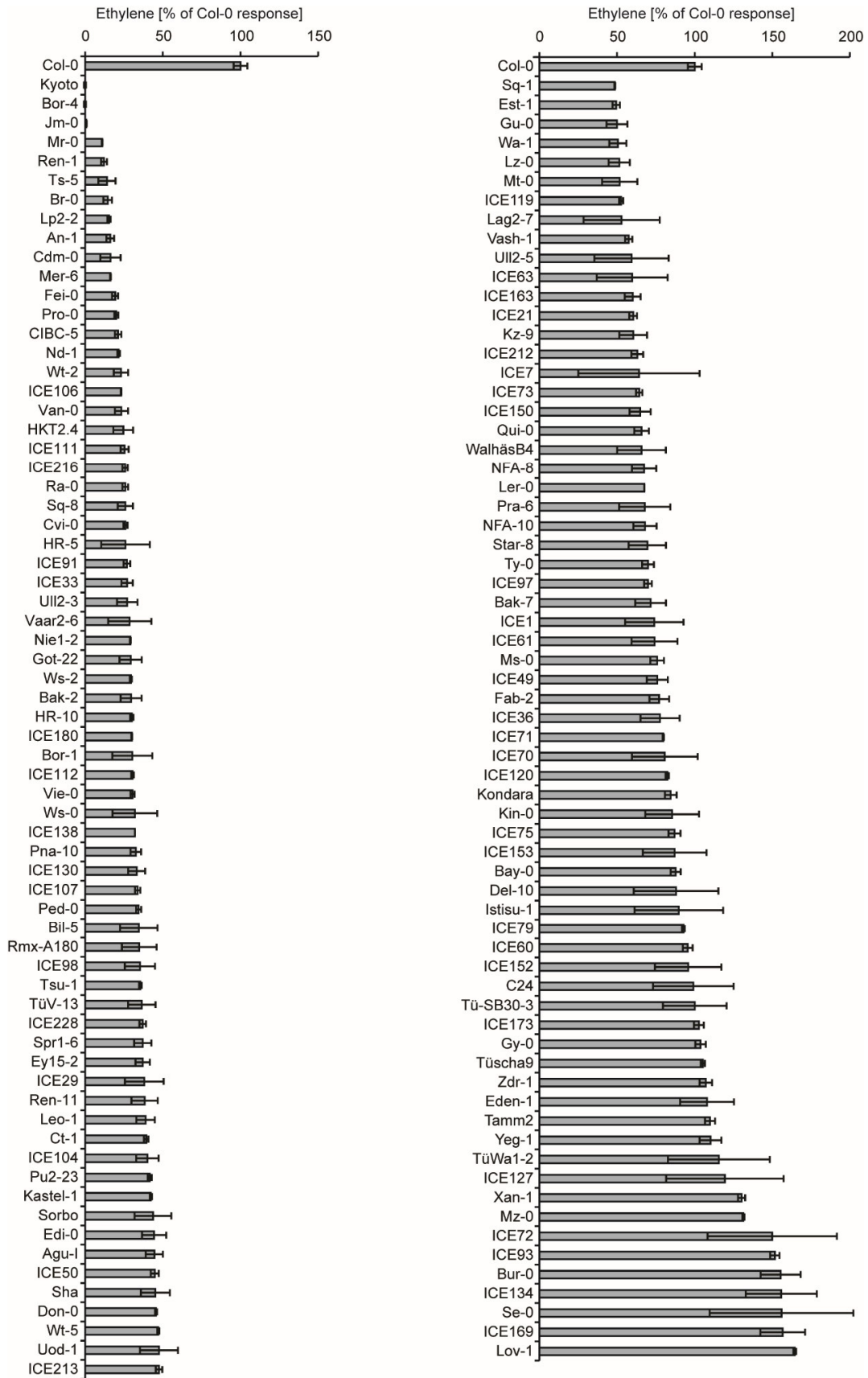
### 3.2.1.2 Identification of nlp20-insensitive *Arabidopsis* accessions

In the past, several orphan PAMPs could be assigned to their corresponding receptors via forward genetic approaches, like in screening and mapping attempts of insensitive *Arabidopsis* accessions (Gomez-Gomez and Boller 2000, Jehle, *et al.* 2013b, Zhang, *et al.* 2013). Hence, natural genetic variation in different accessions might serve as genetic tool to identify PRRs of solitary ligands.

In a screen of 135 *Arabidopsis* accessions, three turned out to be impeded in ethylene formation (Fig. 3-11, ethylene formation in % of Col-0 response). All of the three insensitive ecotypes Kyoto, Bor-4 and Jm-0 exhibited the same frame-shift mutations within LRR 13 leading to an early stop codon, what explains the lack of responsiveness of these ecotypes towards treatment with nlp20.

These results reinforce the assumption of RLP23 being the receptor for nlp20.

## Results



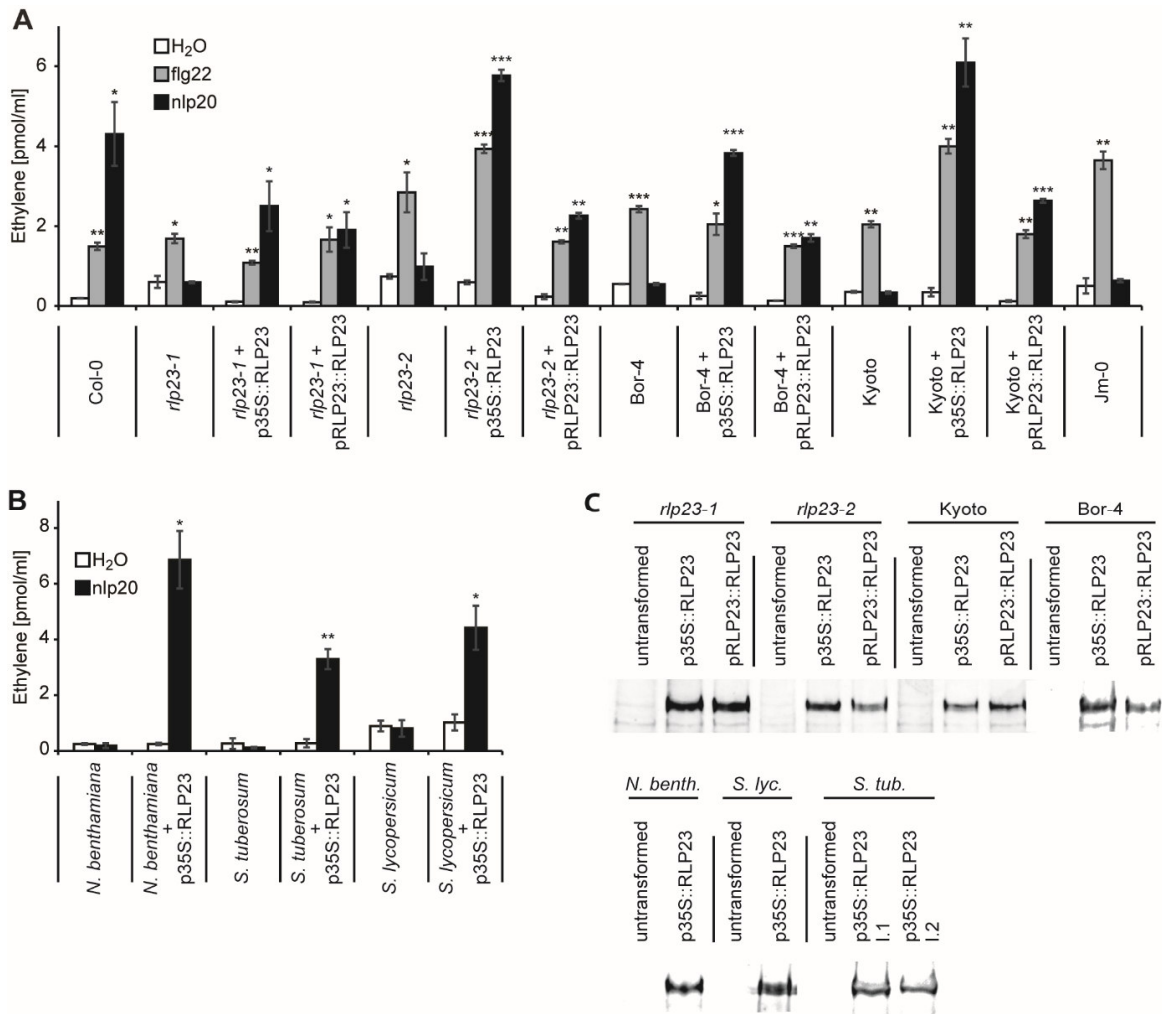
**Figure 3-11: Responsiveness of *Arabidopsis thaliana* accessions towards nlp20 treatment.**

Ethylene production of 135 accessions 4 hours after treatment with 1 μM nlp20. Bars represent means of two samples ± SD given in percent of the response determined for Col-0.

### 3.2.1.3 Complementation of insensitive plants with functional RLP23

To confirm that the insensitivity towards nlp20 of RLP23 T-DNA mutant plants and accessions Kyoto, Bor-4 and Jm-0 is due to the lack of functional RLP23 protein, complementation via stable expression of RLP23 was conducted. Expression of C-terminally GFP-tagged RLP23 under the control of a 35S promoter (*p35S::RLP23:GFP*) restored sensitivity to nlp20 of the mutant genotypes *rlp23-1* and *rlp23-2*, as well as of the ecotypes Bor-4 and Kyoto (formation of ethylene, Fig. 3-12 A). The same applied for the expression of GFP-tagged RLP23 under the control of the native promoter (*pRLP23::RLP23:GFP*), whereas sensitivity to flg22 remained unaltered (Fig. 3-12 A). Moreover, expression of *p35S::RLP23:GFP* in *Nicotiana benthamiana*, potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*), three solanaceous plants previously identified as insensitive to nlp20 (Fig. 3-8), transformed these plants to ethylene responders when treated with the ligand (Fig 3-12 B). Expression of RLP23 could be confirmed in all transgenic plants via western blotting with GFP-specific antiserum (Fig 3-12 C).

In summary, these findings show that RLP23 is needed for nlp20-specific recognition and immune activation in *Arabidopsis* and confers sensitivity to solanaceous species across genus boundaries.

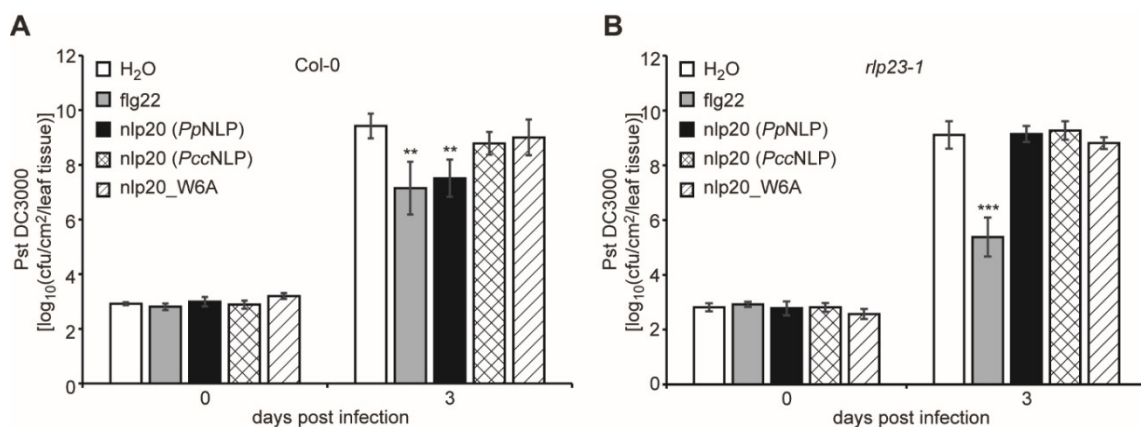


**Figure 3-12: Complementation of nlp20-insensitive plants with RLP23.**

Accumulation of ethylene upon induction with 1  $\mu$ M nlp20, flg22 or water as control in *rlp23* mutants, insensitive *Arabidopsis* accessions and in stably transformed p35S::RLP23:GFP and pRLP23::RLP23:GFP plants (A) and in transgenic p35S::RLP23:GFP *N. benthamiana*, *S. tuberosum* and *S. lycopersicum* plants (B). Bars show means  $\pm$  SD of three replicates. Asterisks indicate significant differences over control treatment determined by Student's t test, \*P $\leq$ 0,05, \*\*P $\leq$ 0,01, \*\*\*P $\leq$ 0,001. Experiments were performed in triplicate. Western blot analysis of RLP23 expression in stably transformed p35S::RLP23:GFP and pRLP23::RLP23:GFP *rlp23* mutant genotypes, insensitive accessions and complemented *N. benthamiana*, *S. lycopersicum* and *S. tuberosum* plants with GFP-specific antiserum (C).

### 3.2.2 RLP23 is required for nlp20-induced resistance against the bacterium *Pseudomonas syringae* pv. *tomato* DC3000

Nlp20 is able to prime a defense response in *Arabidopsis* Col-0 ecotypes leading to reduced disease symptoms upon infection with the bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (Fig. 3-6 A, Fig. 3-13 A). However, biologically inactive peptide derivatives nlp20 (*PccNLP*) and nlp20\_W6A (peptide 20, Table 3-1) were not able to induce a priming effect and bacterial growth remained comparable to the growth observed in mock-treated plants (Fig. 3-13 A). Interestingly, nlp20 did not exert such a priming effect on *rlp23-1* mutant plants and bacterial growth rates were as high as for mock-treated plants, or treatment with the two inactive peptide derivatives nlp20 (*PccNLP*) and nlp20\_W6A (Fig 3-13 B). Flg22-mediated enhanced resistance was not affected in *rlp23-1* mutant plants, highlighting once more the specificity of RLP23-mediated perception of nlp20 in *Arabidopsis*.



**Figure 3-13: RLP23-mediated priming effect of nlp20.**

Bacterial growth rates in *Arabidopsis* Col-0 (A) and *rlp23-1* (B) genotypes 0 and 3 dpi with *Pseudomonas syringae* pv. *tomato* DC3000. Priming of plants with 1  $\mu$ M nlp20 (*PpNLP*) or immunogenically inactive peptides nlp20 (*PccNLP*) or nlp20\_W6A was performed 24 hours prior infection. Water and flg22 served as controls. Bars represent means of six replicates, asterisks indicate significant differences over water control treatment, \*\* $P \leq 0,01$ , \*\*\* $P \leq 0,001$ . One out of three representative experiments is shown.

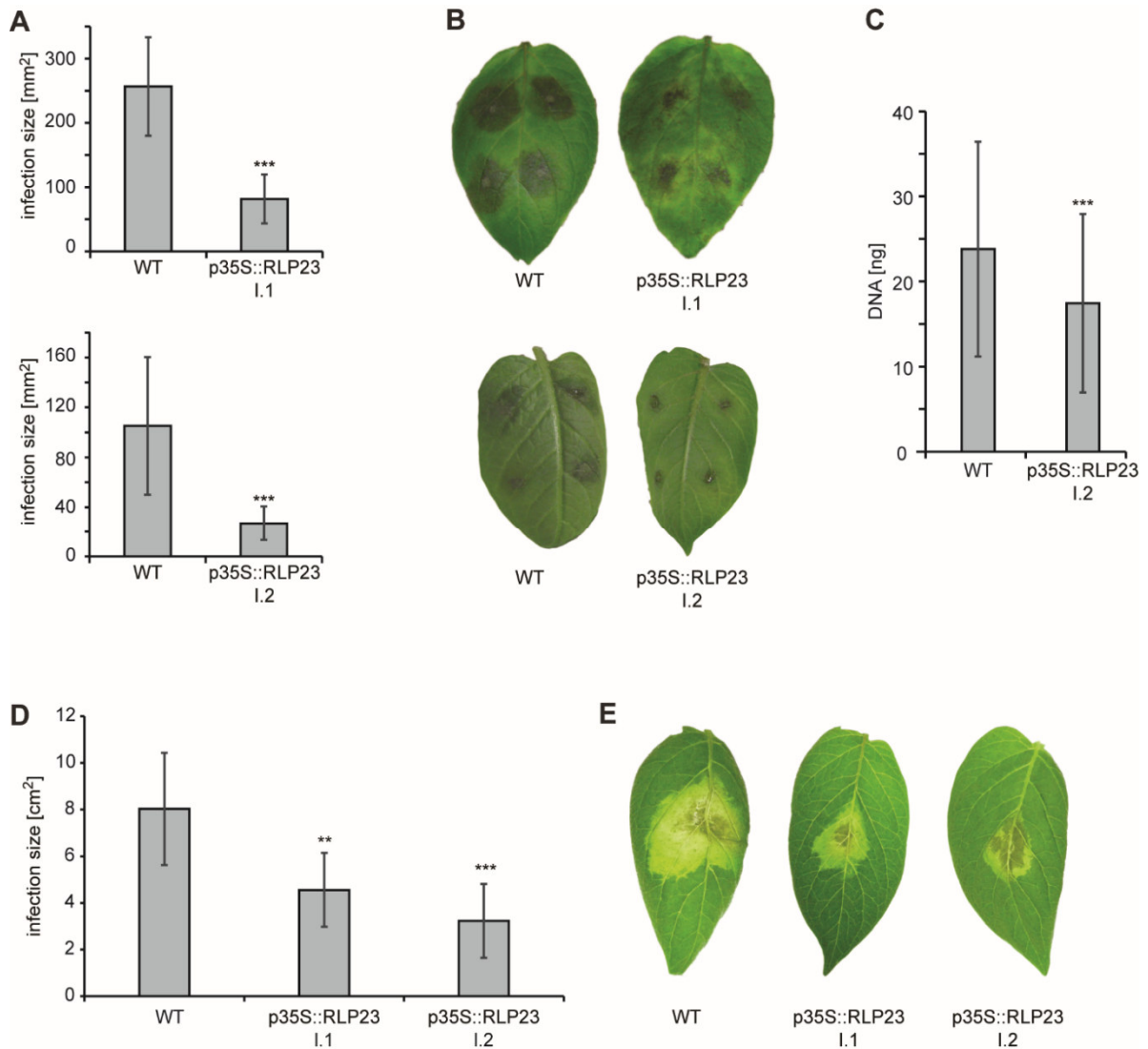


### 3.2.3 Stable expression of RLP23 in potato confers enhanced disease resistance to pathogens from different kingdoms of life

Transfer of PRRs between different plant families or cultivars involves the potential of creating crops with strikingly increased disease resistance against detrimental pathogens as shown for the bacteria *Xanthomonas oryzae*, *X. campestris* or *X. axonopodis* (Wang et al. 1996, Mendes et al. 2010, Tripathi et al. 2014, Schwessinger et al. 2015), *Ralstonia solanacearum* (Lacombe et al. 2010), the fungi *Verticillium dahliae* or *V. albo-atrum* (Fradin et al. 2011) or the oomycete *Phytophthora infestans* (Du, et al. 2015b). To investigate whether transfer of RLP23 into other plant species reveals such an effect, transgenic potato plants expressing RLP23 were challenged with pathogens expressing NLPs with an elicitor active nlp20 motif. As model pathogens the oomycete *Phytophthora infestans* (strain 88069) and the fungus *Sclerotinia sclerotiorum* (strain 1980) were chosen. *Sclerotinia sclerotiorum* expresses two NLPs (NPP1 SS1G\_11912.3; NPP2 SS1G\_03080.3) of which the orthologous nlp20 (*PpNLP*) sequences were tested as elicitor active in ethylene formation in *Arabidopsis* (nlp20 (*SsNLP1*): GIMYAWYFPKDQPAAGNVVGGHR and nlp20 (*SsNLP2*): GLMYSWYMPKDEPSPGIGHR, data not shown). No bacterial contender could be found, since no NLP-expressing bacteria containing an elicitor active nlp20 motif exist which are suitable for laboratory plant-pathogen assays.

Two transgenic potato lines stably expressing RLP23 (line I.1 and I.2) under the control of an 35S promoter exhibited an increased disease resistance with significantly smaller infection sites as wild-type potato leaves, when infected with the devastating pathogen *Phytophthora infestans* (Fig. 3-14 A, B). Moreover, qPCR-based quantification of the oomycete DNA revealed a significantly lower DNA content in RLP23-expressing potato leaves of line I.2 than in wild-type samples (Fig. 3-14 C). Strikingly, the same observations could be made for infections with the fungus *Sclerotinia sclerotiorum*, as potato leaves of transgenic lines I.1 and I.2 showed significantly smaller lesion sizes when compared to untransformed control leaves (Fig. 3-14 D, E).

These observations show that expression of RLP23 in the crop potato confers increased resistance to destructive oomycete and fungal pathogens.



**Figure 3-14: RLP23-mediated pathogen resistance.**

Lesion size development of *Phytophthora infestans* strain 88069 4 days after drop inoculation of a  $5 \times 10^4$  zoospores ml<sup>-1</sup> solution on transgenic p35S::RLP23:GFP *S. tuberosum* plants. Bars represent means  $\pm$  SD of  $n = 24$  samples (I.1) or  $n = 32$  samples (I.2) (A). 2 representative leaves of line I.1 or line I.2 are shown (B). qPCR-based quantification of the oomycete DNA in line I.2, bars represent means of  $n = 60$  samples (C). Infection size assessment 2 dpi of transgenic potato lines I.1 and I.2 with the fungus *Sclerotinia sclerotiorum* strain 1980. Bars represent means of  $n = 9$  leaves per line (D). Representative leaves are shown (E). Asterisks indicate significant differences over untransformed wild-type plants, \*\* $P \leq 0,01$ , \*\*\* $P \leq 0,001$ , determined by Student's t test. All experiments were performed three times with similar results.

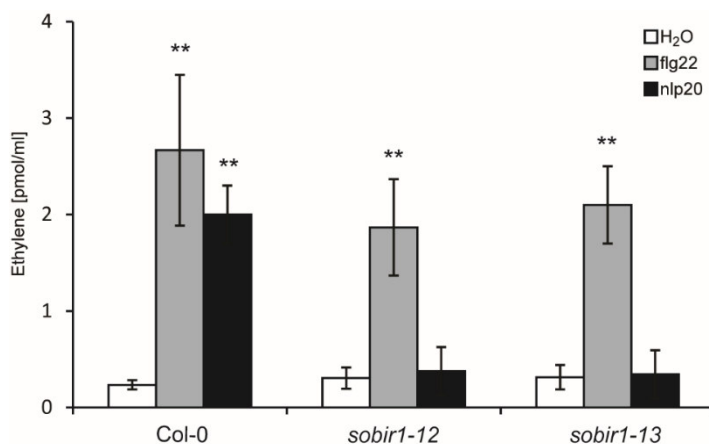
### 3.2.4 Characterization of the RLP23 receptor complex

LRR-RPs like RLP23 are proteins with a small cytoplasmic tail only, lacking any obvious signaling domains. Therefore it was investigated, if RLP23 recruits signaling partners with kinase domains to enable downstream signaling.

#### 3.2.4.1 The LRR-RK SOBIR1 is involved in nlp20-sensing

The LRR-RK SOBIR1/EVR (SUPPRESSOR OF BIR1-1/EVERSHED) has been implicated in LRR-RP function (Gust and Felix 2014). In tomato for example, SOBIR1 interacts with the receptor-like proteins Ve1, Cf-2, Cf-4, Cf-9 and EIX2, some of which are involved in resistance of tomato against fungal infection (Liebrand, *et al.* 2013). SOBIR1 was furthermore shown to be involved in immune signaling mediated by LRR-RLPs or to interact with them in *Arabidopsis*, such as RLP1/REMAX, RLP30 and RLP42/RBPG1 (Jehle, *et al.* 2013a, Zhang, *et al.* 2013, Zhang, *et al.* 2014). To test whether SOBIR1 is involved in nlp20-mediated immune signaling, two *Arabidopsis* T-DNA insertion mutants of SOBIR1 (*sobir 1-12*: SALK\_050715 and *sobir 1-13*: SALK\_009453) were subjected to nlp20 treatment and analysed for ethylene production. Both mutant lines responded normally to treatment with flg22 whereas responsiveness towards nlp20 was completely abrogated (Fig. 3-15).

These findings suggest an involvement of SOBIR1 in nlp20-mediated immune signaling in *Arabidopsis*.



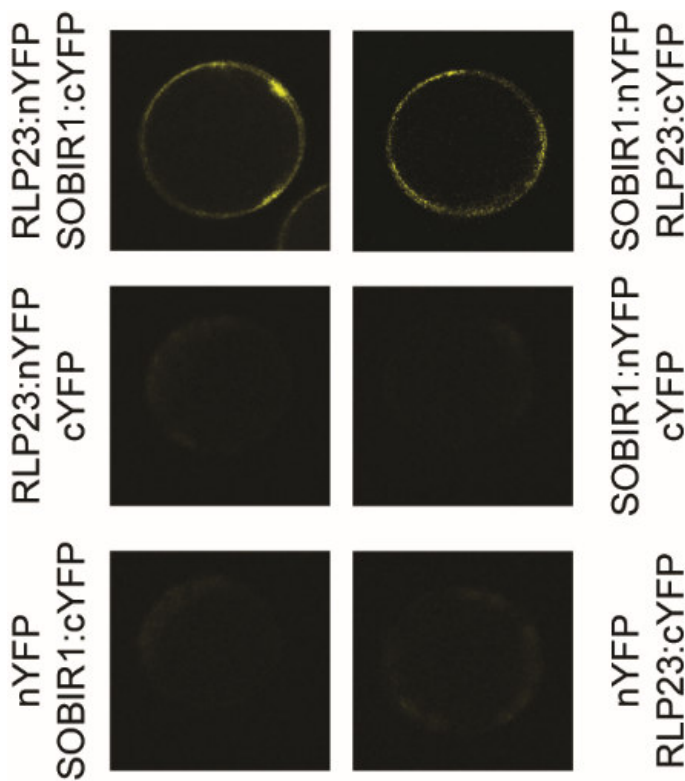
**Figure 3-15: Sensitivity of SOBIR1 towards nlp20.**

Ethylene formation 4 hours after induction with 1  $\mu$ M nlp20. Water and flg22 served as controls. Bars represent means  $\pm$  SD of three replicates. Asterisks indicate significant differences over water control treatment as determined by Student's t test, \*\*P $\leq$ 0,01.

### 3.2.4.2 RLP23 and SOBIR1 physically interact with each other

To demonstrate a physical interaction of RLP23 and SOBIR1, a bimolecular complementation assay (BIFC) was conducted. Here, a fluorescent signal is only obtained in case of a very close proximity of the two proteins. Therefore, *Arabidopsis* protoplasts were co-transformed with RLP23:nYFP and SOBIR1:cYFP or SOBIR1:nYFP and RLP23:cYFP –constructs respectively, and investigated by confocal laser scanning microscopy. A strong and distinct YFP signal could be observed for both construct combinations, whereas control transformations with empty vector constructs did not show any signal (Fig. 3-16).

These images confirm, that RLP23 and SOBIR1 directly interact with each other at the plasma membrane.



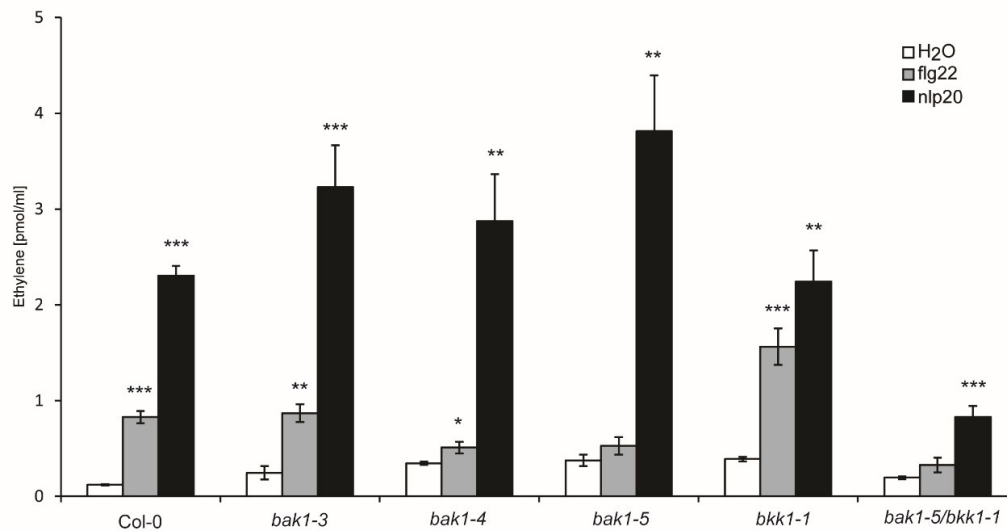
**Figure 3-16: RLP23 and SOBIR1 directly interact with each other.**

Bimolecular fluorescence complementation assay with protoplasts isolated from *Arabidopsis* transiently expressing RLP23:nYFP and SOBIR1:cYFP or SOBIR1:nYFP and RLP23:cYFP. Confocal laser scanning microscopy was applied to detect fluorescence of complemented YFP. nYFP and cYFP vector constructs were used as controls.

### 3.2.4.3 BAK1, another LRR-RK is also involved in nlp20-mediated immune signaling together with BKK1

The LRR-RK BRI1-ASSOCIATED KINASE1 (SERK3/BAK1) is a member of the subclass of subfamily II LRR-RKs with five closely related members and a well-known interactor of the LRR-RKs FLS2 and EFR, mediating immune signaling upon perception of bacterial flg22 or elf18 in *Arabidopsis* (Chinchilla, *et al.* 2007, Schwessinger, *et al.* 2011). In tomato, genetic evidence suggests that the LRR-RP Ve1 requires BAK1 for resistance against *Verticillium dahliae*. Likewise, EIX1/2-mediated signaling is dependent on BAK1 and a direct interaction of EIX1 and BAK1 could be shown (Fradin, *et al.* 2009, Bar, *et al.* 2010). More recently, BAK1 was found to be involved in SCFE1-mediated signaling together with RLP30 and the LRR-RK SOBIR1 in *Arabidopsis* (Zhang, *et al.* 2013). Therefore it was investigated if BAK1 also plays a role in nlp20-mediated signaling. *Bak1-3* (SALK\_034523) and *bak1-4* (SALK\_116202) are both T-DNA insertion lines exhibiting impairment in cell-death control, immunity-associated responses and brassinosteroid signaling (Nam and Li 2002, Chinchilla, *et al.* 2007, Kemmerling, *et al.* 2007). Another mutant genotype, *bak1-5*, is not affected in brassinosteroid signaling or cell-death control but immune signaling mediated by FLS2 and EFR is severely compromised (Schwessinger, *et al.* 2011), (Fig. 3-17). However, all of the mutants responded normally to treatment with nlp20 in an ethylene assay (Fig. 3-16). Due to functional redundancy of BAK1 and BAK1-like1 (SERK4/BKK1), a BKK1 mutant (*bkk1-1*, SALK\_057955) was subjected to nlp20 treatment. This mutant showed a normal ethylene response upon induction with nlp20, whereas the double mutant *bak1-5/bkk1-1* was strongly impaired in ethylene formation (Fig. 3-17).

This suggests that BAK1 and BKK1 might complement each other to gain full signaling strength and hereby play a role in nlp20-mediated immune signaling.



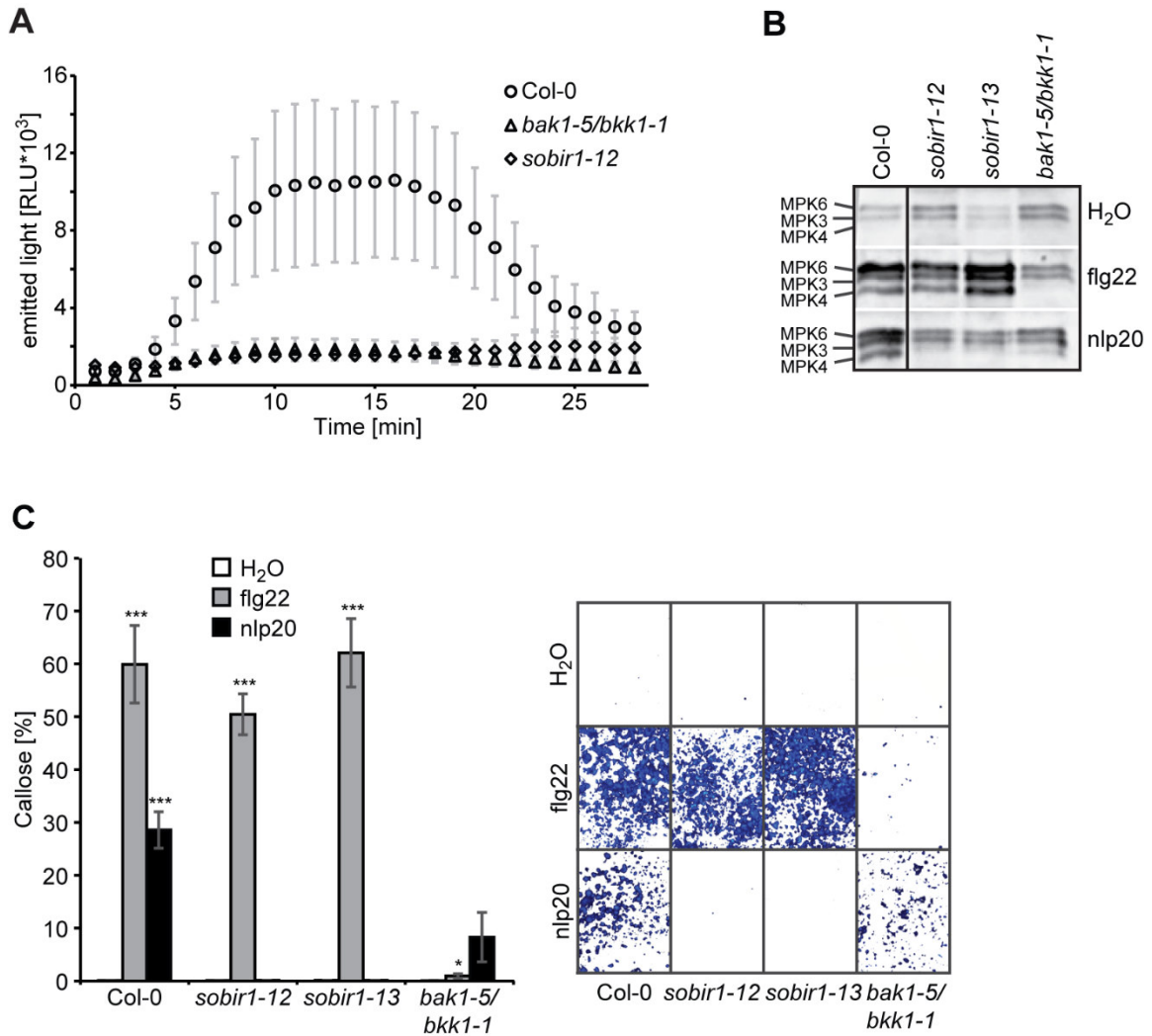
**Figure 3-17: Sensitivity of SERK3/BAK1 and SERK4/BKK1 towards nlp20.**

Production of ethylene of 3 different *bak1* mutant genotypes, a *bkk1* mutant and a *bak1/bkk1* double mutant after 4 hours of incubation with 1  $\mu$ M nlp20. Water and flg22 served as controls. Bars represent means  $\pm$  SD of three replicates, asterisks indicate significant differences over water control determined by Student's t test, \* $P \leq 0,05$ , \*\* $P \leq 0,01$ , \*\*\* $P \leq 0,001$ .

### 3.2.4.4 SOBIR1 and BAK1/BKK1 are essential for nlp20-mediated immune signaling

To confirm a role of SOBIR1 and BAK1/BKK1 in nlp20-mediated signaling in *Arabidopsis*, mutant *sobir1-12*, *sobir1-13* and *bak1-5/bkk1-1* lines were analyzed for a comprehensive set of nlp20-induced marker immune responses. In a luminol-based oxidative burst assay, no production of reactive oxygen species could be observed for *sobir1-12* and *bak1-5/bkk1-1* mutants when incubated with 1  $\mu$ M nlp20 peptide (Fig. 3-18 A). Furthermore, *sobir1-12* and *sobir1-13* mutants showed a strong activation of MAP kinases upon leaf infiltration with flg22, whereas MAP kinase activations in nlp20-treated samples were as sparsely as in water treated plants (Fig. 3-18 B). Likewise, nlp20 triggered only very little MAP kinase activation in *bak1-5/bkk1-1* mutants, and also activation mediated by flg22 was strongly diminished and comparable to control mock treatment (Fig. 3-18 B). A complete abrogation of callose deposition in both *sobir* mutants corroborated these findings, while some residual callose deposition could be observed in *bak1-5/bkk1-1* mutant plants (Fig 3-18 C, callose deposition in % of the image section, and representative image section shown).

This data set further strengthens the assumption, that SOBIR1 and BAK1/BKK1 are essential components in nlp20-mediated immunity acting as co-receptors of RLP23 to facilitate signaling in *Arabidopsis*.



**Figure 3-18: Nlp20-mediated defense responses are abrogated in *sobir* and *bak/bkk* mutant genotypes.**

Production of reactive oxygen species (A), activation of MAP kinases (B) and callose deposition (C) were determined of mutant genotypes *sobir1-12*, *sobir1-13* and *bak1-5/bkk1-1* upon induction with 1  $\mu$ M nlp20 peptide. Water and flg22 were used as control treatments. Callose deposition is determined as percentage of the image section and calculated in pixels (one out of three representative pictures shown in the right panel). Bars and data points represent three replicates  $\pm$  SD, asterisks mark significant differences over control treatment determined by Student's t test, \* $P \leq 0,05$ , \*\*\* $P \leq 0,001$  (C). Experiments were conducted in triplicate with similar results.

### 3.2.5 RLP23 forms a complex with SOBIR1, BAK1 and other SERK family members

To investigate whether RLP23 forms a complex not only with SOBIR1 (3.2.4.2) but also with BAK1 and if complex assembly is nlp20-dependent, co-immunoprecipitations in *N. benthamiana* were conducted. These plants transiently co-expressed the C-terminally tagged receptor proteins. GFP-trap beads were used to pull-down RLP23:GFP and to co-immunoprecipitate SOBIR1:HA and BAK1:Myc protein. Three minutes after leaf infiltration of 1  $\mu$ M nlp20, BAK1:Myc gets recruited into a complex with RLP23:GFP independently of co-expression of SOBIR1:HA (Fig. 3-19 A). However, endogenous SOBIR1 of *N. benthamiana* might take over the function due to its close homology to *Arabidopsis*-derived SOBIR1 and it cannot be stated here if SOBIR1 would be an essential component to form a RLP23/BAK1 complex. Unlike BAK1:Myc, SOBIR1:HA could be co-immunoprecipitated independently of ligand treatment, suggesting the existence of a pre-formed RLP23-SOBIR1 complex (Fig. 3-19 A). Also here, no statement can be made about the requirement of BAK1 for the interaction of RLP23 and SOBIR1 due to potentially functional redundant BAK1 proteins from *Arabidopsis* and *N. benthamiana*.

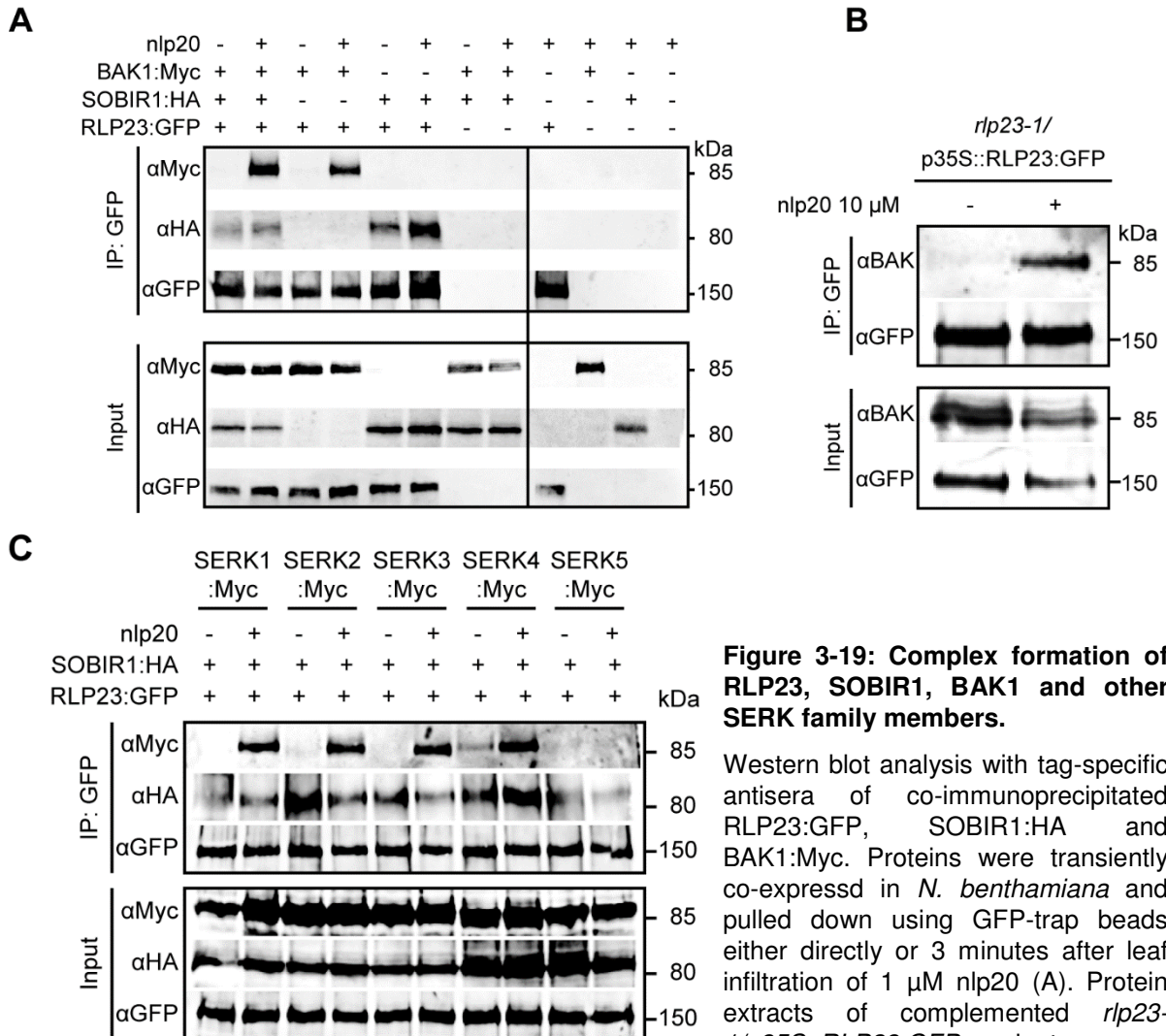
In addition, native BAK1 could be co-immunoprecipitated in transgenic *rlp23-1/p35S::RLP23:GFP Arabidopsis* plants upon induction with 10  $\mu$ M nlp20, confirming a ligand-dependent recruitment of BAK1 into a complex with RLP23 even under native BAK1 levels (Fig. 3-19 B).

Functional redundancy among members of the SERK protein family is a known phenomenon (Albrecht *et al.* 2008). For instance, immune signaling mediated by flg22, elf18 and the damage-associated molecular pattern (DAMP) AtPEP1 is dependent on both, SERK3/BAK1 and SERK4/BKK1, and ligand-dependent interaction with FLS2 or EFR could be shown for SERK1-4 in transient expression assays in *N. benthamiana* (Roux, *et al.* 2011). Moreover, results from 3.2.4.3 and 3.2.4.4 show that both, BAK1 and BKK1 are involved in nlp20-induced signaling events. Therefore it was investigated, if other members of the SERK protein family than SERK3/BAK1 get recruited into a RLP23-SOBIR1 complex upon stimulation with nlp20. SERK4/BKK1 could be co-immunoprecipitated as could be SERK3/BAK1 three minutes after ligand infiltration together with RLP23:GFP and SOBIR:HA transiently co-expressed in



*N. benthamiana* plants (Fig. 3-19 C). The same observations could be made for SERK1 and SERK2, but not for SERK5:Myc.

Altogether, these observations implicate that all three receptor proteins build a complex, whereas RLP23 and SOBIR1 constitute a pre-formed entity in which BAK1 (or other SERK protein family members) get recruited after induction by nlp20 only.



**Figure 3-19: Complex formation of RLP23, SOBIR1, BAK1 and other SERK family members.**

Western blot analysis with tag-specific antisera of co-immunoprecipitated RLP23:GFP, SOBIR1:HA and BAK1:Myc. Proteins were transiently co-expressed in *N. benthamiana* and pulled down using GFP-trap beads either directly or 3 minutes after leaf infiltration of 1 μM nlp20 (A). Protein extracts of complemented *rlp23-1/p35S::RLP23:GFP* plants were subjected to a GFP-trap pull-down and analysed via western blotting using GFP- or BAK1- specific antisera. Leaf material was taken either directly or 3 minutes after infiltration of 10 μM nlp20 peptide (B). SERK1-5:Myc proteins were transiently co-expressed with RLP23:GFP and SOBIR1:HA in *N. benthamiana* and protein extracts were made either directly or 3 minutes after infiltration of 1 μM nlp20. GFP-trap beads were used to pull-down proteins and the western blot was probed with tag-specific antisera. Experiments were performed in triplicate with similar results.

## 4. Discussion

### 4.1 Nlp20- a novel immunogenic pattern within the widespread NLP toxin family

NEP1-like proteins are microbial virulence factors with a broad taxonomic distribution, exerting toxin activity on dicot plant species. Simultaneously, these proteins are potent inducers of plant immunity and mutational analysis within bacterial *PccNLP* revealed a causal link between cell death inducing and immunogenic activity (Küfner, *et al.* 2009, Ottmann, *et al.* 2009). Thus, toxin-induced release of molecules representing self or modified self is assumed to be the agent for activation of plant immunity. Here, the mechanism of action of how NLPs trigger immunity in plants was elucidated and revealed a novel immunogenic feature beside toxin activity. The results discussed in this chapter are published in Böhm *et al.* (2014b) and Oome *et al.* (2014).

#### 4.1.1 Differences in the immunogenic potential of *PccNLP* and *PpNLP* lead to the identification of *nlp20*

Comparative studies on recombinant bacterial *PccNLP* and oomycete-derived *PpNLP* confirmed cytotoxic potential of both proteins when applied in a calcein release assay using purified *Arabidopsis* membrane vesicles (Fig. 3-1). Also, leaf infiltration of *Arabidopsis* plants led to NLP-characteristic necrotic symptoms. At the same time, typical defense-associated responses are exerted by both toxins, such as PR1::GUS and PAD3 defense gene expression and ethylene biosynthesis. Release of endogenous danger signals (DAMPs) by NLP cytotoxicity and subsequent recognition thereof through plant PRRs is suggested to be the immune-stimulating mode of action (Küfner, *et al.* 2009). Toxin-mediated release of self-representing molecules inducing immunity in plants is also known to be triggered by the host-selective toxin victorin from *Cochliobolus victoriae* (Tada *et al.* 2005), fumonisin B1 (FB1) produced by certain *Fusarium* spp. (Asai *et al.* 2000, Stone *et al.* 2000), the type B trichothecene deoxynivalenol (DON) produced by *Fusarium* spp. (Nishiuchi *et al.* 2006), AAL-toxin

from *Alternaria alternata* (Gechev *et al.* 2004) and fusicoccin from *Fusicoccum amygdali* (Schaller and Oecking 1999) and as such is considered to be a hallmark of innate immunity not only in metazoans (toxin-mediated activation of the inflammasome) (Mariathasan *et al.* 2006), but also in plants.

NLPs exhibit highly conserved regions, like the hepta-peptide motif as a part of the surface exposed, negatively charged cavity which is known to be crucial for toxin activity (see 1.4). Mutational analysis of single amino acids within this region of bacterial *PccNLP* revealed a causal link between toxin activity and immune-stimulating activity (Ottmann, *et al.* 2009), supporting the hypothesis of toxin-mediated release of self-representing signals of danger. Indeed, mutated *PccNLP* is not only rendered non-toxic, but also deficient in triggering immune responses like defense gene expression and ethylene production (Fig. 3-1). Intriguingly, *Phytophthora parasitica*-derived NLP does not lose its immunogenic potential, although cytotoxicity is completely abrogated in *PpNLP\_mut* (double mutation in *PpNLP*, H101A D104A) variants. These investigations led to the assumption, that *PpNLP* must feature two independent immune-stimulating mechanisms; toxin-mediated release of immunogenic molecules and immune activation by a yet unidentified immunogenic epitope within the protein. Two out of thirteen synthetic peptides spanning the whole *PpNLP* sequence are capable of eliciting immune responses in *Arabidopsis* (Fig. 3-2), confirming the presence of an immunogenic peptide pattern within *PpNLP*. The minimal immunogenic epitope spans 20 aa (A<sub>103</sub>-R<sub>122</sub>) designated as nlp20, a length characteristic for typical proteinaceous PAMPs such as flg22 and elf18. Identification of two mechanistically different immunogenic principles within one molecule is unreported in metazoan as well as in plant immunity to date.

#### 4.1.2 Nlp20- redefining dated definitions

Nlp20 fulfills characteristics of a typical PAMP; it is a highly conserved pattern among NLPs and evokes a comprehensive set of typical defense-related immune responses in *Arabidopsis* at low concentrations, such as ethylene biosynthesis, production of reactive oxygen species, activation of MAP kinases, defense gene expression and deposition of callose (Fig. 3-7). Accordingly, pretreatment of *Arabidopsis* with nlp20 establishes a state of induced resistance, enhancing disease resistance against bacterial and fungal infection (Fig. 3-6). This is reminiscent of the

prototypical PAMP flg22, albeit significant differences exist between both peptide patterns. Flg22 induces a strong inhibition of *Arabidopsis* seedling growth and elicits extracellular alkalinization in cell suspensions of *Arabidopsis* (Felix, *et al.* 1999, Gomez-Gomez, *et al.* 1999), whereas nlp20 is not capable of triggering such responses (Fig. 3-7). On the other hand, nlp20 strongly triggers production of salicylic acid and camalexin in a dose- and time-dependent manner, whereas flg22-triggered salicylic acid production is much weaker. Camalexin biosynthesis genes PAD3 and CYP71A13 are highly induced 6 hours after treatment with nlp20 (Wei-Lin Wan, personal communication). This might be due to underlying differences in the perception system of flg22 and nlp20, the latter will be discussed in chapter 4.2.

As such, *PpNLP* is not only a toxin and virulence factor, but also features characteristics of a classical PAMP and differentiation into PAMP or effector seems to be blurred. Also other PAMPs just like the generic PAMPs flagellin (Naito *et al.* 2008, Taguchi *et al.* 2010), LPS (Newman *et al.* 2007) and chitin (Soulie *et al.* 2006) have been shown to fulfill a virulence-promoting function in plant-pathogen interaction. Likewise, PAMPs have been identified within effectors such as EIX, the ethylene inducing xylanase from *Trichoderma viride* which is an important virulence factor for the pathogen (Rotblat *et al.* 2002). The immunogenic pattern within EIX consists of five amino acids exposed on the surface of the protein and is not involved in  $\beta$ -1-4-endoxylanase activity. Pep-13 instead, is a conserved PAMP within TGase of *Phytophthora* spp. and the amino acids required for immunogenic activity are the same required for TGase activity (Nürnberg, *et al.* 1994, Brunner, *et al.* 2002). Further examples of PAMPs within virulence factors are the ion-conducting pore forming harpin HrpZ1 from *Pseudomonas syringae* (Engelhardt *et al.* 2009) or fungal endopolygalacturonases (Zhang, *et al.* 2014).

Recognition of nlp20 by *Arabidopsis* is reminiscent of effector-shaped evolution of plant PRRs, triggering ETI. Here, a conserved virulence factor might have promoted the formation of a recognition system mediating PTI, supporting the concept of a continuum between PTI and ETI (Thomma, *et al.* 2011).

### 4.1.3 Nlp20 and the hepta-peptide motif: two distinct conserved regions

Immunogenic activity of nlp20 can clearly be discriminated from cytotoxicity, since denatured and mutated *PpNLP* still evoke a strong immune response in *Arabidopsis*

although toxin activity is completely abrogated (Fig.3-1). Likewise, nlp20 is neither able to provoke cytotoxic symptoms upon leaf infiltration nor a calcein release of *Arabidopsis* membrane vesicles (Fig.3-3). The hepta-peptide motif of *PpNLP* is localized within a negatively charged surface-exposed cavity spanning parts of  $\beta$ 6 and L2 (Ottmann, *et al.* 2009) (Fig. 3-5 B), presumably facilitating binding to certain structures of the plant plasma membrane. This mechanism is deduced from structurally related molecules for which membrane attachment via similar surface topologies is known, like actinoporins and lectins (Küfner, *et al.* 2009). Nlp20 instead spans  $\beta$ 5 and L2, whereas  $\beta$ 5 comprises the amino acids obligatory for immunogenic activity (Fig. 3-5 A left panel, table 3-1). Interestingly, this region is not surface-exposed but rather lies within the protein (Fig 3-5 A right panel). Thus, perception of nlp20 by a PRR must be enabled by structural reorganization of *PpNLP*. Crystal and electron microscopy structures of sticholysin II (StnII), which is an actinoporin structurally similar to NLPs, show that the N-terminal  $\alpha$ -helix and a flexible loop undergo significant conformational changes upon oligomerization (Mancheño, *et al.* 2003). Due to obvious structural similarities of actinoporins and NLPs, structural reorganization of *PpNLP* upon membrane binding is quite likely; thereby exposing the nlp20 motif which can then get recognized by a PRR. Degradation of NLPs by plant proteases is also conceivable, releasing peptide fragments with immunogenic nlp20.

#### 4.1.4 Distribution of nlp20 across kingdom boundaries

No nlp20 orthologous immunogenic sequence is present in *PccNLP* (3.1.5) what explains the loss of immune-stimulating capacity occurring upon mutation of the toxin motif of the protein (Fig 3-1). Instead, sequences orthologous to nlp20 can be found in another oomycete-derived NLP (from *Pythium aphanidermatum*), and also in fungal (*Fusarium oxysporum*, *Botrytis cinerea*) and bacterial (*Bacillus halodurans*, *Bacillus subtilis*) microorganisms (Table 3-2). All of these peptides are capable of inducing a complex set of immune responses in *Arabidopsis* (Fig. 3-7), unveiling a unique distribution pattern among prokaryotic and eukaryotic microbes. The most prominent PAMPs are not distributed across lineages, but can be rather found within kingdom boundaries. Peptidoglycan, flg22 and lipopolysaccharides are generic PAMPs of bacterial origin, whereas chitin or  $\beta$ -glucan structures are typical fungal- or oomycete-derived PAMPs respectively (1.3.1 and 1.3.2). Using the *PpNLP* sequence as a query,

1.091 NLP sequences can be found in databases presently, whereas 221 sequences are from bacterial, 558 from fungal and 312 from oomycete origin belonging to 156, 96 and 10 species respectively. At an average this means, that bacteria possess 1,4, fungi 3,8 and oomycetes 31 NLPs. This is not only a calculated number, but reflects facts; the bacterium *Pectobacterium carotovorum* for example harbors only one NLP (Mattinen, *et al.* 2004), the fungus *Moniliophthora perniciosa* five NLPs (Mondego *et al.* 2008) and the oomycete *Phytophthora sojae* encodes for 33 NLPs (Dong, *et al.* 2012). Screening the database-retrieved sequences for the nlp20 pattern and for the presence of the four amino acids (I<sub>104</sub>, Y<sub>106</sub>, W<sub>108</sub> and Y<sub>109</sub>) within nlp20 which are crucial for immunogenic activity (Table 3-1), revealed that only about 10 % of bacterial NLP sequences are presumably immunogenic. In contrast, almost all of the oomycete and 85 % of the fungal species have NLPs with an immunogenic nlp20 motif. The expansion of NLPs within certain taxonomic groups could be ascribed to horizontal gene transfer and gene duplication (Oome and Van den Ackerveken 2014). Considerable expansion of NLP genes within oomycetes suggests an important role, also of non-toxic NLPs for the pathogen's lifestyle (see also 1.4). The obligate biotroph *Hyaloperonospora arabidopsidis* solely expresses non-toxic NLPs and is the causal agent for downy mildew on *Arabidopsis*. Six *Hyaloperonospora*-derived nlp20 orthologous synthetic peptides strongly induce ethylene formation in *Arabidopsis* leaves and four do so to a lower extent (Oome, *et al.* 2014). Rapid expansion and diversification of the NLP gene family obviously does not provoke selection of severely mutated nlp20 motifs yet, albeit the nlp20 orthologous peptide nlp24 derived from HaNLP3 is capable of inducing a strong priming effect in *Arabidopsis* leading to drastically reduced growth of *H. arabidopsidis* (Oome, *et al.* 2014). However, under natural conditions *H. arabidopsidis* is able to establish an infection on *Arabidopsis*, presumably through secretion of effectors which suppress PTI triggered by immunogenic NLPs. The *H. arabidopsidis* genome encodes for about 130 RXLR effector genes and a lot of effectors from *Hyaloperonospora* have already been characterized as suppressors of PTI (Baxter *et al.* 2010, Fabro *et al.* 2011).

## 4.2 RLP23-BAK1-SOBIR1: The receptor complex mediating recognition of nlp20

Recognition of nlp20 occurs in a rather genus-specific manner, as mainly *Arabidopsis* and close relatives thereof are able to perceive nlp20 (Fig. 3-8). Besides members of the *Brassicaceae* family, only one *Asteraceae* family member can sense nlp20. Lettuce (*Lactuca sativa*) seems to be equipped with the recognition system enabling perception of nlp20 and mounts an ethylene response upon stimulation with the peptide pattern comparable to *Arabidopsis*. So far, lettuce seems to be the only exception, since screening of various other *Aster* family members and further members of unrelated plant families could not identify additional nlp20-sensitive plants (see 3.1.9). Here, the identification of the components of the nlp20 perception system in *Arabidopsis* and characterization of the receptor entity is discussed, enlightening the mechanism of nlp20-mediated LRR-RP and LRR-RK interaction and signaling. The results discussed in this chapter are published in (Albert *et al.* 2015).

### 4.2.1 RLP23 is the receptor for nlp20

Several LRR-RKs and LRR-RPs are sensors for proteinaceous ligands (Monaghan and Zipfel 2012, Böhm, *et al.* 2014a). To identify the receptor for the novel NLP-derived peptide pattern nlp20 in *Arabidopsis*, reverse genetics were applied and screening of RLP and RLK T-DNA insertion mutant collections (Wang, *et al.* 2008, Postel, *et al.* 2010) identified the receptor protein RLP23 as the receptor for nlp20 (Fig. 3-9). *Rlp23* mutants lack the ability to mount typical defense responses which can be observed in Col-0 plants upon treatment with nlp20 such as plasma membrane depolarization, ethylene biosynthesis, production of reactive oxygen species, activation of MAP kinases and deposition of callose ((Albert, *et al.* 2015), Fig. 3-7 and Fig. 3-10). Consequently, RLP23 is needed for an nlp20-induced priming effect conferring enhanced disease resistance towards bacterial and oomycete infection. *Rlp23* mutants are as susceptible as mock-treated plants in contrast to nlp20-primed wild-type plants, which show significantly enhanced disease resistance against infection with *Pseudomonas syringae* or *Hyaloperonospora arabidopsidis* ((Albert, *et al.* 2015) and Fig. 3-13). Identification of three nlp20-insensitive *Arabidopsis* accessions exhibiting a

premature stop codon within LRR 13 of RLP23 and successful complementation of nlp20-insensitive plants further supports the finding that RLP23 is the receptor mediating nlp20-induced immunity in *Arabidopsis*.

Functional RLP23 protein seems to be necessary for nlp20 recognition and downstream signaling, since none of the immunity-related defense responses are elicited in *rlp23*-deficient plants, *rlp23* mutants cannot be primed by nlp20 and nlp20-insensitive plants show frame-shift mutations within the LRR of RLP23. In fact, no RLP23 transcript accumulates in *rlp23* mutant plants and no RLP23 protein is detectable upon expression of the Bor-4 *RLP23* allele in *N. benthamiana* (Albert, *et al.* 2015). RLP23 is thus the receptor for nlp20 and necessary for initiation of NLP-induced PAMP signaling in *Arabidopsis*.

Some peptide patterns are known to physically and specifically interact with the LRR-domains of their cognate RLPs. Fungal ethylene-inducing xylanase EIX physically interacts with tomato *S/EIX2* and endopolygalacturonase PG3 from *Botrytis cinerea* interacts specifically with RLP42/RBPG1 (Ron and Avni 2004, Zhang, *et al.* 2014). As reported in Albert, *et al.* (2015), nlp20 specifically binds to and physically interacts with RLP23 in vitro and in planta. Resin-bound GST-tagged nlp20 exclusively precipitates recombinant RLP23 ectodomains and not FLS2-LRR, this interaction is competed by excess amounts of soluble nlp20 and RLP23 ectodomains are not precipitated by a structurally unrelated peptide. Similarly, in vivo cross-linking experiments show specific binding of biotinylated nlp20 to RLP23 at concentrations similar to the EC<sub>50</sub> value of nlp20 and this binding is competed by excess of unlabeled nlp20. This competition only occurs when a biologically active nlp20 peptide is used, stressing the specificity of the binding event between nlp20 and RLP23. Likewise, no binding of nlp20 can be observed in *Arabidopsis rlp23* mutants or with the structurally related LRR-RP RLP30.

These findings demonstrate that RLP23 is the receptor for nlp20 and that ligand and receptor physically and specifically interact with each other. Specific and direct interaction of a LRR-RP-PAMP pair has been shown for only two examples; the binding of EIX to *S/EIX2* and *BcPG3* to *AtRLP42/RBPG1*, making the nlp20-RLP23 system to another model for further studies on LRR-RP-peptide pattern interaction. Here it would be interesting, to determine the interacting residues within the LRRs of RLP23 and the nlp20 peptide in detail. Regarding the peptide, three aromatic residues Y<sub>106</sub>, W<sub>108</sub> and Y<sub>109</sub> and I<sub>104</sub> can be assigned as crucial for immunogenicity (table 3-1) and thus most



likely for proper binding to RLP23. Substitution of Y<sub>106</sub>, W<sub>108</sub> and Y<sub>109</sub> by other aromatic amino acids like phenylalanine, tyrosine or tryptophan do not have any impact on peptide activity whereas substitution of Y<sub>109</sub> by histidine or lysine and substitution of Y<sub>106</sub> by lysine drastically reduces peptide activity (data not shown). This is in agreement with the strong conservation of Y<sub>106</sub>, W<sub>108</sub> and Y<sub>109</sub> among nlp20 orthologous peptides assigned as immunogenic. Next to the peptides listed in table 3-2, four oomycete-derived (*Phytophthora infestans*, *Phytophthora sojae*, *Plasmopara viticola* and *Hyaloperonospora arabidopsidis*) and eight fungal (*Mycosphaerella graminicola*, *Verticillium dahliae* NLP1, 3 and 4, *Magnaporthe grisea*, *Moniliophthora perniciosa* and *Sclerotinia sclerotiorum* NLP1 and 2) orthologous peptides are tested as immunogenic (data not shown). All exhibit the presence of Y<sub>106</sub>, W<sub>108</sub> and Y<sub>109</sub> or in case of *Verticillium dahliae* NLP3 and NLP4 a W<sub>108</sub> to Y substitution within the peptide pattern. Also I<sub>104</sub> is highly conserved among all patterns assigned as immunogenic, with the exception of *Sclerotinia sclerotiorum* NLP2 and *Moniliophthora perniciosa* NLP (I<sub>104</sub>L) and *Phytophthora sojae* NLP (I<sub>104</sub>F). This is reminiscent of the bacterial pattern flg22, where all amino acids crucial for binding to the LRR-RK FLS2 are conserved among FLS2-activating bacteria. Here, hydrophobic contacts and hydrogen bonds are shown to mediate binding of conserved flg22 residues to the receptor FLS2 (Sun, *et al.* 2013). Thus, also conserved amino acids I<sub>104</sub>, Y<sub>106</sub>, W<sub>108</sub> and Y<sub>109</sub> within nlp20 could be essential to mediate proper binding to RLP23 LRRs.

Regarding RLP23, successive deletion of the LRRs and transient expression thereof in *N. benthamiana* lead to a complete loss of nlp20-mediated immune signaling as soon as LRR3 is missing, uncovering this LRR as one interaction site of RLP23 and nlp20 (analysis done together with Dr. Isabell Albert, data not shown). Further mutational analysis of RLP23 LRRs or crystallization of RLP23 in complex with nlp20 would refine the exact binding sites and events happening between nlp20 and RLP23.

#### **4.2.2 Nlp20-mediated signaling is dependent on RLP23-interacting LRR-RKs SOBIR1 and BAK1**

RLPs like RLP23 are proteins lacking a cytoplasmic kinase domain required for mediating downstream signaling upon pattern perception. As such, most RLPs involved in immune responses are considered to interact with the LRR-RK SOBIR1,

forming functional receptor kinase equivalents (Gust and Felix 2014). This is supported by genetic evidence, as signaling through *Arabidopsis* RLP1/ReMAX or RLP42/RBPG1 induced by eMax or PGs respectively, is dependent on SOBIR1 (Jehle, *et al.* 2013a, Zhang, *et al.* 2014). In tomato, SOBIR1 is required for Cf-2 and Cf-4-mediated immunity and furthermore interacts with Ve1 and EIX2, mediating signaling upon perception of Ave1 and EIX respectively (Liebrand, *et al.* 2013). Interestingly, Ave1 not only requires SOBIR1 but also BAK1 for proper downstream signaling (Fradin, *et al.* 2009) and also EIX1 interacts with BAK1, attenuating immune response output mediated by the EIX2/SOBIR1 complex (Bar, *et al.* 2010). Complex formation of RLPs and RLKs forming modules resembling functional RLKs not only occurs with PRRs with an extracellular LRR domain, but also with LysM domain proteins. LYM1 and LYM3 mediate PGN-induced signaling by forming a functional receptor kinase equivalent with the LysM-RK CERK1 in *Arabidopsis* (Willmann, *et al.* 2011). Another generic example of complex formation between LysM-RPs and -RKs is the chitin induced dimerization of rice CEBIP RLPs with subsequent recruitment of the RLK CERK1, thereby establishing a signaling active  $\alpha_2\beta_2$  heterotetramer (Hayafune, *et al.* 2014).

*Arabidopsis* RLP30, a receptor protein structurally related to RLP23, requires SOBIR1 as well as BAK1 for SCFE1-mediated immune signaling (Zhang, *et al.* 2013). In this work it could be shown, that also nlp20-mediated signaling in *Arabidopsis* is dependent on SOBIR1 and BAK1 and conceivably on other members of the SERK protein family like SERK4/BKK1. *Sobir1* mutants display complete insensitivity towards nlp20 treatment in early as well as in late defense responses (Fig. 3-15 and 3-18). *Bak1* mutant plants still induce a strong ethylene response upon nlp20 treatment whereas *bak1/bkk1* mutants are significantly impaired in responsiveness, indicating functional redundancy between BAK1 and BKK1. This phenomenon has also been shown for the PAMPs flg22 and elf18 and the DAMP AtPEP1 for which immune signaling is dependent on both proteins, BAK1 and BKK1 (Roux, *et al.* 2011). BAK1 and BKK1 seem to represent an ohnologous gene pair originated by whole genome duplication (WGD) and might as such be functionally redundant (Liebrand *et al.* 2014). Early defense responses elicited by nlp20 like production of reactive oxygen species are completely abrogated in *bak1/bkk1* mutant genotypes whereas some residual callose deposition is detectable (Fig. 3-18). This implies that also other SERK proteins

might partially overtake the function of BAK1 and BKK1 in nlp20-mediated immune signaling.

Genetic evidence for the roles of SOBIR1 and BAK1 in nlp20-induced immune signaling is further strengthened as MS/MS spectroscopy identifies both proteins as interactors of RLP23 (Albert, *et al.* 2015). SOBIR1 interaction with RLP23 is represented by 15 SOBIR1-derived peptides, found upon co-immunoprecipitations independently of nlp20-treatment. This confirms ligand-independent and constitutive interaction of SOBIR1 with RLP23 as described recently by Bi *et al.* (2014). SOBIR1 is not only assumed to form heteromeric complexes with RLP-type immune receptors (Liebrand, *et al.* 2013, Gust and Felix 2014), but also to serve receptor protein stability as shown for Ve1 and Cf-4 proteins (Liebrand, *et al.* 2013). Preliminary results indicate that RLP23 stability is not dependent on the presence of SOBIR1, as no quantifiable differences in accumulation of RLP23 protein in *sobir-1* mutant and Col-0 protoplasts are measurable (data not shown). Interaction of SOBIR1 with LRR-RPs such as Cf-4, Ve1 and EIX2 is considered to be mediated by the GxxxG dimerization motif also referred to as 'glycine zipper' within the transmembrane domain of SOBIR1 (Bi *et al.* 2015). This motif has been assigned as key factor for dimerization of two transmembrane proteins via helix-helix interactions (Cymer *et al.* 2012, Fink *et al.* 2012). Mutations within the RLP23 GxxxG motif do obviously not severely influence the interaction with *Nb*SOBIR1, as sensitivity towards nlp20 is not abrogated when tested in transient expression experiments in *N. benthamiana* (preliminary analysis, performed by Dr. Isabell Albert). To describe the role of the GxxxG motif in RLP23-SOBIR1 interaction more precisely, mutational analysis within both proteins and co-immunoprecipitations need to be performed. So far, it cannot be excluded that interaction with the *Arabidopsis*-derived SOBIR1 would be disturbed quantitatively upon mutation of the RLP23 GxxxG motif, or vice versa. *Arabidopsis* LRR-RLPs behave differently regarding their compatibility with SOBIR1 of *N. benthamiana*. RLP1/ReMAX and RLP30 for example are not compatible with *Nb*SOBIR1, as transient expression of the RLPs in *N. benthamiana* do not lead to sensitivity towards the corresponding ligands eMax and SCFE1 (Jehle, *et al.* 2013a, Zhang 2013). RLP23 instead is able to signal, thus to interact with SOBIR1 from *N. benthamiana*, *S. lycopersicum* and *S. tuberosum* (Fig. 3-12). Other regions than the GxxxG motif might also play a role in RLP-SOBIR1 interaction. The extracellular juxtamembrane of SOBIR1 for example is rich of positively charged lysine residues, possibly mediating

contact to the negatively charged extracellular juxtamembrane of RLPs by ionic interactions. An interaction via LRR domains of SOBIR1 and RLPs is also conceivable (Gust and Felix 2014).

In the MS/MS analysis, BAK1 fragments are solely detectable with precipitated RLP23 protein upon treatment of the plants with nlp20 (Albert, *et al.* 2015). Constitutive complex formation of SOBIR1 and RLP23 and ligand-dependent recruitment of BAK1 into the entity can also be investigated in co-immunoprecipitation assays in *N. benthamiana*, regardless of whether RLP23-GFP or SOBIR1-GFP proteins are precipitated (Fig. 3-19) (Albert, *et al.* 2015). Also SERK1, SERK2 and SERK4/BKK1 are pulled-down in a nlp20-dependent manner. This is reminiscent of the receptor kinases FLS2 and EFR, recruiting the same SERKs in a flg22 or elf18-dependent manner respectively (Roux, *et al.* 2011). Nlp20-dependent recruitment of BAK1 also works under native BAK1 levels, as shown in a co-immunoprecipitation assay using *rlp23-1/p35S::RLP23:GFP* transgenic plants (Fig. 3-19). Ligand-dependent recruitment of BAK1 might be a general characteristic of RLP-type immune receptors, since BAK1 can be co-immunoprecipitated with RLP30 and SOBIR1 solely after treatment with the corresponding ligand, SCFE1 (Albert, *et al.* 2015).

SOBIR1 constitutively interacts with RLP23 in a fashion independent of nlp20 treatment, and this is a physical interaction as ratiomeric bimolecular fluorescence complementation assays (BIFC) in *Arabidopsis* protoplasts show a yellow fluorescent signal upon expression of SOBIR1 and RLP23 split-YFP constructs (Fig. 3-16). Likewise, a complemented YFP signal of interacting SOBIR1 and RLP23 proteins proves direct interaction of the proteins in *N. benthamiana* (Albert, *et al.* 2015). In vitro gel filtration experiments with recombinant ectodomains of RLP23 and BAK1 reveal that also these proteins physically interact with each other, since co-migration and co-elution occurs in the presence of nlp20 (Albert, *et al.* 2015). This is reminiscent of the proposed mechanism for the interaction of BAK1 with the RLK FLS2, in which the ligand flg22 acts as 'molecular glue' between both proteins (Sun, *et al.* 2013). RLP23, SOBIR1 and BAK1 are thus in very close physical vicinity, likely forming a tripartite receptor entity to mediate nlp20-induced signaling. Crystallization of the complex is in progress in collaboration with Jijie Chai (Tsinghua University Beijing), and will reveal new insight into ligand-induced LRR-RP-LRR-RK complex assembly.

### 4.2.3 Expression of RLP23 confers broad-spectrum disease resistance in potato

Nowadays, deployment of agrichemicals is an indispensable tool to control pests and microbial diseases on plants, preventing crop shortfalls and saving global food security. Many pesticides are toxic and pose serious health and environmental risks. Thus, other solutions are urgently in demand and research on plant-pathogen interactions within the last years has revealed many genes that are promising candidates for genetic engineering of pest resistant crops. However, attempts to engineer resistant crops often fail due to detrimental side effects on plant development and growth or crop yield (Hammond-Kosack and Parker 2003, Gurr and Rushton 2005).

Potato is a crop playing an important role in the world's food production and supply. 386 million tons were produced in 2013 worldwide, whereas two thirds are directly for human consumption and the rest is used as animal feed or for the production of starch (Food and Agriculture Organization of the United Nations). It is a cheap and nutritious crop, growing under most diverse climate conditions and altitudes and thereby requires little water. The United Nations named the year 2008 as the International Year of the Potato, with the potential to fight the hunger of an ever growing world population. *Phytophthora infestans* is one of the most devastating potato pathogens and the causal agent for late blight. Fungicides are the means of choice to fight the pathogen and to avoid crop failures, but still 20 % of the potato crop loss worldwide can be assigned to *P. infestans*. In the last years, researchers identified R genes from wild potato cultivars conferring enhanced resistance against *P. infestans* upon transfer into cultivated potato (Bradeen *et al.* 2009, Foster *et al.* 2009). The delicate part of R gene mediated resistance is that no durability is guaranteed, since effectors might evolve quite rapidly and positive selection leads to growth of pathogens circumventing recognition by the plant. Recently, transfer of the *Solanum microdontum* LRR-RP ELR, the receptor for elicitor, has been shown to be a valuable tool for engineering resistance in potato (Du *et al.* 2015a). Elicitors are evolutionary conserved proteins among several *Phytophthora* species, increasing the potential to confer durable resistance against *P. infestans* through ELR transfer into susceptible crops.

Many of the oomycete and fungal pathogens listed in the top 10 in molecular plant pathology express NLPs and constitute a serious threat for crop yields such as

*Phytophthora* species, *Magnaporthe oryzae* or *Fusarium* species (see 1.3). The broad taxonomic distribution of NLPs among bacteria, omycetes and fungi, the fact that various destructive pathogens express NLPs with the immunogenic nlp20 motif, and the rather genus-specific occurrence of RLP23 predestines the receptor for engineering disease resistance in economically important crops. Transfer of RLP23 into solanaceous plants like *N. benthamiana*, *S. lycopersicum* and *S. tuberosum* confers responsiveness towards nlp20 (Fig. 3-12), suggesting the presence of RLP23-compatible components needed for downstream signaling such as SOBIR1 and BAK1/BKK1. Transgenic potato plants expressing RLP23 exhibit increased disease resistance against the devastating pathogens *Phytophthora infestans* and *Sclerotinia sclerotiorum* (Fig. 3-14), turning RLP23 into a valuable instrument for engineering broad-spectrum disease resistance in crops.

This is reminiscent of the successful transfer of *Arabidopsis* EFR, a receptor-like kinase recognizing the highly conserved bacterial elongation factor Ef-Tu or its epitope elf18, into solanaceous plants such as tomato leading to broad-spectrum disease resistance towards bacterial pathogens (Lacombe, *et al.* 2010). Dicotyledoneous-derived EFR also confers responsiveness and enhanced disease resistance upon transfer into monocot species like rice and wheat, suggesting that both classes share conserved immune signaling components (Schoonbeek *et al.* 2015, Schwessinger, *et al.* 2015). Likewise, the rice receptor-like kinase Xa21 can be transferred into dicot species such as orange, banana and tomato cultivars conferring resistance to *Xanthomonas* and *Pseudomonas* species (Mendes, *et al.* 2010, Afroz *et al.* 2011, Tripathi, *et al.* 2014). Xa21 confers robust resistance against most strains of *Xanthomonos oryzae* pv. *oryzae* and recognizes the conserved RaxX protein (Pruitt, *et al.* 2015). Virtually all crop plants are affected by *Xanthomonas* species, making Xa21 to a meaningful object of research in transgenic engineering of resistant crops. In spite of all that, *raxX* allelic variants of economically important *Xanthomonas* species are discovered, which evade recognition by Xa21. This means that novel Xa21 variants need to be engineered which recognize the altered RaxX proteins to confer resistance against certain *Xanthomonas* strains.

Breeding crops with resistance genes like Xa21 (or variants thereof), ELR, EFR (Gust *et al.* 2010, Dangl *et al.* 2013) or RLP23 recognizing highly conserved structures might lead to extremely durable disease resistance, since the recognized patterns are not subject to rapid evolution like most effector molecules. Pyramiding of such

receptors with R genes would lead to maximized quantitative and qualitative immune output, presumably establishing crop plants with a durable, broad-spectrum disease resistance.

## 5. Summary

NEP1-like proteins are phytotoxins and microbial virulence factors secreted by bacteria, oomycetes and fungi capable of exerting immune responses in dicot plant species. Previously, immunogenic activity has been linked to cytotoxicity, supposedly through toxin-mediated release of endogenous signals of danger called DAMPs.

Comparative analysis of *Pectobacterium carotovorum* and *Phytophthora parasitica*-derived NLPs revealed a second, toxin-independent immune stimulating mechanism within the *Pp*NLP protein. This concerns a 20 amino acid stretch designated as nlp20. Orthologous immunogenic sequences can be found in NLPs of bacteria, oomycetes and fungi; a unique distribution pattern among all known PAMPs to date. Nlp20 and its orthologs elicit a comprehensive set of immune responses in *Arabidopsis*. Besides some closely related *Arabidopsis* species, lettuce could be identified as nlp20-sensitive. Nlp20 is localized rather inside of *Pp*NLP than being exposed on the surface of the protein. Presumably, structural reorganization of the protein upon binding to the plant plasma membrane takes place, exposing the pattern for recognition by a PRR. NLPs with the novel PAMP nlp20 are paradigmatic for the discussed continuum between ETI and PTI, since usual classification into PAMP or effector seems to be blurred.

Nlp20 is perceived by the *Arabidopsis* LRR-RP RLP23, and ligand and receptor physically interact with each other. Likely, interaction is mediated by the highly conserved amino acids Y<sub>106</sub>, W<sub>108</sub> and Y<sub>109</sub> and I<sub>104</sub> of the nlp20 motif. The LRR-RKs SOBIR1 and BAK1/BKK1 are required as co-receptors for facilitating nlp20-induced downstream signaling. RLP23 interacts constitutively and independent of nlp20 with SOBIR1, whereas BAK1 is recruited into the receptor complex in a nlp20-dependent fashion. This is reminiscent of the 'molecular glue' flg22 stabilizing FLS2 and BAK1 interaction. All three receptors are in close physical proximity, likely forming a tripartite receptor complex. Interfamily transfer of RLP23 into the important crop potato confers broad-spectrum disease resistance against fungi and oomycetes, turning RLP23 into a promising candidate for engineering durable disease resistance against an ample spectrum of pathogens in crops.



## 6. Zusammenfassung

NEP1-ähnliche Proteine sind Phytotoxine und mikrobielle Virulenzfaktoren welche von Bakterien, Oomyzeten und Pilzen sekretiert werden und imstande sind Immunantworten in dikotylen Pflanzen auszulösen. Bislang wurde die immunogene Aktivität mit der Zytotoxizität verknüpft, vermutlich ausgelöst durch Toxin-induzierte Freisetzung endogener Gefahrensignale welche DAMPs genannt werden.

Vergleichsstudien von NLPs aus *Pectobacterium carotovorum* und *Phytophthora parasitica* deckten einen zweiten, toxinunabhängigen Mechanismus der Immunstimulation innerhalb des PpNLP Proteins auf. Hierbei handelt es sich um ein 20 Aminosäuren langes Stück welches als nlp20 bezeichnet wird. Orthologe, immunogene Sequenzen können in NLPs aus Bakterien, Oomyzeten und Pilzen gefunden werden; ein einzigartiges Verteilungsmuster unter allen bisher bekannten PAMPs. Nlp20 und dessen Orthologe lösen eine ganze Reihe an Immunantworten in *Arabidopsis* aus. Neben ein paar eng verwandten *Arabidopsis* Spezies konnte eine Lattich-Art als nlp20-sensitiv identifiziert werden. Nlp20 ist vielmehr im Inneren von PpNLP lokalisiert als exponiert auf der Oberfläche des Proteins zu liegen. Vermutlich finden nach erfolgter Bindung an die Plasmamembran strukturelle Umgestaltungen des Proteins statt, wobei das Erkennungsmuster zur Erkennung durch einen PRR freigelegt wird. NLPs mit dem neuen PAMP nlp20 sind beispielhaft für das derzeit diskutierte Kontinuum zwischen ETI und PTI, da die gebräuchliche Klassifizierung in PAMP oder Effektor unklar erscheint.

Nlp20 wird von dem *Arabidopsis* LRR-RP RLP23 erkannt, wobei Ligand und Rezeptor direkt miteinander interagieren. Wahrscheinlich wird die Interaktion durch die stark konservierten Aminosäuren Y<sub>106</sub>, W<sub>108</sub> and Y<sub>109</sub> und I<sub>104</sub> des nlp20 Motivs vermittelt. Die LRR-RKs SOBIR1 und BAK1/BKK1 werden als Korezeptoren benötigt um die nlp20-induzierte Signalweiterleitung zu ermöglichen. RLP23 interagiert konstitutiv und unabhängig von nlp20 mit SOBIR1, während BAK1 in einer nlp20-abhängigen Art und Weise in den Rezeptorkomplex rekrutiert wird. Dies erinnert an den „molekularen Kleber“ flg22, der die Interaktion von FLS2 mit BAK1 stabilisiert. Alle drei Rezeptoren befinden sich in unmittelbarer Nähe zueinander, wobei vorraussichtlich ein dreiteiliger Rezeptorkomplex gebildet wird. Ein interfamiliärer Transfer von RLP23 in die bedeutende Feldfrucht Kartoffel vermittelt

Krankheitsresistenz mit einem breiten Wirkspektrum gegen Pilze und Oomyceten, was RLP23 zu einem vielversprechenden Kandidaten macht um langlebige Resistenz gegen ein umfassendes Spektrum an Pathogenen in Kulturpflanzen zu entwickeln.

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## 8. Appendix

### Abbreviations

<i>At</i>	<i>Arabidopsis thaliana</i>
ATP	Adenosine triphosphate
BAK1	BRI1-associated kinase 1
<i>Bc</i>	<i>Botrytis cinerea</i>
<i>Be</i>	<i>Botrytis elliptica</i>
<i>Bh</i>	<i>Bacillus halodurans</i>
BIK1	Botrytis-induced kinase 1
BIR	Baculovirus inhibitor repeat
BIR2	BAK1-interacting receptor-like kinase 2
BKK	BAK1-like kinase
BLB	Bacterial leaf blight
BR	Brassinosteroid
BRI	Brassinosteroid insensitive 1
<i>Bs</i>	<i>Bacillus subtilis</i>
BSK1	BR-signaling kinase 1
B-type	Bulb-type
C6	Chitin hexamer
CARD	Caspase-recruitment domain
CBB	Cassava bacterial blight
CBEL	<i>Phytophthora</i> cellulose-binding elicitor lectin
CC	Coiled-coil
CDPK	Calcium-dependent protein kinase
CEBIP	Chitin elicitor-binding protein
CERK1	Chitin elicitor receptor kinase 1
<i>Cf</i>	<i>Cladosporium fulvum</i>
cfu	Colony forming unit
CLR	C-type lectin receptor
CpG	Cytosin-phosphatidyl-guanin
CRD	Carbohydrate recognition domain
CRN	Crinkling and necrosis
Csp	Cold-shock protein
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
DORN1	Does not respond to nucleotides 1
EFR	Ef-Tu receptor
Ef-Tu	Elongation factor thermo unstable
EGF	Epidermal growth factor
EIX	Ethylene-inducing xylanase
elf18	Peptide from EF-Tu with the sequence SKEKFERTKPHVNVGTIG
ELR	Elicitin response
eMax	Enigmatic MAMP of Xanthomonas
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility

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flg22	Peptide from flagellin with the sequence QRLSTGSRINSAKDDAAGLQIA
FLS2	Flagellin-sensing 2
<i>Fo</i>	<i>Fusarium oxysporum</i>
GBP	$\beta$ -glucan binding protein
GlcNAc	N-acetylglucosamine
GUS	$\beta$ -glucuronidase
<i>Ha</i>	<i>Hyaloperonospora arabidopsidis</i>
HG	Heptagluco-side
HMGB1	High mobility group box 1
HR	Hypersensitive response
HST	Host-selective toxin
IRAK	Interleukin-1 receptor-associated kinase
JA	Jasmonic acid
KAPP	Kinase-associated protein phosphatase
LORE	Lipooligosaccharide-specific reduced elicitation
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LysM	Lysin motif
MAMP	Microbe-associated molecular pattern
MAP kinase	Mitogen-activated protein kinase
MurNAc	N-acetylmuramic acid
MyD88	Myeloid differentiation primary response gene 88
<i>Myg</i>	<i>Mycosphaerella graminicola</i>
NADPH	Nicotinamide adenine dinucleotide phosphate
NBS	Nucleotide-binding site
NEP1	Necrosis- and ethylene-inducing peptide 1
NF	nodulation factor
NF- $\kappa$ B	Nuclear factor-KappaB
NLP	NEP1-like protein
nlp20	Peptide from <i>Pp</i> NLP with the sequence AIMYSWYFPKDSPVTGLGHR
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
OGs	Oligogalacturonides
<i>Os</i>	<i>Oryza sativa</i>
PAD3	Phytoalexin deficient 3
PAMP	Pathogen-associated molecular pattern
<i>Pcc</i>	<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>
PGN	Peptidoglycan
<i>Pp</i>	<i>Phytophthora parasitica</i>
PP2C	Protein phosphatase 2C
PR1	Pathogenesis-related 1
PRR	Pattern recognition receptor
PTI	PAMP-triggered immunity
pv.	Pathovar
<i>Pya</i>	<i>Pythium aphanidermatum</i>
PYR	Pyrin
RaxX	Required for activation of Xa21 X
rbcl	Ribulose bisphosphate carboxylase large chain
RBOHD	Respiratory burst oxidase-D



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RBPG 1	Responsiveness to <i>Botrytis</i> polygalacturonases 1
ReMAX	Receptor of eMax
RIG I	Retinoic acid inducible I
RIN4	RPM1-interacting protein 4
RK	Receptor kinase
RLCK	Receptor-like cytoplasmic kinase
RLK	Receptor-like kinase
RLP	Receptor-like protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP	Receptor protein
R-protein	Resistance protein
SA	Salicylic acid
SAR	Systemic acquired resistance
SCFE1	Sclerotinia culture filtrate elicitor 1
SERK	Somatic embryogenesis receptor
<i>S/</i>	<i>Solanum lycopersicum</i>
SOBIR1/EVR	Suppressor of BIR1-1/evershed
spp.	Species pluralis
ssRNA	Single-stranded RNA
StnII	Sticholysin II
T3SS	Type 3 secretion system
TAL	Transcription activator-like
TIR	Toll/interleukin 1-receptor
TLR	Toll-like receptor
VBP	Victorin binding protein
WAK1	Wall-associated kinase 1
WGD	Whole genome duplication

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**Curriculum Vitae**


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**Hannah Undine Böhm**


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26.05.1985 / Friedrichshafen, Germany **Date / Place of Birth**

Single **Family Status**


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**University Career**

University of Tübingen, GERMANY 09 / 2011- 12 / 2015  
 ZMBP, Plant Biochemistry  
 PhD thesis adviser: Prof. Dr. Thorsten Nürnberger  
 - Identification and characterization of a novel PAMP from a  
 widespread microbial virulence factor and its perception system  
 in *Arabidopsis* -

Julius- Maximilians- University of Würzburg, GERMANY 10 / 2007 - 01 / 2011  
 Diploma of Biology  
 - Biotic and abiotic stress are inversely regulated by *Arabidopsis*  
*thaliana* Plat Domain Protein1 -

University of Hamburg, GERMANY 04 / 2005 - 10 / 2007  
 Intermediate Diploma of Biology

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**Research stays abroad**

John Innes Centre, The Sainsbury Laboratory, Norwich, UK 09 / 2012  
 - Handling the oomycete *Phytophthora infestans*:  
 Infection assays and evaluation of disease symptoms -

Karl- Franzens- University of Graz, Institute of Plant Sciences, 02 / 2010 – 11 / 2010  
 AUSTRIA  
 - Biotic and abiotic stress are inversely regulated by *AtPDP1* -

National University of Singapore, Dept. of Biological Sciences, 01 / 2009 - 05 / 2009  
 SINGAPORE  
 - From cloning to visualization: *HOG1* and *HOG2-GFP* in  
*A.thaliana* -

National Institute of Plant Genome Research, New Delhi, INDIA 11 / 2008 - 12 / 2008  
 - Dissecting mitogen activated protein kinase (MAPK)  
 cascade in rice -

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	<b>Publications</b>
<b>Albert and Böhm et al. (2015):</b> An RLP23-SOBIR1-BAK1 complex mediates NLP-triggered immunity.	<i>Nature Plants</i>
<b>Hyun et al. (2015):</b> The <i>Arabidopsis</i> PLAT Domain Protein1 promotes abiotic stress tolerance and growth in Tobacco.	<i>Transgenic Research</i>
<b>Hyun et al. (2014):</b> The <i>Arabidopsis</i> PLAT Domain Protein1 is critically involved in abiotic stress tolerance.	<i>PLOS ONE</i>
<b>Böhm and Albert et al. (2014):</b> A conserved peptide pattern from a widespread microbial virulence factor triggers pattern-induced immunity in <i>Arabidopsis</i> .	<i>PLOS Pathogens</i>
<b>Oome et al. (2014):</b> Nep1-like proteins from three kingdoms of life act as a microbe-associated molecular pattern in <i>Arabidopsis</i> .	<i>PNAS</i>
<b>Böhm et al. (2014):</b> Immune receptor complexes at the plant cell surface.	<i>COPB</i>