Activation of plant immunity by microbial Nep1-like protein patterns

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Activation of plant immunity by microbial Nep1-like protein patterns
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Activation of plant immunity by microbial Nep1-like protein patterns

Activatie van het immuunsysteem van planten
door een patroon van Nep1-achtige eiwitten

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter

General introduction

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Abstract

Extracellular recognition of pathogens by plants constitutes an important early detection system in plant immunity. Microbe-derived molecules, also named patterns, can be recognized by pattern recognition receptors (PRRs) on the host cell membrane that trigger plant immune responses. Most knowledge on extracellular pathogen detection by plants comes from research on bacterial and fungal pathogens. For oomycetes, that comprise some of the most destructive plant pathogens, mechanisms of extracellular pattern recognition have only emerged recently. These include newly recognized patterns, e.g., cellulose-binding elicitor lectin, necrosis and ethylene-inducing peptide 1-like proteins (NLPs), and glycoside hydrolase 12, as well as their receptors, e.g., the putative elicitin PRR elicitin response and the NLP PRR receptor-like protein 23. Immunity can also be triggered by the release of endogenous host-derived patterns, as a result of oomycete enzymes or damage. In this introductory chapter, we will describe the types of patterns, both pathogen-derived exogenous and plant-derived endogenous ones, and what is known about their extracellular detection during (hemi-) biotrophic oomycete infection of plants.

Introduction

Most plant pathogens are able to penetrate host tissues but essentially grow in the plant apoplast or extracellular space. Even haustoria, feeding structures formed by many biotrophic fungi and oomycetes that invaginate host cells, remain separated from the plant cell cytoplasm by the plant-derived extrahaustorial membrane (Parniske, 2000). It, therefore, comes as no surprise that a first line of pathogen recognition is extracellular and mediated by membrane-bound receptors that detect microbe- or host damage-derived molecules or patterns. Over the last decades, many receptors mediating immunity to molecules of bacteria and fungi have been reported. Well-known examples include the *Arabidopsis* receptor-like kinase (RLK) FLAGELLIN-SENSING 2 (FLS2) that mediates recognition of bacterial flagellin, and the rice lysin motif (LysM)-receptor-like protein (RLP), chitin elicitor-binding protein (CEBiP) involved in detection of fungal chitin (Zipfel, 2014). Flagellin and chitin are considered microbe-associated molecular patterns (MAMPs), while their cognate receptors are termed pattern-recognition receptors (PRRs; Jones and Dangl, 2006; Hein et al., 2009; Dodds and Rathjen, 2010).

MAMPs are generally considered conserved molecules that occur in all species of a given taxon. There are, however, many examples of patterns that are species-specific or that are less well conserved, e.g., apoplastic effectors that are recognized by cognate resistance gene-encoded membrane-bound receptors (Thomma et al., 2011). In this review we, therefore, refer to all extracellular molecules that trigger immunity as patterns (Cook et al., 2015). In older papers the term "elicitor" is most often used, but many of these can be regarded as patterns too (Boller and Felix, 2009; Cook et al., 2015). Although numerous oomycete patterns have been described, knowledge on the mechanism of their extracellular recognition has only emerged recently for some of them.

Oomycetes are filamentous organisms that belong to the Stramenopiles, a taxon that also encompasses the diatoms and brown algae. Many oomycetes are free-living saprobes in soils or aquatic environments. The best-known oomycetes, or the most infamous ones, are species that are pathogenic on plants, e.g., the potato late blight pathogen *Phytophthora infestans* and the grape downy mildew *Plasmopara viticola* (Haas et al., 2009; Kamoun et al., 2015). Five main taxa of phytopathogenic oomycetes can be distinguished: (i) the genus *Phytophthora*, (ii) the downy mildews, (iii) the white blister rusts, (iv) the genus *Pythium*, and (v) the genus *Aphanomyces* (Thines and Kamoun, 2010).

In this chapter, we focus on the extracellular recognition of (hemi-)biotrophic oomycetes, on patterns that trigger immunity, and on mechanisms of pattern recognition. A broad range of molecules or patterns is released during oomycete infection of plants, either exogenous ones derived from the pathogen, or endogenous ones that are released from the plant host (Figure 1). The distinction between exogenous and endogenous signals

can also be referred to as non-self and modified-self patterns (Schwessinger and Zipfel, 2008). Endogenous patterns, also known as damage-associated molecular patterns (DAMPs), either result from oomycete enzyme activities, or from lysis or disruption of host cells during the infection process. Oomycete patterns and other elicitors can be grouped based on their cellular origin (oomycete cell wall/membrane, or pathogen secreted). In this chapter, we will review the different patterns, their cellular origin, and what is known about the detection mechanisms that have evolved to recognize such patterns, and trigger the plant immune system.

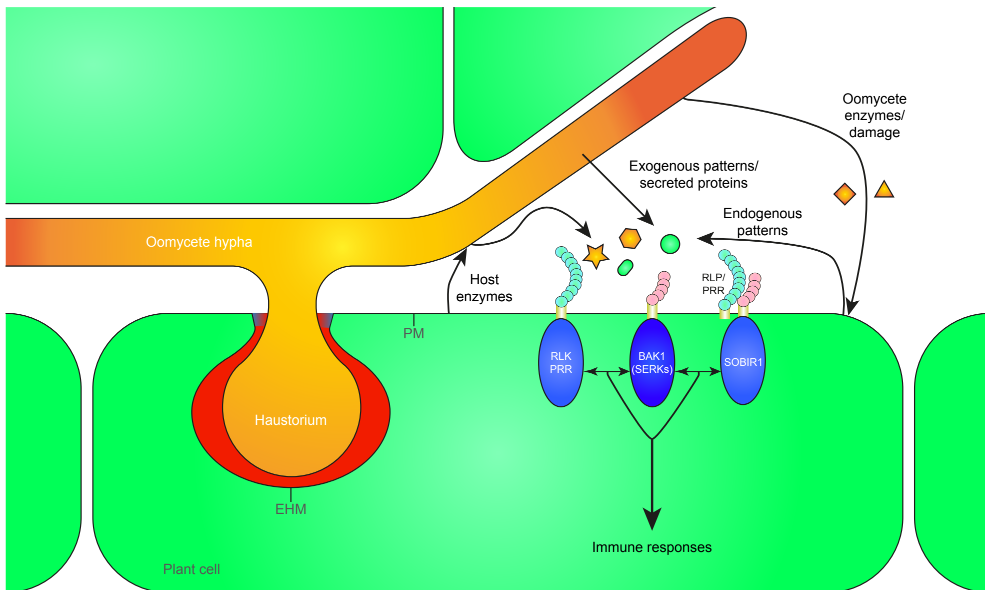

FIGURE 1 | Recognition of exogenous and endogenous patterns during oomycete infection leads to the activation of plant immunity. Oomycete pathogens secrete proteins in the apoplast (white) and extrahaustorial matrix (red) that can be perceived as exogenous patterns by pattern recognition receptors (PRRs) in the plant plasma membrane (PM) or extrahaustorial membrane (EHM). Furthermore, pathogen-derived cell wall or membrane fragments are released during infection, possibly by host enzymes, and recognized as patterns by the host. Mechanical damage or damage caused by oomycete secreted enzymes can release endogenous patterns that trigger immunity. The receptor-like kinase (RLK) BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), a member of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family, functions as a central hub of RLK and receptor-like protein (RLP) triggered immunity. RLPs form a bimolecular receptor kinase with the RLK SUPPRESSOR OF BIR1 1 (SOBIR1). RLKs and RLPs bound to SOBIR1 associate with BAK1 to activate pattern-triggered immunity upon the perception of exogenous or endogenous patterns. The haustorial callosic neckband that is sometimes formed in oomycete–plant interactions is depicted in blue. Oomycete-derived patterns and proteins are depicted in orange, plant-derived patterns in green.

Oomycete Patterns Triggering Immunity

Plants can sense a wide variety of extracellular oomycete-derived patterns. These molecules can be secreted by oomycetes during infection, or released from the invading pathogens by host-derived enzymes (Table 1). Several oomycete patterns are derived from the pathogen's cell wall or membrane, whereas others are secreted to the extracellular environment before being detected by the plant immune system. Below we discuss the different extracellular patterns, where they derive from, and what is known about their function.

TABLE 1 | Oomycete patterns that activate plant immunity.

Elicitor[a]	Source	Type	(Putative) Receptor[b]	Receptor type[c]	Co-receptors[d]	References
ß-glucans	Cell wall	Carbohydrate	GBP, additional components required	GH16		Fesel and Zuccaro, 2016
Glucan-chitosaccharides	Cell wall	Carbohydrate	Unknown			Nars et al., 2013
Pep-13	Cell wall	Peptide	Unknown monomeric 100 kDa integral plasma membrane protein			Reiss et al., 2011
Eicosapolyenoic acids	Membrane	Fatty acid	Unknown			Robinson and Bostock, 2015
GH12 (XEG1)	Secreted protein	Protein	Unknown		SERK3/BAK1 required	Ma et al., 2015
nlp20/nlp24	Secreted protein	Peptide	RLP23	RLP	BAK1 and SOBIR1 required	Albert et al., 2015
Elicitins	Secreted protein	Protein	ELR	RLP	BAK1 and SOBIR1 required	Du et al., 2015
CBM1/CBEL	Secreted protein	Protein	Unknown		partially requires BAK1	Larroque et al., 2013
OPEL	Secreted protein	Protein	Unknown			Chang et al., 2015

[a] GH12 = glycoside hydrolase family 12; XEG1 = xyloglucanspecific endo-β-1,4-glucanase; nlp = necrosis and ethylene-inducing peptide 1-like protein; CBM1 = carbohydrate binding module 1; CBEL = cellulose-binding elicitor lectin.
[b] GBP = Glucan Binding Protein; RLP23 = receptor-like protein 23; ELR = elicitin response.
[c] GH16 = glycoside hydrolase family 16; RLP = receptor-like protein.
[d] SERK3 = SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 3; BAK1= BRI1-ASSOCIATED RECEPTOR KINASE; SOBIR1 = SUPPRESSOR OF BIR1 1.

Cell Wall/Membrane-Derived Patterns

ß-Glucans

The most abundant constituents of oomycete cell walls are glucans, polysaccharides that consist of linked glucose units (Aronson et al., 1967; Sietsma et al., 1969). β-1,3 and β-1,6-glucan are the major components of oomycete cell walls, whereas cellulose, a β-1,4-glucan, forms a relatively small fraction (Aronson et al., 1967). β-1,6-Glucan is only found in oomycetes and fungi, whereas cellulose and β-1,3-glucan are present in plant cell walls too (Fesel and Zuccaro, 2016).

A β-glucan-triggered response, i.e., the accumulation of the phytoalexin glyceollin, was first observed when soybean (*Glycine max*) was treated with glucans isolated from cell walls of *Phytophthora sojae* (previously *P. megasperma* f. sp. *glycinea* and *P. megasperma* var. *sojae*; Ayers et al., 1976). β-Glucans also trigger phytoalexin production in several other fabaceous species, and in potato (*Solanum tuberosum*), although this is a weaker response (Cline et al., 1978; Cosio et al., 1996). A purified β-1,3/1,6-glucan heptaglucoside was found to be one of the active molecules in eliciting production of phytoalexins in soybean (Sharp et al., 1984a, 1984b). Laminarin, an oligomeric β-1,3-glucan with β-1,6-glucan branches isolated from the marine brown alga *Laminaria digitata*, is another pattern that can induce a plethora of defense-associated responses in tobacco *(Nicotiana tabacum)*, grapevine (*Vitis vinifera*), and the monocots rice (*Oryza sativa*) and wheat (*Triticum aestivum*; Inui et al., 1997; Klarzynski et al., 2000; Aziz et al., 2003). Furthermore, *Arabidopsis thaliana* is responsive to the β-glucan laminarin, although it does not respond to the oomycete-derived heptaglucoside elicitor. *Arabidopsis* responses to laminarin are mediated by the plant hormone ethylene and do not seem to involve the well-known defense hormone salicylic acid (SA). In contrast, when *Arabidopsis* or tobacco plants are treated with a sulfated form of laminarin the expression of the SA-responsive marker gene *PR-1* is induced (Ménard et al., 2004). Taken together, responses to β-glucans vary greatly depending on the specific β-glucan and plant species. Therefore, different plant species might have different receptors involved in the recognition of different β-glucan patterns.

Phytophthora-derived β-1,3-glucan was shown to bind soybean membranes (Yoshikawa et al., 1983). The glucan-binding protein (GBP) from soybean was identified and it was demonstrated that, when expressed in tobacco and *Escherichia coli*, GBP conferred β-glucan-binding activity. Furthermore, an antibody raised against GBP inhibited β-glucan-binding activity in soybean and reduced phytoalexin accumulation (Umemoto et al., 1997). Interestingly, GBP also shows β-glucanase activity and might release β-glucans from the pathogen’s cell wall (Fliegmann et al., 2004). After heterologous expression of soybean GBP in tomato, high-affinity binding of the β-1,3/1,6-glucan heptaglucoside was observed. However, this did not result in activation of downstream defense responses in tomato (Mithöfer et al., 2000; Fliegmann et al., 2004). These data suggest that additional,

probably membrane-bound, proteins are required to recognize the β-glucan patterns (Mithöfer et al., 2000).

Glucan-Chitosaccharides

Recently, glucan-chitosaccharides were isolated from the cell wall of the root oomycete *Aphanomyces euteiches* and were found as novel patterns that triggered calcium oscillations in the nucleus of root cells and induced defense genes in *Medicago truncatula* (Nars et al., 2013). How these molecules are perceived is not yet known, but there is a role for the nod factor perception (NFP) protein, LysM-RLK. NFP is involved in the recognition of microbial *N*-acetylglucosamine patterns and is required for nodule formation in interaction with *Rhizobium* bacteria. An *nfp* mutant was more susceptible to *A. euteiches*, whereas overexpression of *NFP* led to increased resistance, demonstrating its involvement in the perception of *A. euteiches* by *M. truncatula* (Rey et al., 2013). However, NFP was not required for the glucan-chitosaccharide-induced calcium oscillations, suggesting a regulatory function in defense for NFP rather than direct recognition (Nars et al., 2013).

Transglutaminases (Pep-13)

Transglutaminases (TGases) are a widespread family of enzymes, found in prokaryotes and eukaryotes, that facilitate cross-linking between glutamine and lysine residues in proteins, thereby strengthening structures, e.g., cell walls (Lorand and Graham, 2003; Martins et al., 2014). The formation of a covalent bond between amino acid residues confers high resistance to proteolysis (Reiss et al., 2011). In oomycetes, TGases could protect cell walls from hydrolytic host enzymes. A 42-kDa TGase cell wall glycoprotein (GP42) of *P. sojae* functions as a potent elicitor of phytoalexin synthesis in the non-host parsley (*Petroselinum crispum*; Parker et al., 1991). A 13-amino acid peptide fragment (Pep-13) derived from GP42 was found responsible for triggering immunity and was shown to bind to purified plasma membranes of parsley. Furthermore, Pep-13 elicits a multitude of defense responses, e.g., expression of defense-related genes and phytoalexin production (Nürnberger et al., 1994, 1995; Hahlbrock et al., 1995). Interestingly, Pep-13 treatment of potato resulted in a similar defense activation, with the distinct difference that it induced a hypersensitive response (HR; Halim et al., 2004).

GP42 homologs are only found in oomycetes and some marine bacteria belonging to the genus *Vibrio* that are pathogenic on fish and several marine invertebrates (Reiss et al., 2011). It is thought that an ancestral oomycete, from which species of *Phytophthora*, *Pythium* and downy mildews have evolved, acquired GP42 from *Vibrio* bacteria through horizontal gene transfer, giving a selective advantage over oomycetes that lack this TGase (Reiss et al., 2011). A 100 kDa monomeric plasma membrane protein from parsley was shown to bind to the Pep-13 ligand and thus may be part of the putative receptor complex (Nennstiel et al., 1998).

Eicosapolyenoic Acids

Application of mycelial extracts from *P. infestans* to potato tubers led to necrosis and accumulation of phytoalexins, predominantly rishitin and lubimin. The molecules responsible for triggering this response were identified as the eicosapolyenoic acids (EPs), arachidonic acid (AA), and eicosapentaenoic acid (EPA; Bostock et al., 1981). Treating potato tuber slices with AA greatly reduced or even arrested growth of *P. infestans* (Bostock et al., 1982). EPs are components of *Phytophthora* cells that are seemingly not present in other microbial classes nor are they produced by higher plants (Robinson and Bostock, 2015). Interestingly, the downy mildew *Hyaloperonospora arabidopsidis* has lost the genes required for AA synthesis (Baxter et al., 2010). It is tempting to speculate that *H. arabidopsidis* has lost this ability through evolution as a way to avoid recognition.

Eicosapolyenoic acids induce the accumulation of antimicrobial compounds in many plant species, ranging from many solanaceous species, e.g., potato and tomato, to bean (*Phaseolus vulgaris*) and avocado (*Persea americana*; Longland et al., 1987; Romero-Correa et al., 2014; Robinson and Bostock, 2015). Furthermore, in potato application of AA induced accumulation of reactive oxygen species (ROS), that could be involved in mediating the synthesis of the phytoalexin rishitin from lubimin (Yoshioka et al., 2001).

EPs are able to trigger systemic acquired resistance in several plants species to different pathogens. The hormonal regulation of these responses seems to differ among plant species; in some, the SA pathway is elicited, whereas in other species responses seem to rely on jasmonic acid (JA) or ethylene. It is postulated that this may be due to the concentration of EPs in the treatment (Robinson and Bostock, 2015). For example, *Arabidopsis* plants made to produce low levels of EP showed increased resistance to *Botrytis cinerea*, *P. capsici* and aphid feeding, but higher susceptibility to *Pseudomonas syringae* pv. *tomato* DC3000. This was associated with higher levels of JA and enhanced expression of JA-related genes, but decreased SA levels and reduced expression of SA-related genes. Furthermore, low levels of AA administered to tomato leaves resulted in increased JA levels and decreased SA levels and higher resistance against *B. cinerea* (Savchenko et al., 2010).

How, exactly, EPs are perceived remains to be resolved. EPs could be recognized directly by a membrane-bound receptor, leading to the activation of plant immunity. Another possibility is that plant membranes that readily incorporate AA (Ricker and Bostock, 1992), are perturbed leading to the release of endogenous patterns from the host cell cytoplasm. Or alternatively, AA can be used as a substrate for lipoxygenases, e.g., the potato LOX1, thereby producing oxylipin signals that trigger plant immunity. In the latter two scenarios recognition would be independent of plant PRRs (Robinson and Bostock, 2015).

Interestingly, treating potato with a combination of AA and β-1,3-glucans strongly increased the response to AA. β-glucans alone, however, did not trigger a response in potato (Preisig and Kuć, 1988).

Secreted Proteins

Glycoside Hydrolase 12 Proteins

Recently, the XEG1 (xyloglucan-specific endo-β-1,4-glucanase) protein was isolated from *P. sojae* culture filtrates (Ma et al., 2015). This secreted protein elicits cell death in *N. benthamiana*, *N. tabacum*, pepper (*Capsicum annuum*), tomato (*Solanum lycopersicon*) and soybean but not in maize (*Zea mays*) and cotton (*Gossypium hirsutum*). Analysis of the XEG1 protein sequence revealed that it belongs to the glycoside hydrolase GH12 family that is widespread amongst prokaryotic and eukaryotic microbes, especially in plant-associated microorganisms. Within the *Phytophthora* genus, many GH12 proteins are found of which half trigger cell death in *N. benthamiana.* The downy mildew *H. arabidopsidis* also has three GH12 genes, however, none of them encode a protein that elicits cell death (Ma et al., 2015). Previously, it was demonstrated that fungal GH12 proteins are able to degrade β-glucan (Karlsson et al., 2002) and xyloglucan, a hemicellulose found in the plant cell wall (Master et al., 2008). Recombinant XEG1 protein partially released reducing sugars from both glucans, but was most active with a xyloglucan substrate. Mutations in the catalytic site of XEG1 strongly decreased xyloglucanase activity and abolished β-glucanase activity. In contrast, XEG1 enzyme activity was not required for the induction of cell death in *N. benthamiana* and soybean. Moreover, active and inactive recombinant XEG1 were able to induce resistance against *P. sojae* and *Phytophthora parasitica* var. *nicotianae* to a similar extent in soybean and *N. benthamiana*, respectively. Silencing as well as overexpression of XEG1 in *P. sojae* both led to reduced virulence on soybean through distinct mechanisms. Silenced *P. sojae* lines showed reduced virulence, but did not activate a stronger defense response in soybean, suggesting that XEG1 has a role in virulence, possibly through the breakdown of cell wall components. XEG1 overexpression transformants induced more ROS accumulation and callose deposition compared to wild-type *P. sojae*, confirming the idea that XEG1 acts as a molecular pattern. A XEG1 PRR has not been identified but XEG1 requires the co-receptor SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 3/BRI1-ASSOCIATED RECEPTOR KINASE (SERK3/BAK1) for triggering cell death, suggesting that a SERK3/BAK1-associated RLK or RLP recognizes XEG1 (Ma et al., 2015).

Necrosis and Ethylene-Inducing Peptide 1 (Nep1)-Like Proteins

Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) form a family of secreted proteins mainly found in plant-associated microorganisms, and cytotoxic members are well known to induce necrosis and ethylene production in dicot plants (Bailey, 1995; Oome and Van den Ackerveken, 2014). Three types of NLPs have been identified: type 1 NLPs are found in bacteria, oomycetes, and fungi, type 2 NLPs are found in fungi and bacteria and the newly identified type 3 NLPs are only present in fungi (Oome and Van den Ackerveken, 2014). Although many members of the NLP family

are cytotoxic to plants, in recent years many non-cytotoxic NLPs have been identified in fungal and oomycete species with a (hemi-)biotrophic lifestyle (Cabral et al., 2012; Dong et al., 2012; Zhou et al., 2012). In search of the function of 10 non-cytotoxic NLPs of the obligate biotrophic downy mildew *H. arabidopsidis* (HaNLPs), it was found that NLPs activate plant immunity in *Arabidopsis* (Oome et al., 2014). Expression of *HaNLPs* in *Arabidopsis* led to a severe growth reduction and increased resistance to *H. arabidopsidis* for 7 out of 10 HaNLPs. Only a small fragment of the tested HaNLP3 protein was sufficient to activate plant defense responses and immunity to downy mildew. This 20–24 amino acid fragment (nlp20/nlp24) contains two conserved regions. The second region is the heptapeptide motif GHRHDWE which is highly conserved in all NLPs (Oome and Van den Ackerveken, 2014). The first motif that starts with the AIMY amino acid sequence is highly conserved in type 1 NLPs (Oome et al., 2014). Treatment of *Arabidopsis* plants with synthetic nlp24 peptides corresponding to an oomycete, fungal and bacterial type 1 NLP resulted in the increased production of the defense-related phytohormone ethylene and high resistance to downy mildew. Conversely, a synthetic peptide of a type 2 NLP from the bacterial pathogen *Pectobacterium carotovorum* that lacks the AIMY motif was unable to elicit a response in *Arabidopsis*. Taken together, this demonstrated that the first motif contains the immunogenic part of nlp24 (Oome et al., 2014). Furthermore, nlp20, a peptide based on *Pp*NLP, a cytotoxic *P. parasitica* type 1 NLP, was sufficient for MAPK activation, production of ROS, and increased callose deposition in *Arabidopsis*, but did not have any cytotoxic effect (Böhm et al., 2014). Other plant species were tested for their ability to respond to nlp peptides, revealing that nlp-triggered ethylene production was observed in several closely related Brassicaceae species, and also in more distantly related lettuce plants (*Lactuca sativa*), but not in solanaceous species such as tomato, potato, and *N. benthamiana* (Böhm et al., 2014).

In a screen for nlp20 sensitivity, a collection of T-DNA insertion mutants corresponding to 29 RLKs and 44 RLPs were tested for loss of nlp20-induced ethylene production. Furthermore, 135 natural accessions of *Arabidopsis* were also tested for the loss of nlp20 sensitivity. Two T-DNA insertion alleles of *RLP23*, *rlp23-1*, and *rlp23-2* that were unable to express the receptor-like protein as well as three *Arabidopsis* accessions that carried a frameshift mutation resulting in a premature stop codon in *RLP23* coding sequence were insensitive to nlp20. It was shown that the RLP23 LRR domain physically interacts with nlp20 *in vitro* and *in planta* (Albert et al., 2015). RLP23 lacks a cytoplasmic signaling domain but was shown to require the RLK SUPPRESSOR OF BIR1 1 (SOBIR1) for signaling. RLP23 and SOBIR1 interact in the absence of nlp peptides (Bi et al., 2014; Albert et al., 2015), whereas a second RLK, BAK1, was recruited only in presence of the ligand (Albert et al., 2015). *Arabidopsis sobir1* and *bak1-5/bkk1* mutants lost nlp20-responsiveness, indicating that SOBIR1 and BAK1 are required for RLP23 to function. Moreover, it was demonstrated that RLP23 is required for nlp peptide-induced resistance. Unlike wild-type

Arabidopsis, nlp24 treatment of *rlp23* mutants did not result in an increased resistance to *H. arabidopsidis* (Albert et al., 2015).

Elicitins

Many oomycete pathogens secrete small 10 kDa proteins called elicitins. The first proteins from this family that were identified were cryptogein and capsicein from *Phytophthora cryptogea* and *Phytophthora capsici*, respectively. These proteins were found to elicit necrosis, induce resistance, and cause increased production of ethylene as well as the phytoalexin capsidiol in tobacco plants (Ricci et al., 1989; Milat et al., 1991). Elicitin responses were observed in all tested *Nicotiana* spp., but not in other solanaceous species, such as tomato and eggplant. Furthermore, some Brassicaceae species also respond to elicitin; most radish cultivars (*Raphanus sativus*) and one turnip cultivar (*Brassica campestris*), but not *Arabidopsis*, showed necrosis after elicitin treatment (Kamoun et al., 1993). The gene encoding for *P. infestans* elicitin INF1 was found to be downregulated during early infection of potato. However, in the necrotrophic phase of infection *inf1* expression was upregulated (Kamoun et al., 1997). Interestingly, *N. benthamiana*, a nonhost of *P. infestans*, gained susceptibility after silencing of *inf1*, demonstrating that the recognition of INF1 contributes to resistance (Kamoun et al., 1998).

Members of the Peronosporales, e.g., *Phytophthora* spp. and downy mildews are unable to synthesize sterols and must, therefore, acquire them during pathogenesis. Dehydroergosterol binding activity was shown for several elicitins *in vitro*. Furthermore, elicitins are able to catalyze sterol transfer between liposomes (Mikes et al., 1998). However, *in vivo* sterol-binding activity of elicitins has not been demonstrated. Elicitin and elicitin-like sequences are also found in downy mildew pathogens, but no functional analysis has been performed on these proteins (Baxter et al., 2010; Cabral et al., 2011; Stassen et al., 2012; Sharma et al., 2015). Interestingly, the oomycete pathogen *A. euteiches* is able to synthesize sterols and seems to lack elicitin genes (Gaulin et al., 2008, 2010).

The putative elicitin receptor was recently cloned from a wild potato (*Solanum microdontum*) that responds to the *P. infestans* elicitin INF1. A *S. microdontum* ecotype showed a clear cell-death response when *inf1* was transiently expressed. Crosses with an unresponsive *S. microdontum* subspecies and further screening and genetic mapping resulted in the identification of the RLP ELR (elicitin response). Stable expression of *ELR* in *S. tuberosum* cv. Désirée conferred the cell death response after expression of *inf1*. Furthermore, ELR mediated a broad-spectrum response to elicitins of oomycetes: most tested elicitins induced a cell-death response in transgenic ELR potato, even though there is often low sequence similarity between elicitins (Du et al., 2015). Recognition might, therefore, be based on structural similarity rather than on a small conserved peptide. ELR was shown to bind to SERK3/BAK1, but binding of the putative receptor to the RLP

adaptor protein SOBIR1 or the elicitin ligand was not tested (Du et al., 2015). Intracellular perception, however, cannot be ruled out as elicitins have, anecdotally, been reported to be detected inside plant cells, e.g., the immunocytochemical localization of the elicitin quercinin in oak (*Quercus robur*) root cells infected with *P. quercina* (Brummer et al., 2002). ELR is thought to mediate extracellular recognition of elicitins, but direct binding to confirm the receptor function of ELR still needs to be demonstrated (Du et al., 2015). Previously, studies in tobacco suggested that INF1 binds to the cytoplasmic domain of a lectin RLK from *N. benthamiana*, NbLRK1 (Kanzaki et al., 2008). Silencing of *NbLRK1* resulted in reduced INF1-responsiveness suggesting the RLK contributes to defense signaling. Although no ELR has been identified in tobacco yet, SERK3/BAK1 and SOBIR1 were found to be required for elicitin-triggered cell death in *N. benthamiana* (Chaparro-Garcia et al., 2011; Peng et al., 2015). It is, therefore, likely that ELR acts similar to RLP23 (Albert et al., 2015) and tomato Cf-4 (Postma et al., 2016), in that it requires both a BAK1-like RLK and SOBIR1-like RLK for pattern-triggered immunity.

Cellulose-Binding Elicitor Lectin

A 34 kDa glycoprotein was isolated from *P. parasitica* var. *nicotianae* mycelium that triggered enhanced lipoxygenase activity as well as accumulation of the defense-related cell wall hydroxyproline-rich glycoproteins in tobacco. This protein was localized to the internal and external layers of the hyphal cell wall (Séjalon-Delmas et al., 1997). The protein sequence revealed two cellulose-binding domains belonging to the carbohydrate binding module 1 (CBM1) family similar to that of fungal glycanases (Mateos et al., 1997; Gaulin et al., 2006). This putative function was corroborated by demonstrating protein binding to fibrous cellulose and plant cell walls. Furthermore, the protein was shown to have lectin-like activities; human red blood cells were readily agglutinated by this protein. Therefore, it was designated cellulose-binding elicitor lectin (CBEL). Moreover, CBEL was able to elicit necrosis, activate defense gene expression, and trigger immunity to *P. parasitica* var. *nicotianae*. No enzymatic activities for CBEL were observed, suggesting it acts as a pattern (Mateos et al., 1997).

Silencing of *CBEL* resulted in a severe reduction of adhesive abilities of *P. parasitica* var. *nicotianae* to cellulosic surfaces, but did not affect pathogenicity. Interestingly, knockdown mutants showed dispersed abnormal cell wall thickenings, indicating that CBEL might be involved in cell wall deposition in the pathogen (Gaulin et al., 2002). CBEL activity as a pattern is not limited to tobacco, as infiltration of CBEL in *Arabidopsis* leaves resulted in defense responses differentially dependent on the phytohormones SA, JA, and ethylene (Khatib et al., 2004). CBEL-induced necrosis was lost in JA-insensitive *coi1* and ethylene-insensitive *ein2* mutant plants, whereas *PR-1* and *WAK1* expression, accumulation of hydroxyproline-rich glycoproteins, and peroxidase activity were greatly reduced or abolished in an *Arabidopsis NahG* mutant that metabolizes SA (Khatib et al.,

2004). Transient expression of *CBEL*, as well as infiltration of recombinant CBEL in tobacco leaves, resulted in rapid development of necrotic lesions. Immunocytochemistry revealed that the delivered CBEL was bound to the plant cell wall. Substitution of aromatic residues in CBEL that are possibly involved in cellulose binding reduced the necrosis-inducing activity. Necrosis-induction in tobacco was lost for three recombinant CBEL proteins (Y52A, Y188A, and Y52A_Y188A), that were also unable to induce defense-related genes at similar concentrations as native CBEL. Recently, it was shown that CBM1-1 is the main determinant in the interaction with cellulose; a mutation in CBM1-2 (Y188A) only showed a slight decrease in cellulose binding compared to wild-type CBEL, whereas a mutation in CBM1-1 (Y52A) strongly decreased the binding capacity of CBEL and the double mutant (Y52A_Y188A) entirely lost the ability to bind cellulose (Martinez et al., 2015). Taken together, these data show amino acids in the two CBM1s, that were predicted to be important for cellulose binding, are important for elicitor activity.

To define the minimum CBEL pattern that triggers immunity, synthetic peptides of CBM1-1 and CBM1-2 were generated. CBM1-1synt and CBM1-2synt were sufficient to activate plant defense in tobacco and *Arabidopsis*, respectively. Intriguingly, recombinant CBEL but not recombinant CBEL_Y52A_Y188A, induced calcium fluxes in tobacco cells but not in protoplasts. This demonstrates that the plant cell wall and unmodified CBM1s are important for CBEL perception (Gaulin et al., 2006).

CBM1s are probably not essential for pathogens with an obligate biotrophic lifestyle; only one was detected in the *Albugo laibachii* genome and no clear CBM1-encoding genes were found in *H. arabidopsidis*, whereas *Pythium ultimum* and *Phytophthora* spp. contain multiple CBM1-encoding genes (Larroque et al., 2012). It has been proposed that adhesion of CBEL or its CBM1s perturb the cellulose status, and the perception of this disturbance leads to defense activation, but this remains to be proven (Dumas et al., 2008). The fact that BAK1 and RESPIRATORY BURST OXIDASE HOMOLOGUE (RBOH) D and F proteins are required for some of the CBEL-induced defense responses suggests that a PRR might be involved (Larroque et al., 2013). The oxidative burst triggered by pattern recognition is mediated by the NADPH oxidases RBOH D and F (Suzuki et al., 2011). Necrosis induction by CBEL in *bak1-4* and the *rbohD/F* double mutant was similar to the Col-0 *Arabidopsis* wild type. However, no ROS production was detected in *bak1-4*and *rbohD/F* and activation of MAP kinases was reduced in *bak1-4* and delayed in *rbohD/F* compared to Col-0. The expression of JA-responsive genes *WRKY11* and *PDF1.2*, but not the expression of the SA-responsive gene *PR-1*, was also reduced in these mutant lines (Larroque et al., 2013). The dependence of some CBEL-induced responses on BAK1 suggests a role for an RLK or RLP in the perception of CBEL. Three *Arabidopsis* accessions were found that are unresponsive to CBEL, and may, therefore, offer a way to decipher CBEL-triggered immunity (Larroque et al., 2013).

OPEL

A secreted apoplastic protein from *P. parasitica* called OPEL was recently discovered to trigger a plant immune response (Chang et al., 2015). OPEL contains a thaumatin-like domain, a glycine-rich domain, and a glycosyl hydrolase (GH) domain that has a putative laminarinase active site. OPEL seems to be oomycete specific; homologues were only found in *Phytophthora* spp. and other oomycetes such as *H. arabidopsidis, Py. ultimum* and *A. laibachii*. OPEL is expressed during early infection stages of *P. parasitica*, rapidly increasing transcript levels within 12 hours after inoculation on *N. benthamiana*. Furthermore, infiltration of *N. tabacum* with recombinant OPEL protein resulted in cell death, increased callose deposition, ROS accumulation, induction of defense-related genes and systemic acquired resistance against several pathogens. Moreover, transient expression of *OPEL* in *N. benthamiana* enhanced resistance to *P. parasitica*. It was shown that the GH domain was essential for the increased callose deposition and increased accumulation of ROS in *N. tabacum*. Although the OPEL GH domain contains a laminarinase signature active site motif, no laminarin or β-1,3-glucan enzymatic activity was detected in OPEL recombinant protein. Mutation of the putative laminarinase active site motif in the predicted GH domain abolished elicitor activity of OPEL, which suggests that the enzymatic activity of OPEL is required to trigger the defense response (Chang et al., 2015). The OPEL substrate has not been identified but is likely a polysaccharide in the plant cell wall. OPEL-released degradation products might, therefore, be perceived by plants as DAMPs.

Endogenous Patterns

Next to exogenous patterns, host-derived molecules that are released upon pathogen infection can serve as danger signals (Table 2). Several endogenous patterns, also known as DAMPs, have been described that are plant cell wall derived or that are released from the host cytosol (Boller and Felix, 2009; Yamaguchi and Huffaker, 2011). The release of these patterns is promoted by a plethora of hydrolytic enzymes that are produced by pathogens (Baxter et al., 2010; Blackman et al., 2015). Interestingly, the downy mildew *H. arabidopsidis* has fewer hydrolases than the hemibiotrophic *Phytophthora* spp., probably as an adaptation to its obligate biotrophic lifestyle (Baxter et al., 2010).

Oligogalacturonides (OGs) are released from the plant cell wall after mechanical damage or by pathogen-secreted hydrolytic enzymes through degradation of homogalacturonan (Ferrari et al., 2013). OGs bind to several members of the cell wall-associated kinase (WAK) family, which consequently leads to the activation of immunity (Brutus et al., 2010; Ferrari et al., 2013). Also, cutin, the main constituent of the plant cuticle (Heredia, 2003), can be degraded to cutin monomers by pathogen released cutinases. Cutin monomers are potent elicitors of defense in several plant species (Schweizer et al., 1996; Fauth et al., 1998). However, it remains unknown how cutin monomers are recognized by plants.

Damage patterns could also be released from the plant cytosol during oomycete infection. These include members of the plant elicitor peptide (Pep) family. The cytosolic precursors of Peps, PROPEPS are released and cleaved when the plant cell is damaged, resulting in the production of endogenous patterns. The receptors for Peps have been identified, the RLKs PEP1 RECEPTOR 1 (PEPR1) and PEP1 RECEPTOR 2 (PEPR2) recognize Peps and contribute to immune responses against several pathogens (Yamaguchi et al., 2006, 2010; Krol et al., 2010; Yamaguchi and Huffaker, 2011; Albert, 2013; Bartels et al., 2013; Bartels and Boller, 2015).

Furthermore, extracellular adenosine triphosphate (eATP) could be perceived as a damage pattern. Treatment of *Arabidopsis* with ATP induced a similar set of genes as wounding did (Choi et al., 2014). In a screen for ATP-insensitivity, a *dorn1* (Does Not Respond to Nucleotides 1) mutant was identified that is defective in the lectin receptor kinase LecRK-I.9. LecRK-I.9 binds to ATP with high affinity and is required for the activation of several ATP-induced responses, demonstrating it is an ATP receptor (Choi et al., 2014). Previously, *lecrk-I.9* mutants were shown to be more susceptible to two *Phytophthora* species than wild-type *Arabidopsis*. Conversely, overexpression of *LecRK-1.9* led to increased resistance to *P. brassicae* (Bouwmeester et al., 2011).

Finally, it has been proposed that recognition of the exogenous pattern β-1,3-glucan could have evolved as an endogenous danger signal; callose could be degraded by host or pathogen-derived β-1,3-glucanases, thereby eliciting a defense response (Klarzynski et al., 2000).

TABLE 2 | Plant-derived patterns that trigger plant immunity.

Elicitor[a]	Type	Receptor[b]	Receptor type[c]	Source	References
Oligogalacturonides	Carbohydrate	WAK1	EGF-like	Cell wall	Ferrari et al., 2013
Cutin monomers	Fatty alcohol	Unknown		Cell wall	Fauth et al., 1998
Peps	Peptide	PEPR1/PEPR2	RLK	Cytosol	Bartels and Boller, 2015
Extracellular ATP	Nucleoside triphosphate	DORN1/LecRK-I.9	LecRK	Cytosol	Choi et al., 2014

[a] ATP = Adenosine triphosphate.
[b] WAK1 = CELL WALL-ASSOCIATED KINASE1; PEPR1/PEPR2 = PEP1 RECEPTOR 1/PEP1 RECEPTOR 2; DORN1 = Does Not Respond to Nucleotides 1; LecRK-I.9 = lectin receptor kinase clade 1.9.
[c] EGF = epidermal growth factor; RLK = receptor-like kinase; LecRK = lectin receptor kinase

Putative Receptor Proteins

Plant genomes encode many RLKs and RLPs. The *Arabidopsis* genome, for example, encodes more than 600 RLKs and 57 RLPs (Shiu et al., 2004; Wang et al., 2008). For most of these proteins, the function is unknown. We expect that several of these receptor proteins have a role in the perception of oomycete pathogens. Recently, it was shown that many *RLP* genes are upregulated after treatment with *P. infestans* and the *P. infestans* NLP NPP1, suggesting a role for these RLPs during oomycete infection (Wu et al., 2016). Several RLKs are also reported to affect the interaction with oomycete pathogens. For example, other LecRKs, next to the aforementioned LecRK-I.9 and NbLRK1, influence the defense response against *Phytophthora* in *Arabidopsis*, tomato and *N. benthamiana* (Wang et al., 2014, 2015a, 2015b). Silencing of several LecRKs in tomato and *N. benthamiana* led to increased susceptibility to *P. capsici* and *P. infestans*, respectively (Wang et al., 2015b). Two *Arabidopsis* LecRKs from the same clade (IX) were shown to affect *Phytophthora* resistance in a similar way (Wang et al., 2015a). Finally, the *Arabidopsis* LecRK-VI.2A positively regulates the pattern-triggered immunity response (Singh et al., 2012). Although some RLKs and RLPs partly regulate the defense response against oomycetes, the patterns or molecules that are recognized by these proteins are still largely unknown.

Conclusions and Perspectives

Recent discoveries in extracellular recognition of oomycete patterns have provided new insight in how plants detect early infection of these (hemi-)biotrophic pathogens. Novel PRRs for elicitins and NLPs have been identified and mechanisms of how these exogenous patterns are perceived by plants have been elucidated. The scientific progress described in this review provides interesting leads for resistance breeding of crops. For example, transgenic expression of the PRRs ELR and RLP23 in cultivated potato resulted in increased resistance to the late blight pathogen *P. infestans* that is known to produce elicitins and NLPs (Haas et al., 2009; Albert et al., 2015; Du et al., 2015). Classical resistance breeding has mainly focused on the introgression of resistance genes encoding cytoplasmic NB-LRR receptors, which are rapidly broken by new emerging strains of the pathogen. The use of PRRs, many of which recognize conserved microbial patterns, for breeding a new generation of disease resistant crops could offer a more durable solution, especially if PRRs and resistance genes are stacked (Dangl et al., 2013; Schwessinger et al., 2015). A great example is the expression of the *Arabidopsis* PRR EFR in tomato that resulted in broad spectrum resistance to different bacterial pathogens that all produce the EF-Tu pattern that is recognized by EFR (Lacombe et al., 2010). As many of the described oomycete patterns are broadly distributed, expression of the cognate PRRs in crops could reduce plant disease and aid in securing our future food.

Thesis Outline

In this thesis, I describe my research on the recognition of extracellular NLPs by the plant immune system. I aimed to elucidate how plant immunity is activated by microbial NLP patterns. My research was supported by a Less is More grant of the Netherlands Organization for Scientific Research (NWO) with the title "Dissecting oomycete-induced immunity in *Arabidopsis* deploying a non-toxic NEP1-like protein as PAMP".

In this introductory chapter, I gave an overview of oomycete-related patterns that can trigger immunity. The discovery of a novel microbial pattern, NLPs, which are also found in many oomycete species is described in chapter 2. While trying to decipher the function of non-cytotoxic NLPs of the downy mildew *H. arabidopsidis* (HaNLPs), it was serendipitously found that NLPs act as a pattern. Transgenic *Arabidopsis* lines that express *HaNLP*s were created and, surprisingly, this resulted in severe growth reduction of the transgenic lines that was associated with activation of immunity. A conserved peptide fragment of 24 amino acids, called nlp24, was sufficient to trigger a strong immune response in *Arabidopsis*. Strikingly, nlp24 patterns based on NLPs from fungal and bacterial origins were potent elicitors too. Thus, NLPs are the first microbial patterns identified that occur in three kingdoms of life (Chapter 2).

Next, we characterized the receptor complex that recognizes the nlp24 patterns. By screening for natural variation in nlp-responsiveness in *Arabidopsis,* and by a reverse genetic screening, the receptor-like protein RLP23 was identified as the NLP PRR. Furthermore, the adaptor protein SOBIR1 was shown to be crucial for NLP-triggered immunity (NTI). Interestingly, besides local activation of immunity, nlp24 also triggers systemic immunity, requiring RLP23 and SOBIR1. We further found that the nlp24-induced systemic response is similar to systemic acquired resistance (SAR); previously identified components required for SAR, are needed for systemic NTI as well. The final part of chapter 3 describes the study of a possible role of HaNLPs in virulence. In order to test this, transgenic *HaNLP* overexpression lines were generated in the *Arabidopsis rlp23* mutant background. Strikingly, expression of *HaNLP*s did not contribute to a change in disease susceptibility to the pathogens *H. arabidopsidis* and *B. cinerea*. The function of non-cytotoxic (Ha)NLPs in pathogenicity, therefore, remains elusive (Chapter 3).

Chapter 4 concerns the description of a forward genetic screen for *decreased NTI* (*dni*) mutants, with the goal to identify novel *DNI* genes that encode proteins required for NLP-induced signalling or responses. An estradiol-inducible *NLP Arabidopsis* line was EMS mutagenized and the *M2* generation was screened for loss of NLP-induced growth reduction. This resulted in the discovery of new null alleles of *RLP23* and *SOBIR1* impaired in NTI. Finally, four *dni* mutants with functional *RLP23* and *SOBIR1* were genome sequenced and verification of *DNI* candidate genes is ongoing.

Recognition of NLPs is not a unique trait of *Arabidopsis*, but is also observed in other plant species, e.g. in lettuce. NTI in lettuce is not only triggered by the nlp24 peptide but also by nlp26, derived from a type 2 NLP that does not activate NTI in *Arabidopsis*. Wild lettuce varieties were identified that do not show nlp24-induced ethylene production. Interestingly, most wild accessions were nlp24-unresponsive. To further dissect the genetic basis of nlp24 recognition in cultivated lettuce, a backcross inbred (BIL) population and an F2 population of cultivated and wild lettuce were tested for nlp24-responsiveness. All BILs were sensitive and only 4 nlp24-insensitive F2 were found out of 93 tested plants. Therefore, it is likely that two redundant dominant PRRs in cultivated lettuce can recognize nlp24 (Chapter 5).

In the final chapter, the work in this thesis is summarized and put into a broader perspective. Possible applications of the research herein are discussed as well as important open questions that need to be addressed in future investigatory endeavours.

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Chapter

Nep1-like proteins from three kingdoms of life act as a microbe-associated molecular pattern in *Arabidopsis*

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Abstract

Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) are secreted by a wide range of plant-associated microorganisms. They are best known for their cytotoxicity in dicot plants that leads to the induction of rapid tissue necrosis and plant immune responses. The biotrophic downy mildew pathogen *Hyaloperonospora arabidopsidis* encodes 10 different non-cytotoxic NLPs (HaNLPs) that do not cause necrosis. We discovered that these non-cytotoxic NLPs, however, act as potent activators of the plant immune system in *Arabidopsis thaliana*. Ectopic expression of *HaNLP3* in *Arabidopsis* triggered resistance to *H. arabidopsidis*, activated the expression of a large set of defense-related genes, and caused a reduction of plant growth that is typically associated with strongly enhanced immunity. N- and C-terminal deletions of HaNLP3, as well as amino acid substitutions, pinpointed to a small central region of the protein that is required to trigger immunity, indicating the protein acts as a microbe-associated molecular pattern (MAMP). This was confirmed in experiments with a synthetic peptide of 24 amino acids, derived from the central part of HaNLP3 and corresponding to a conserved region in type 1 NLPs that induces ethylene production, a well-known MAMP response. Strikingly, corresponding 24-amino acid peptides of fungal and bacterial type 1 NLPs were also able to trigger immunity in *Arabidopsis*. The widespread phylogenetic distribution of type 1 NLPs makes this protein family (to our knowledge) the first proteinaceous MAMP identified in three different kingdoms of life.

Significance

Peptide fragments of Nep1-like proteins (NLPs), occurring in diverse microorganisms of three different kingdoms of life, were found to trigger immunity in the model plant *Arabidopsis*, indicating that they act as a microbe-associated molecular pattern (MAMP). A synthetic peptide of 24 amino acids from the central part of the downy mildew HaNLP3 protein was found to activate the plant immune system and trigger resistance to this pathogen. Strikingly, not only peptides of oomycete NLPs, but also those of bacteria and fungi were shown to act as a MAMP. This unprecedented broad taxonomic distribution demonstrates that the occurrence of a MAMP is not necessarily restricted to a class of microorganisms but can occur in a wide range of species from the tree of life.

Introduction

Immune responses in plants generally start by receptor-mediated detection of non-self molecules that are conserved among different classes of microbes, both beneficial and pathogenic (Zamioudis and Pieterse, 2012). These molecules often have essential functions in microbial fitness (Thomma et al., 2011) and are known as microbe-associated molecular patterns (MAMPs). Upon their perception by the plant, MAMPs trigger basal immune responses (Boller and Felix, 2009), e.g., ethylene biosynthesis, production of reactive oxygen species, release of antimicrobial compounds (Tsuda and Katagiri, 2010), and in certain cases programmed cell death (Thomma et al., 2011). Collectively, these responses contribute to resistance against non-adapted pathogens (MAMP-triggered immunity [MTI]).

MAMPs of plant-infecting microbes have been described for bacteria, fungi, and oomycetes. Three characterized bacterial MAMPs are flagellin (Felix et al., 1999), EF-Tu (Kunze et al., 2004), and peptidoglycan (Gust et al., 2007). Flagellin is the main protein of the bacterial flagellum, which is used by eubacteria for movement. A highly conserved fragment of 22 amino acids, named flg22 (Felix et al., 1999), is sufficient to activate MTI in *Arabidopsis* and other plant species. Elongation factor thermo unstable (EF-Tu) is an abundant and conserved bacterial protein that plays a central role in the elongation phase of protein synthesis. An 18-amino acid domain of EF-Tu, named elf18, is recognized as a MAMP in *Brassicaceae* species, but not in other tested plant families (Kunze et al., 2004). Peptidoglycan (PGN), the third characterized bacterial MAMP, is a major structural component of most bacterial cell walls. PGN, consisting of strands of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues, triggers immunity in *Arabidopsis* (Gust et al., 2007). An important fungal MAMP is chitin, a structural component of all fungal cell walls. Plants are able to recognize chitin, and fragments of 4–10 *N*-acetylglucosamine residues are the most potent inducers of defense (Felix et al., 1993). Recently, a second fungal MAMP was identified, a secreted polygalacturonase of *Botrytis cinerea* that triggers immunity in *Arabidopsis* (Zhang et al., 2014).

Four oomycete-derived MAMPs have been identified to date (Hein et al., 2009): (*i*) heptaglucoside fragments, originating from branched β-glucans that are major cell wall polysaccharides, and that trigger defense responses in many Fabaceous plants (Fliegmann et al., 2004); (*ii*) glycoprotein 42, a calcium-dependent transglutaminase that functions in irreversible protein cross-linking and is abundant in *Phytophthora* cell walls, and a 13-amino acid peptide fragment (Pep-13) thereof that elicit MTI responses in parsley (Nürnberger et al., 1994) and potato (Brunner et al., 2002); (*iii*) elicitins, secreted proteins with sterol-binding activity (Kamoun, 2006), which provoke necrosis in *Nicotiana* plants through induction of cell death (Sasabe et al., 2000); and (*iv*) the *Phytophthora* cellulose-binding elicitor lectin, which is thought to cause perturbation of the cell wall cellulose status, thereby triggering necrosis and MTI in tobacco and *Arabidopsis* (Séjalon-Delmas et

al., 1997; Gaulin et al., 2006). Other groups of cell death-inducing proteins may also qualify as MAMPs based on their widespread occurrence among different pathogens (Thomma et al., 2011), e.g., the Crinklers and the cytotoxic necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs; Hein et al., 2009).

Two major NLP types are found in bacteria, fungi, and oomycetes (Gijzen and Nürnberger, 2006; Oome and Van den Ackerveken, 2014) and are known to cause rapid necrosis and ethylene production in many dicot, but not in monocot plant species (Bailey, 1995; Gijzen and Nürnberger, 2006). Type 2 NLPs differ from type 1 by an additional conserved second cysteine bridge and putative calcium-binding domain (Oome and Van den Ackerveken, 2014). In *Arabidopsis*, cytotoxic NLPs were found to activate immunity-related gene expression, which strongly overlapped with that induced by flg22 (Bae et al., 2006; Qutob et al., 2006). However, it was suggested that immune responses resulted from cytotoxicity. Moreover, necrosis was only induced upon treatment with the complete NLP protein (Fellbrich et al., 2002). In vitro, cytotoxic NLPs cause rapid leakage of dicot membrane-derived vesicles, suggesting a direct cytolytic activity (Ottmann et al., 2009). The immunogenic effect of NLPs was therefore suggested to result from direct cellular damage (Ottmann et al., 2009), or release of damage-associated molecular patterns (Boller and Felix, 2009).

Several plant-infecting oomycetes have large expansions of *NLP*s in their genomes (Mattinen et al., 2004; Seidl et al., 2011; Cabral et al., 2012), suggesting that these proteins play an important role in the pathogen's lifestyle. A clear virulence function was observed for NLP_{Pcc} of the rot bacterium *Pectobacterium carotovorum* (Mattinen et al., 2004). Also, individual deletion of two *NLP* genes in the fungus *Verticillium dahliae* resulted in reduced virulence on different host plants (Santhanam et al., 2013). Five other *NLP* genes in this fungus encode non-cytotoxic proteins (Zhou et al., 2012), a phenomenon that is also observed in oomycetes. When tested by transient expression in tobacco, necrosis was only induced by one out of 3 tested NLPs of *Phytophthora infestans* (Kanneganti et al., 2006), eight out of 33 NLPs of *Phytophthora sojae* (Dong et al., 2012), whereas not a single one of 10 NLPs of *Hyaloperonospora arabidopsidis* tested caused necrosis (Cabral et al., 2012). In contrast to cytotoxic *NLPs* that are mainly expressed during necrotrophic stages of infection, non-cytotoxic *NLPs* appear to be expressed early during infection (Kanneganti et al., 2006; Cabral et al., 2012), suggesting they serve an, as-yet-unknown, function during penetration or initial colonization of the host.

In our search for the biological function of non-cytotoxic NLPs of *H. arabidopsidis*, transgenic *HaNLP*-expressing *Arabidopsis* plants were generated that were severely stunted. In this paper, we show that *Arabidopsis* responds to non-cytotoxic HaNLPs and small peptide fragments thereof that are highly conserved in type 1 NLPs. The peptides activate ethylene production and other typical MAMP-triggered defense responses, but

not tissue necrosis, indicating they act as a MAMP. NLPs are not restricted to a single class of microbes but present in a broad range of mostly plant-associated microbes (bacteria, fungi, and oomycetes) belonging to three kingdoms of life, making this a MAMP with an unprecedented broad taxonomic occurrence.

Results

***HaNLP* Expression in *Arabidopsis* Leads to Severe Growth Reduction and Resistance to Downy Mildew**

H. arabidopsidis, the downy mildew pathogen of *Arabidopsis*, has an expanded family of 10 different *NLP* genes that encode non-cytotoxic secreted proteins (Cabral et al., 2012). To determine whether the HaNLPs would facilitate the infection process and enhance disease susceptibility of *Arabidopsis*, transgenic *HaNLP* overexpression lines were created. Surprisingly, overexpression of 7 of the 10 *NLP* genes (*HaNLP2*, *3*, *4*, *5*, *6*, *9*, and *10*) resulted in transgenic plants showing severely reduced growth, compared with control plants transformed with *Yellow Fluorescent Protein* (*YFP*; Figure 1*A*). Plants expressing *HaNLP1*, *7*, and *8* showed no, or limited growth reduction, which was not significantly different from the *YFP*-expressing control. All other transgenic lines, except for *HaNLP5*-expressing plants, produced seeds and were tested in the next generation (T3) by weighing the aerial parts of 10 seedlings per *NLP*-expressing line. NLP-induced weight reduction confirmed the growth effects observed on individual T3 plants (Figure 1*B*).

HaNLP-expressing plants showed strongly reduced susceptibility to the downy mildew *H. arabidopsidis* (Figure 1*C*), and strikingly, these same lines also showed severe growth reduction. There was a strong correlation (R^2= 0.89) between the level of susceptibility and the fresh weight of the transgenic lines expressing different *HaNLP* genes. In literature, there is a multitude of examples of plant growth inhibition as a result of activation of plant immunity (Bolton, 2009). The fact that the level of immunity of the *HaNLP*-expressing plants is well correlated to their growth inhibition, therefore, suggests that activation of plant immunity causes the observed growth reduction.

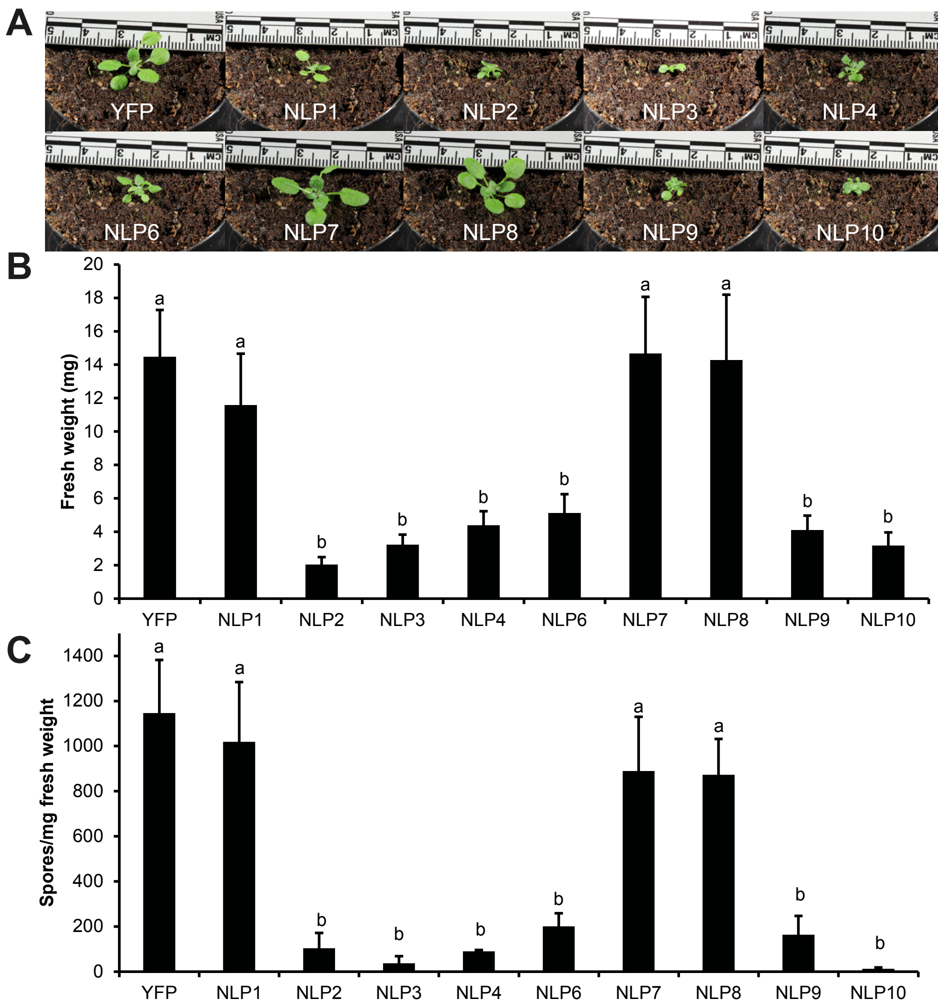

FIGURE 1 | *HaNLP* expression in *Arabidopsis* leads to growth reduction and enhanced resistance to downy mildew. (***A***) The result of reduced growth is visible as the smaller sizes of representative T3 transgenic *Arabidopsis* lines (21 days old) expressing *HaNLP2*, *3*, *4*, *6*, *9*, and *10*, but not of those expressing *HaNLP1*, *7*, *8*, and the *YFP* control. (***B***) The reduction in growth was quantified as fresh weight of the aerial parts of T3 seedlings [*n* = 10, with standard deviation (SD)]. *Arabidopsis* plants overexpressing *HaNLP5* died before day 21. (***C***) Transgenic T3 lines that showed growth reduction also showed enhanced resistance to the downy mildew *H. arabidopsidis* isolate Waco9, as measured by counting the number of spores per milligram of fresh-weight above-ground tissue (with SD). Plants were inoculated at 14 days after germination, and spores counted 6 days postinoculation (*n* = 10; the experiment was repeated three times with similar results). Significance of differences in the level of sporulation was assessed with the Tukey's honestly significant difference (HSD) test and indicated with "a" and "b" (α = 0.05).

HaNLP Is a Potent Activator of Plant Immunity

As the observed activation of plant immunity in 35S:*HaNLP*-expressing plants could be the result of continuous overexpression, we created an estradiol-inducible line (containing an XVE:*HaNLP3* construct). *HaNLP3* was chosen for this as we studied this protein in more detail previously (Cabral et al., 2012). A transgenic line was selected that showed no detectable *HaNLP3* expression in untreated plants and a strong induction upon treatment with estradiol. When sprayed with estradiol every 2 days for a period of 2 weeks, these plants showed strongly reduced growth, similar to that of the 35S:*HaNLP3* lines, whereas non–estradiol-treated plants developed normally (Figure S1). A control estradiol-inducible *YFP* line (XVE:*YFP*) did not show any growth reduction upon estradiol treatment. These data clearly indicate that growth reduction indeed results from exposure of plants to HaNLP3. The same lines were next used to investigate the effect of *HaNLP3* expression on *H. arabidopsidis* infection. For this, the inducible XVE:*HaNLP3* and XVE:*YFP* lines, which were phenotypically identical, were sprayed with water or estradiol 24 hours before inoculation. A very strong reduction in susceptibility was observed in the estradiol-induced *HaNLP3* line, but not in the *YFP* control line or water-treated *HaNLP3* line (Figure 2*A*). These data strongly support the idea that HaNLP3 triggers the plant immune system, resulting in resistance to *H. arabidopsidis*.

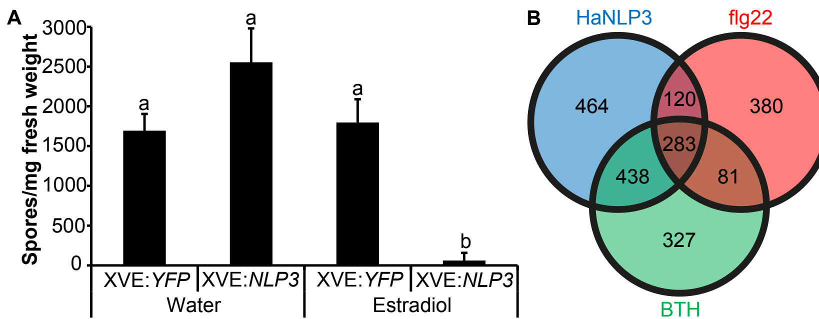

FIGURE 2 | Estradiol-induced expression of *HaNLP3* in *Arabidopsis* results in the activation of immunity to downy mildew and defense-associated gene expression. (***A***) Susceptibility of estradiol-inducible *HaNLP3* and *YFP* lines of *Arabidopsis* to *H. arabidopsidis* as measured by counting the number of spores per milligram of fresh-weight above-ground tissue (with SD). *Arabidopsis* seedlings were sprayed with either water or 100 µM estradiol 24 hours before inoculation with *H. arabidopsidis* Waco9. Spore counts were performed 6 days after inoculation. Significance of differences in the level of sporulation was assessed with a Tukey's HSD test ($n = 4$; the experiment was repeated three times with similar results). Significant differences between the lines is indicated with "a" and "b" ($\alpha = 0.05$) (***B***) Venn diagram showing the overlap in *Arabidopsis* genes that are activated in response to different inducers of plant defense responses with the 1,305 genes that are activated by HaNLP3 (blue). flg22-induced genes (864; red) are up-regulated 1 hour and/or 4 hours after treatment with flg22 peptide (Qutob et al., 2006). BTH-induced genes (1,129; green) are activated at 24 hours after treatment with BTH (Wang et al., 2006).

The question why transient expression of *HaNLP3* leads to immunity to *H. arabidopsidis* was addressed by analyzing gene expression changes at 24 hours after induction of the *HaNLP3* transgene with estradiol. The expression of *HaNLP3* resulted in a strong transcriptional response (Dataset S1); 2,586 genes were significantly (*q* value, <0.05) differentially expressed (at least fourfold) between estradiol- and water-treated seedlings of XVE:*HaNLP3*, of which 1,305 genes showed enhanced expression (more than fourfold up) and 1,281 genes were down-regulated (more than fourfold down). Comparing the 1,305 HaNLP3-induced gene set to other publicly available data showed that there was a strong overlap with genes up-regulated in response to the flagellin-derived MAMP flg22 (Qutob et al., 2006), and to BTH (Wang et al., 2006), a salicylic acid analog that activates plant immune responses (Figure 2*B*). The fact that HaNLP3 activates immunity-related gene expression, as well as resistance to downy mildew, strongly suggests that the protein acts as a MAMP.

Defense Induction in *Arabidopsis* by Recombinant NLPs

The observed induction of defense could be caused by artificial *in planta* production of high levels of the secreted HaNLP3 protein. We therefore tested whether recombinant HaNLP3 protein, delivered extracellularly in the leaf intercellular space, would activate plant immune responses, e.g., the defense gene *PR-1*. For this, HaNLP3 was produced in *Pichia pastoris* and the purified protein infiltrated in leaves of an *Arabidopsis* promoter *PR-1*:*GUS* reporter line. Leaves infiltrated with HaNLP3 showed a high β-glucuronidase (GUS) activity, indicating that the *PR-1* promoter is strongly activated, similar to leaves infiltrated with the flg22 peptide that is a potent MAMP in *Arabidopsis* (Figure 3). In contrast, the control sample, PIC3 (*P. pastoris* empty vector control, purified in the same way as HaNLP3), as well as the buffer control, showed very little GUS activity. This experiment clearly shows that extracellular exposure of plant cells to HaNLP3 activates plant immune responses.

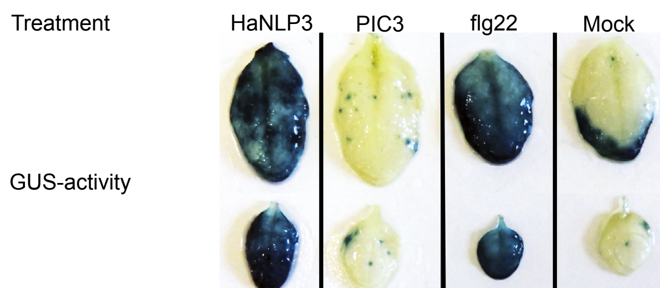

FIGURE 3 | Recombinant HaNLP3 protein activates *PR-1* expression. (***A***) Induction of defense in *Arabidopsis* leaves was measured by staining for GUS expression in leaves of *pPR-1:GUS Arabidopsis* plants infiltrated with recombinant HaNLP3 protein (0.5 µM), a control sample (PIC3), flg22 peptide (0.5 µM), and water (Mock). GUS staining was performed at 24 h after infiltration.

A Fragment of HaNLP3 Is Sufficient to Induce Plant Growth Reduction

Proteinaceous MAMPs, e.g., flagellin and EF-Tu, are often recognized through smaller protein epitopes. To test whether smaller NLP fragments can still act as MAMPs, we made N- and C-terminal deletions and substitutions in HaNLP3 and expressed them in transgenic *Arabidopsis* lines, measuring plant growth reduction as a proxy for activation of immune responses (Figure 4*A*). Disruption of the disulfide bridge, which is essential for toxicity of cytolytic NLPs (Fellbrich et al., 2002), by substitution of the first cysteine residue by serine (C79S), did not reduce the growth-inhibiting effect of HaNLP3. Deletion of a 26-amino acid region between the two conserved cysteine residues also did not affect HaNLP3-induced growth reduction. Transgenic expression of successive C-terminal deletions of HaNLP3 resulted in a reduced growth phenotype for fragments 1–4, whereas further C-terminal deletions did no longer have a negative effect on plant growth (fragments 5–8). This suggested that sequences N-terminal of the heptapeptide motif are important for HaNLP3-induced growth reduction. Fragment 4, which ends with the heptapeptide motif, was further reduced in size by successive N-terminal deletions while leaving the signal peptide intact. Expression of fragments 9–12 in *Arabidopsis* showed that fragments 9 and 10, but not 11 and 12, reduced growth when expressed in transgenic plants. A 28-amino acid fragment of HaNLP3 (fragment 10) is thus sufficient to cause the growth effect. This fragment contains two regions that are highly conserved in type 1 NLPs (Figure 4*B*): conserved region I starting with the AIMY amino acid sequence, and conserved region II matching the heptapeptide motif. The corresponding conserved region I in the structure of NLP_{Pya} is fully located inside of the protein, whereas the heptapeptide motif (conserved region II) is partly surface exposed (Figure S2). Nevertheless, native recombinant HaNLP3 protein induces ethylene production in *Arabidopsis*, a well-known MAMP response (Figure S3). Interestingly, heat-denatured HaNLP3 (boiled for 1 hour) was an approximately three times more potent inducer of ethylene production (EC_{50} = 0.2 µM) than native recombinant protein (EC_{50} = 0.5 µM), suggesting the immunogenic epitope is not fully exposed in the native protein.

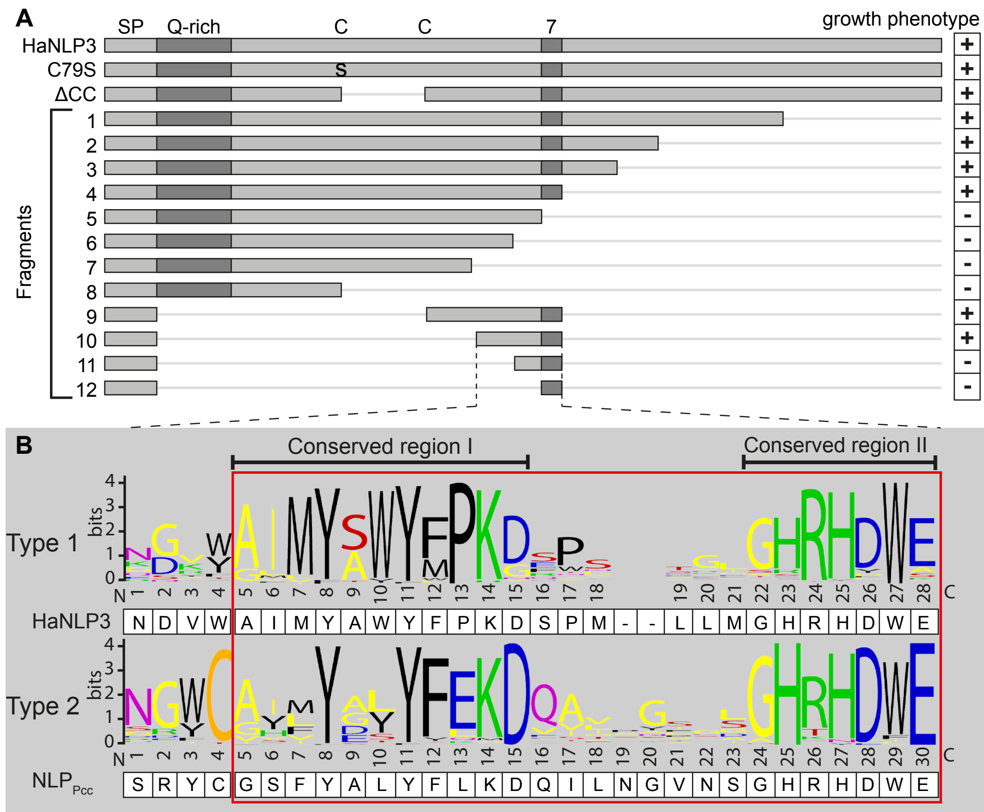

FIGURE 4 | A conserved region from the central part of HaNLP3 is sufficient for MAMP-associated growth reduction. (***A***) Schematic representation of substituted and deleted versions of the HaNLP3 protein (showing the signal peptide, "SP"; glutamine-rich region, "Q-rich"; cysteine residues, "C"; and heptapeptide motif, "7") and their effect on growth when overexpressed in transgenic *Arabidopsis* seedlings. Multiple T1 lines per construct were scored for growth reduction (with "+" indicating strong growth reduction and "–" indicating no growth reduction) following transformation of the different 35S:*HaNLP3* variants. Fragment 10 contains the minimal region of 28 amino acids that is still able to induce MAMP-associated growth reduction. (***B***) A 24-amino acid peptide (red-lined box) is conserved in type 1 NLPs from oomycetes, fungi, and bacteria. The 11-residue conserved region I is less conserved in type 2 NLPs. The second conserved region in the 28-amino acid fragment is the GHRHDWE heptapeptide that is characteristic for the NLP family, and that is conserved in both type 1 and type 2 NLPs. The WebLogo is based on 378 type 1 NLP sequences and 122 type 2 NLP sequences (Oome and Van den Ackerveken, 2014).

Synthetic NLP Peptides Trigger Immunity

A synthetic peptide of 24 residues (nlp24) was made that contains both conserved region I and II, but lacks the first 4 amino acids of the 28-amino acid peptide that are not conserved in type 1 NLPs (Figure 4*B*). nlp24 appeared to be a strong inducer of ethylene production in *Arabidopsis* (Figure 5*A*), confirming that this HaNLP3 peptide is sufficient to trigger an immune response. To investigate whether peptide fragments of other microbial NLPs

also act as MAMPs in *Arabidopsis*, the corresponding nlp24 peptides of BcNEP2 (of the fungus *B. cinerea*) and BsNPP1 (of the bacterium *Bacillus subtilis*) were tested and found to induce ethylene production in *Arabidopsis* (Figure 5*A*). In contrast, the corresponding 26-amino acid peptide of the type 2 NLP of *P. carotovorum* (NLP_{Pcc}) did not induce ethylene production. Similarly, the nlp24 peptide of HaNLP3, but not the nlp26 peptide of NLP_{Pcc}, was a strong inducer of GUS expression in the *Arabidopsis* promoter *PR-1*:*GUS* reporter line (Figure S4). It is striking to see that nlp24 is the most conserved part (containing both conserved region I and II) in type 1 NLPs of bacteria, fungi, and oomycetes, as illustrated by alignment of HaNLP3, BcNEP2, and BsNPP1 (Figure S5). In contrast, conserved region I of type 2 NLPs differs at several amino acid positions from that of type 1 NLPs, whereas the heptapeptide motif is highly conserved in the two NLP types (Figure 4*B*).

To study whether nlp24 peptides also trigger immunity in *Arabidopsis*, we pretreated leaves with 100 nM peptide 1 day before inoculation with the downy mildew *H. arabidopsidis* Noco2. The nlp24 peptides corresponding to HaNLP3, BcNEP2, and BsNPP1 induced a strong immune response, resulting in resistance to Noco2. In contrast, treatment with the peptide of NLP_{Pcc} did not reduce susceptibility to downy mildew, but resulted in sporulation levels similar to that of mock-treated leaves (Figure 5*B*). Our data show that peptides derived from type 1 NLPs of microbes occurring in three kingdoms of life are recognized as MAMPs by *Arabidopsis* and trigger effective immune responses.

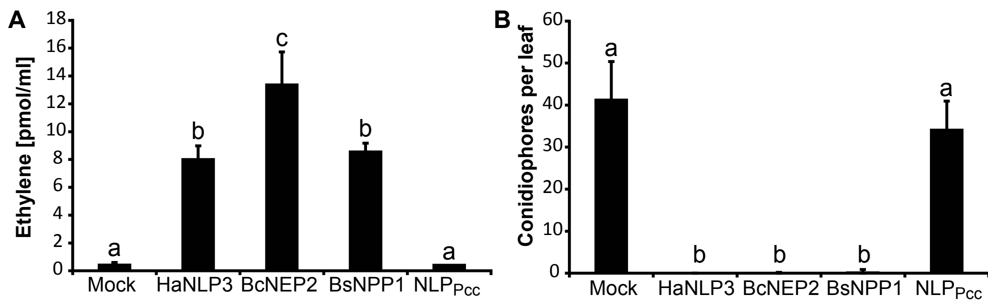

FIGURE 5 | Synthetic 24-amino acid NLP peptides (nlp24) induce MAMP responses and trigger immunity to downy mildew. (***A***) Ethylene production in *Arabidopsis* is induced in response to nlp24 peptides (1 μM) of HaNLP3, BcNEP2, and BsNPP1, but not to the nlp26 fragment of the type 2 NLP_{Pcc}. Leaf pieces were incubated for 4 hours in buffered peptide solution before ethylene concentrations were determined ($n = 3$; SD is indicated and the experiment was performed three times with similar results). (***B***) Resistance to *H. arabidopsidis* in *Arabidopsis* is induced by nlp24 (100 nM) of HaNLP3, BcNEP2, and BsNPP1, but not of the type 2 NLP_{Pcc}. The numbers of conidiophores per leaf is a measure of susceptibility. Leaves of 4.5-week–old *Arabidopsis* plant were infiltrated with nlp24 peptides 1 day before inoculation with downy mildew isolate Noco2. Conidiophore counts were performed 10 days after inoculation. Significance of differences in the level of sporulation (with SE) was assessed with a Tukey's HSD test ($n = 44$) and significant differences between lines are indicated with "a" and "b" ($\alpha = 0.01$).

nlp24 Peptides Are Diverse and Tolerant to Substitutions

The *HaNLPs*, when transgenically expressed in *Arabidopsis*, trigger different levels of immunity. To test whether this is caused by differences in affinity, the EC_{50} values for ethylene production were determined (Table 1). For NLP2, 4, 5, 6, and 10, the EC_{50} values were in the range of 0.1–0.2 μM, similar to that obtained for nlp24 of HaNLP3, and of the heat-denatured HaNLP3 protein (0.2 μM). However, the EC_{50} values were higher for HaNLP1, 7, 8, and 9 (range of 0.4–0.6 μM). Their reduced activity could explain the lower effect on growth in transgenic plants transformed with HaNLP1, 7, and 8, but not for HaNLP9 (Figure 1).

We next tested the minimal peptide length and composition by measuring ethylene production in response to truncated peptides and alanine-substituted versions of nlp24 (HaNLP3; Table 1). Activity was not affected when the first 2 amino acids (AI) were not included (nlp22), but was strongly reduced when the first 4 amino acids (AIMY) were absent (nlp20). Deletion of the C-terminal heptapeptide motif from nlp24 (in nlp17) did not increase the EC_{50} value. Further C-terminal truncated forms were still active, including an 11-amino acid peptide with the sequence AIMYAWYFPKD (nlp11) that corresponds to conserved region I (Figure 5*B*) and even had a slightly lower EC_{50} of 0.1 μM. Removal of the first 2 amino acids of nlp11 resulted in a peptide (nlp9) that was 40 times less active. Ethylene-inducing activity of alanine substitutions in the conserved region I of nlp24 showed that methionine at position 3 of the peptide is required for full activity. Two other substitutions, of tyrosine 7 and aspartic acid 11, resulted in peptides with slightly reduced activity. Substitution of histidine 19, which is highly conserved in NLPs and is required for necrosis induction by cytolytic NLPs (Ottmann et al., 2009), did not result in a decreased EC_{50} value, confirming that conserved region I, but not II, is required for MAMP activity of NLPs.

The bacterial BsNPP1 and fungal BcNEP2 peptides are 5–10 times more potent triggers of ethylene production than nlp24 of HaNLP3. In contrast, the 26-amino acid peptide of the type 2 NLP_{Pcc} had a very high EC_{50}value (>10 μM) and is clearly not acting as a MAMP in *Arabidopsis*. This was confirmed with nlp11 peptides that had slightly lower EC_{50} values than HaNLP3 for BcNEP2, BsNPP1, and NLP_{Pya}, but again a very high EC_{50} for NLP_{Pcc} (Table 1). The data presented demonstrate that microbial NLPs, occurring in three kingdoms of life, act as MAMPs, making this an immunity-triggering protein family of unprecedented broad taxonomic distribution.

TABLE 1 | Synthetic Half-maximum effective concentration (EC_{50}) of different nlp24-based peptides for the induction of ethylene biosynthesis in *Arabidopsis*. Values were determined for nlp24 peptides of 10 different HaNLPs, for truncated versions and alanine substitutions of HaNLP3, as well as for nlp24- and nlp11-based peptides for BcNEP2, BsNPP1, NLP_{Pcc}, and NLP_{Pya}. EC_{50} data were based on three measurements for each of six peptide concentrations tested, repeated three times with similar results. aa = amino acids.

Name	Size, aa	Amino acid sequence	EC_{50}, µM
nlp24 (HaNLP1)	24	AIMFAYYFPKSQPRRSVSVRHSWE	0.3
nlp24 (HaNLP2)	24	GIVYAWFFPKDSVRHGIGHRYDWE	0.2
nlp24 (HaNLP4)	24	GIIFAWYFPKDSVRDGVGHRHDWE	0.1
nlp24 (HaNLP5)	24	AIMFSWYFPKGFHDRKASRRHDWA	0.2
nlp24 (HaNLP6)	24	GIVYAWYFPKDSVRDGIGHRYDWE	0.1
nlp24 (HaNLP7)	24	AIAYAYYSPKAHPHQRVWIRHVWN	0.5
nlp24 (HaNLP8)	24	AIMYALYFPKDMKVLNRGYRHAFE	0.5
nlp21 (HaNLP9)	21	AIMYVWYFPKD---NRDDDRHDWE	0.3
nlp24 (HaNLP10)	24	AIMYAWYFPKDAPDEESGQRHDWE	0.1
nlp24 (HaNLP3)	24	AIMYAWYFPKDSPMLLMGHRHDWE	0.2
nlp22	22	--MYAWYFPKDSPMLLMGHRHDWE	0.1
nlp20	20	--MYAWYFPKDSPMLLMGHRHDWE	2.0
nlp17	17	AIMYAWYFPKDSPMLLM-------	0.1
nlp13	13	AIMYAWYFPKDSP-----------	0.1
nlp11	11	AIMYAWYFPKD-------------	0.2
nlp9	9	--MYAWYFPKD-------------	1.0
nlp24_M3A	24	AIAYAWYFPKDSPMLLMGHRHDWE	1.5
nlp24_Y4A	24	AIMAAWYFPKDSPMLLMGHRHDWE	0.2
nlp24_W6A	24	AIMYAAYFPKDSPMLLMGHRHDWE	0.2
nlp24_Y7A	24	AIMYAWAFPKDSPMLLMGHRHDWE	0.3
nlp24_F8A	24	AIMYAWYAPKDSPMLLMGHRHDWE	0.2
nlp24_P9A	24	AIMYAWYFAKDSPMLLMGHRHDWE	0.2
nlp24_K10A	24	AIMYAWYFPADSPMLLMGHRHDWE	0.1
nlp24_D11A	24	AIMYAWYFPKASPMLLMGHRHDWE	0.3
nlp24_H19A	24	AIMYAWYFPKDSPMLLMGARHDWE	0.2
nlp24 (BcNEP2)	24	AIMYSWYMPKDEPSTGIGHRHDWE	0.03
nlp24 (BsNPP1)	24	AIMYSWYFPKDEPSPGLGHRHDWE	0.02
nlp26 (NLP_{Pcc})	26	GSFYALYFLKDQILNGVNSGHRHDWE	>10
nlp11 (BcNEP2)	11	AIMYSWYMPKD	0.07
nlp11 (BsNPP1)	11	AIMYSWYFPKD	0.09
nlp11 (NLP_{Pcc})	11	GSFYALYFLKD	>10
nlp11 (NLP_{Pya})	11	AIMYSWYMPKD	0.07

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Discussion

NLPs Act as MAMPs

The discovery that non-cytotoxic NLPs activate immunity in *Arabidopsis* was made while searching for a virulence function of these proteins in the downy mildew *H. arabidopsidis* (Cabral et al., 2012). When transgenically expressed in *Arabidopsis*, 7 of the 10 *HaNLPs* induced severe growth reduction that resembled that of documented *Arabidopsis* autoimmune mutants, e.g., *cpr1* and *cpr5* (Bowling et al., 1994, 1997), suggesting that the secreted proteins activate plant immunity. Inducible expression of *HaNLP3* resulted in the activation of many well-known immunity-related genes, which are also activated by the flg22 MAMP and the defense hormone salicylic acid (or its analog BTH). By creating C- and N-terminal truncations of HaNLP3, a 28-amino acid fragment was pinpointed as sufficient for MAMP-associated growth reduction. This fragment could be further reduced to a synthetic peptide of 24 amino acids that was sufficient to induce MAMP responses, e.g., ethylene production, and immunity to *H. arabidopsidis*.

The nlp24 peptide is strongly conserved in both cytotoxic and non-cytotoxic type 1 NLPs. Conserved region I (of 11 amino acids) contains the immunogenic part of nlp24. In the fungal VdNLP2 protein, this region was analyzed in more detail by Zhou et al. (Zhou et al., 2012), who observed that alanine substitution of six different amino acid residues resulted in loss or reduction of necrosis induction by this cytotoxic type 1 NLP. The fact that region I is also strongly conserved in non-cytolytic NLPs, in particular those of the *Arabidopsis* pathogen *H. arabidopsidis*, suggest that this region is also important for a, so-far-unknown, non-cytotoxic function related to virulence. A synthetic peptide (nlp26) of the type 2 NLP_{Pcc} does not induce ethylene production in *Arabidopsis*, nor does it trigger immunity to downy mildew. This suggests that perception of NLPs is specific for type 1 NLPs (although we do not rule out that other type 2 NLPs can trigger immunity in *Arabidopsis*). The cause for this may be that conserved region I of type 2 NLPs differs from that of type 1 NLPs (Figure 4*B*).

Activation of immune responses by cytolytic NLPs has always been causally linked to their toxic activity (Fellbrich et al., 2002; Qutob et al., 2006). Ottmann et al. (Ottmann et al., 2009) demonstrated that the purified NLP_{Pp} protein caused membrane leakage in vesicles from dicots, indicating the protein has a cytolytic activity. The immune response was suggested to result from cellular damage, or the release of damage-associated molecular patterns. The finding that *Arabidopsis* mounts an effective immune response to only a small, highly conserved, peptide of non-cytotoxic and cytotoxic type 1 NLPs demonstrates that cellular damage is not required for NLP-triggered immunity in *Arabidopsis*. However, the fact that the type 2 NLP_{Pcc} induces immune responses in *Arabidopsis*, but its internal peptide fragment is not recognized as a MAMP, suggests that cytotoxic NLPs also activate immunity through a different mechanism.

NLP Recognition in *Arabidopsis*

In their natural environment, *Arabidopsis* plants are exposed to a wide range of microbial organisms, a few of which are known to cause disease under field conditions (Coates and Beynon, 2010). Of these natural pathogens, the downy mildew *H. arabidopsidis* is the only one, known so far, that contains *NLP* genes. As pathogens are known to be important in shaping the evolution of host species, it is tempting to speculate that *Arabidopsis* has evolved the capability to detect NLPs as a mechanism to protect itself from downy mildew infection. The NLP-triggered immune response is clearly effective as pretreatment of plants with NLP proteins or peptides provide protection against downy mildew infection. Nevertheless, in untreated plants, *H. arabidopsidis* can overcome these defenses, as it is able to cause disease. We envision that, during its coevolution with *Arabidopsis*, the downy mildew has evolved effectors that suppress NLP-triggered immunity, a specific form of MTI. Candidate effectors of *H. arabidopsidis* for this suppression are the well-known host-translocated RXLR proteins that are encoded by an estimated 130–150 genes in this oomycete (Baxter et al., 2010). A large number of these RXLRs have been identified as effective suppressors of defense responses and MTI (Cabral et al., 2011; Fabro et al., 2011; Anderson et al., 2012; Caillaud et al., 2012, 2013; Badel et al., 2013), and could suppress the early responses induced by NLPs.

MAMPs are generally recognized by pattern recognition receptors (PRRs) that are either receptor-like kinases (RLKs) and/or receptor like-proteins (RLPs; Böhm et al., 2014). A peptide fragment of 10–25 amino acids is, in most cases, sufficient for triggering immunity, e.g., flg22 (Felix et al., 1999), Pep-13 (Brunner et al., 2002), and elf18 (Kunze et al., 2004). The specificity of the ligand is determined by the receptor, but often a coreceptor, e.g., BAK1 (Chinchilla et al., 2007; Shan et al., 2008) or SOBIR1 (Liebrand et al., 2013; Zhang et al., 2014), is required for signal transduction. Other host factors could be required for the recognition of nlp24, as the peptide fragment is predicted not to be surface exposed, but located on the inside of the protein (Figure S2), based on the structure of the type 1 NLP_{Pya} protein (Ottmann et al., 2009). This suggests that it cannot directly be recognized by a cognate receptor, but requires (partial) degradation of the protein, likely by host proteases.

NLP MAMPs Occur in Microorganisms of Three Kingdoms of Life

MAMPs have been defined as “highly conserved molecules within a class of microbes and to contribute to general microbial fitness” (Thomma et al., 2011). Some MAMPs are so important to microbes that they cannot thrive without the associated molecules. In *Phytophthora* and downy mildew species, belonging to the oomycetes, *NLP* genes have considerably expanded in number, suggesting they contribute to the lifestyle of these pathogens. It is striking to see that the NLP immunogenic region of 24 amino acids (nlp24)

is highly conserved in type 1 NLPs. Substitutions in the nlp24 region of the fungal VdNLP2 protein in most cases led to loss of cytotoxicity, indicating the region has an important function (Zhou et al., 2012). The observed conservation of the recognized NLP peptide is important for the efficiency and durability of MTI and makes the application of NLP-triggered immunity to generate resistance to non-adapted phytopathogens promising.

NLPs are unique in their extremely wide taxonomic occurrence, suggesting they are advantageous to many different microbial species. Our finding of NLPs acting as proteinaceous MAMPs in *Arabidopsis* clearly shows that these recognized molecules are not confined to a single class of microbes; they are found in oomycetes, bacteria, and fungi. Therefore, the definition of MAMPs could be broadened to "highly conserved molecules found in microbes." The widespread occurrence of this class of secreted proteins, in particular in plant-associated microorganisms, makes their role as MAMPs highly relevant.

Materials and Methods

Generation of Transgenic Lines

The coding sequences of the *HaNLPs* were amplified from *H. arabidopsidis* (isolate Emoy2) genomic DNA using the gene-specific primers (Table S1), cloned into a pENTRY™/D-TOPO® vector using Gateway cloning (Invitrogen), and verified by PCR and Sanger sequencing. For HaNLP3, fusion 4 was used (Cabral et al., 2012), which has the *PsojNIP* signal peptide instead of the *HaNLP3* signal peptide to secrete the protein more efficiently when expressed *in planta*. All *HaNLPs* cloned into a pENTRY™/D-TOPO® were subsequently recombined into the binary vectors pB7WG2 (Karimi et al., 2002), pFAST (Shimada et al., 2010), or a Gateway-compatible version of XVE (Zuo et al., 2000) that was kindly provided by Dr. A.P. Mähönen, University of Helsinki, Helsinki. Binary vectors were transformed into *Agrobacterium tumefaciens* strain C58C1 (pGV2260) by electroporation. *Arabidopsis* Col-0 plants were transformed using the floral dip method (Clough and Bent, 1998). Transformants were selected for BASTA resistance (pB7WG2 and XVE) or for fluorescence of the seed coat (pFAST). Multiple independent T1 lines showing expression of the transgenes, as analyzed by RT-PCR, were selected for further studies. An estradiol-inducible line with proper induction and no measurable leakage was selected by RT-PCR analysis of *HaNLP3* expression.

Plant Growth Conditions

All plants were grown on potting soil (mix z2254, Primasta B.V., Asten, The Netherlands) at 22 °C, 75% relative humidity. *NLP* (inducible)-overexpressing plants (both full-length and truncated proteins) were grown with 16 hours of light per day. Plants used for ethylene measurements had 5 to 6 weeks old and received 8 hours of light per day. Finally, plants

used for pathogenicity assays after peptide treatment were grown under 10 hours of light per day and were 4.5 weeks old when used. All seeds were stratified for 3 days at 4 °C and subsequently moved to growth chambers.

Pathogenicity Assays

Infection assays on seedlings were performed with *H. arabidopsidis* isolate Waco9 (50 spores per µl) and on adult plants (4.5 weeks) with isolate Noco2 (100 spores per µl). After inoculation, plants were left to dry for ~30 min and were subsequently incubated at 100% humidity at 16 °C with 10 hours of light per day. Five to 10 days after inoculation, the disease severity was quantified. For seedlings, the shoots were cut and suspended in a known volume of water and the number of spores per milligram of plant tissue (fresh weight of aerial parts) was determined. For adult plants, the number of conidiophores per leaf was counted.

Ethylene Measurements

Leaves of 5-week old *Arabidopsis* plants (Col-0) were cut into 3-mm squares and left in deionized water overnight at room temperature. The next day, three leaf pieces were transferred to 5-mL glass tubes containing 400 µl of 20 mM MES, pH 5.7, and the appropriate amount of synthetic peptide. Vials were sealed with rubber septa, and after 4 hours, ethylene accumulation was measured by taking a 1-mL sample from the headspace for analysis by gas chromatography (Felix et al., 1999).

GUS Staining

Expression of β-glucuronidase (GUS) in promoter *PR-1:GUS Arabidopsis* lines was assessed by vacuum infiltrating leaves with GUS-staining solution (1 mM X-Gluc, 100 mM NaPi-buffer, pH 7.0, 10 mM EDTA, and 0.1% [vol/vol] Triton X-100). Leaves were incubated for 24 hours at 37 °C in the GUS-staining solution, and subsequently chlorophyll was removed by repeated washes in 70% ethanol.

Microarray Analysis

Twenty-four hours before harvesting, 10-day-old *Arabidopsis* seedlings containing either XVE:*HaNLP3* or XVE:*YFP* were induced by spraying with estradiol solution (100 µM estradiol in 0.02% Silwet) or 0.02% Silwet as control. RNA was extracted from three biological replicates each using an RNeasy kit (Qiagen) following the manufacturer's instructions. RNA quality was assessed by NanoDrop, and three samples from estradiol-sprayed as well as three samples from control XVE:*HaNLP3* plants were analyzed using ATH1 Affymetrix chips (ServiceXS B.V.). Microarray data were normalized using RMA (Irizarry et al., 2003),

compared with data of estradiol-sprayed and control XVE:*YFP* plants, and differentially expressed genes were selected using the R package Limma (Smyth, 2005).

Protein Production and Peptide Synthesis

HaNLP3 was produced as described previously (Cabral et al., 2012). Peptides were ordered at Genscript and prepared as 10 mM stock solutions in 100% DMSO before use.

Creation of WebLogos

The WebLogos (Crooks et al., 2004) were generated on a total of 378 type 1 NLP sequences (231 oomycete, 135 fungal, and 12 NLPs of bacterial origin), and 122 type 2 NLP sequences (61 fungal and 61 bacterial NLPs; Oome and Van den Ackerveken, 2014).

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Supplemental Data

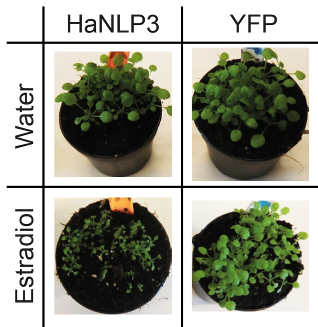

FIGURE S1 | The inducible XVE:*HaNLP3* line shows severe growth reduction when treated with estradiol. From 1 day after germination, XVE:*HaNLP3* and XVE:*YFP* transgenic lines were sprayed every 2 days with either water or 100 µM estradiol. The pictures were taken 14 days after germination, showing only growth reduction of the estradiol-treated XVE:*HaNLP3* line but not of the control XVE:*YFP* line.

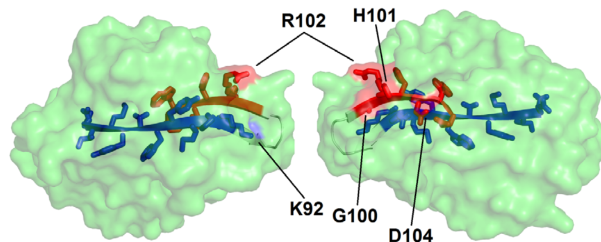

FIGURE S2 | Three-dimensional model of the 24-amino acid immunogenic peptide (nlp24) visualized in NLP$_{Pya}$ (Protein Data Bank ID code 3GNU). The left- and right-hand figure are views of the opposing sites of the protein. The model shows both the individual residues of conserved region I (blue) and conserved region II (red), as well as the surface of the complete protein (green). The less conserved 6-amino acid region connecting the two conserved regions is also in green, and its side chains are not displayed. Of the conserved region I, only the side chain of K92 partly reaches the protein surface (as shown by the paler blue surface), whereas the rest is completely located on the inside of the protein. Of conserved region II, 4 of the 7 amino acids (G100, H101, R102, and D104) are on the surface of the protein (shown by the pink surface). The image was generated with POLYVIEW-3D (Porollo and Meller, 2007).

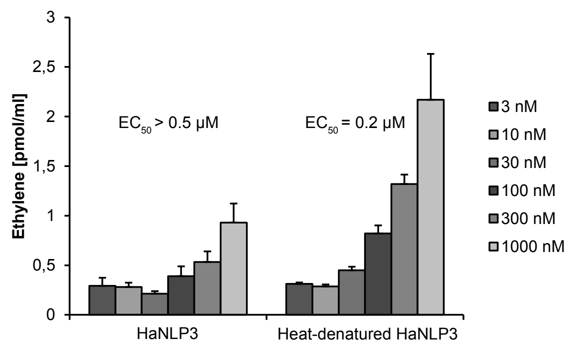

FIGURE S3 | Heat-denatured HaNLP3 protein is a stronger inducer of ethylene production in Arabidopsis than native recombinant HaNLP3. Ethylene accumulation was tested at different protein concentrations of native and heat-denatured (boiled for 1 hour) recombinant NLP protein. The EC$_{50}$ value for the heat-denatured protein was 0.2 µM, similar to that of the nlp24 peptide, and approximately threefold lower than that of the native recombinant HaNLP3 protein.

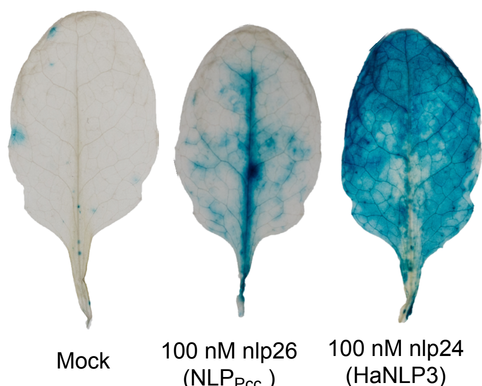

FIGURE S4 | A synthetic nlp24 peptide corresponding to HaNLP3 activates *PR-1* expression. Induction of defense in Arabidopsis leaves was measured by staining for GUS expression in leaves of *pPR1:GUS Arabidopsis* plants infiltrated with the negative control 0.01% DMSO (Mock), 100 nM nlp26 of NLP_{Pcc}, and 100 nM nlp24 of HaNLP3. GUS staining was performed at 24 hours after infiltration. The *PR1* promoter was strongly activated by nlp24, whereas low signals were observed in mock-treated leaves and in response to nlp26 of NLP_{Pcc}.

```
HaNLP3      MKLDGFITTAILAHIPVYARNDYVQEEKQQQLQEPLDGQWKPTTTGHDAIVPFSEPKPVT   60
BcNEP2      --MVAFSKSLQLS-LSVLAS-TVIAIPTPSQLES-------RAVIDSDAVVGFAETVPSG   49
BsNPP1      --------MRKIA-LAVLMS-FFAFISLVPTVN--------AAVIGHDKVVGFDEVVPTT   42
                        :: :.*              ::          :.  . * :* * *   *

HaNLP3      ISEKAGVKFKPLLDVNTGCAPYAAVNAEGETSGGLQTSGDPESGCRGSKYGSQVYGRSTW   120
BcNEP2      TVGTVYEAYKPFLKVVNGCVPFPAVDASGNTGGGLSPTGSSNGGC--SSSTGQVYVRGGQ   107
BsNPP1      IAQKAEKKFQPYLKVYSGCVPFPAVDAQGNTSGGLQPTGAPEGGC--SKHTGQVYSRSTW   100
               ..    ::*  *.*  .**.*:  **:*.*:*.***.  :*   :.**   *    .*** *.

HaNLP3      YNDVWAIMYAWYFPKDSPMLLMGHRHDWENVVVFINDPDEVEPT-ILGCSTSWHSGYIKY   179
BcNEP2      SGSNYAIMYSWYMPKDEPSTGIGHRHDWEGVIVWLSSATATTADNILAVCPSAHGGWD-C   167
BsNPP1      YNGVWAIMYSWYFPKDEPSPGLGHRHDWEGIVVWVDNPSIQNAK-VLSIAYSGHGKFTNV   159
             ..  :****:**:***.*     :*******  ::*::...     .    :*.  .  * *.  :

HaNLP3      APCPTDSINGSSVMIKYEHSFPLNHALNITKDAGAYQDLIMWHQMPDLARRALNDTDFGK   239
BcNEP2      STDGY-SLSGTSPLIKYESIWPVDHSMGLTSTVGGKQPMIAWESLPTAAQTALENTDFGA   225
BsNPP1      QPNEK-NMKDTHPLIAYNSTWPLNHELHISDQVGGTQPLIGWEDLTPEARNALNITDFGK   218
             .      .:..:   :*  *:   :*::*  :  ::.  .*.  *  :*  *..:.   *:  **:  ****

HaNLP3      AITPMNDLNFMEKIEAAWPFKTKKDGA   266
BcNEP2      ANVPFIPAVFTDNLAKATF--------   244
BsNPP1      ANVPFNDPNFTNHLEKAWFR-------   238
            *  .*:      *  :::    *
```

FIGURE S5 | Conservation of the nlp24 peptide in NLPs of microorganisms from three kingdoms of life. A multiple alignment was generated of HaNLP3 of the oomycete *H. arabidopsidis*, BcNEP2 of the fungus *B. cinerea*, and BsNPP1 of the bacterium *B. subtilis*. Signal peptides are indicated in yellow, and the 24-amino acid regions tested for ethylene induction in Arabidopsis are indicated in black.

Table S1 | Primers used in this study.

Primer	Forward	Reverse
HaNLP1	CACCATGAGGACTGGCGCCTTC	CTCATTAAAAAGGCCAAGAAGCG
HaNLP2	CACCATGAAGACCAGTGCCTTC	TCAATAGTCATTGTCCTCGAC
HaNLP3	CACCATGAACCTCCGCCCTGCA	TCATGCTCCATCTTTTTTCGTTTTAAACGG
HaNLP4	CACCATGAAGGCCAGCGCATTCCTG	TTAACTGTCGTAGCTATCTTGGC
HaNLP5	CACCATGAGCTTCCGGGCTCTAGTC	TCAGAATGCCATGCCCAGGC
HaNLP6	CACCATGAAGGCCAGCGCATTC	TCAATCTTGCCTCGCTTAACCT
HaNLP7	CACCATGAGGATAGGTAAGTCCTTGTGC	CTATCCAGCCATTTCGTAAGG
HaNLP8	CACCATGAAGACTTTGTCTTGCTTGTAT	TCACTTCAGCGGTGCAAAAG
HaNLP9	CACCATGAAGACCGGTCTCTTCTTGTA	TCAGCCTTCAACAAAGTCGTA
HaNLP10	CACCATGAAGGCCGTCGCCTTGTTG	CTAGCTAGCTGCGCTCACAT
HaNLP3 C79S	AGTGCACCGTACGCGGCT	GCCCGTATTAACATCGAGCA
HaNLP3 ΔCC	CGTGGATCGAAGTACGGGT	GCCCGTATTAACATCGAGCA
Fragment 1	TGAAAGGGTGGGCGCG	CACCATGATAAGGTCCTGGTAAGCT
Fragment 2	TGAAAGGGTGGGCGCG	ATACTTGATGTAGCCACTGTGCCA
Fragment 3	TGAAAGGGTGGGCGCG	ACCCAAGATCGTCGGCT
Fragment 4	TGAAAGGGTGGGCGCG	CTCCCAGTCATGCCGATGA
Fragment 5	TGAAAGGGTGGGCGCG	CATCAGTAGCATCGGCGAGT
Fragment 6	TGAAAGGGTGGGCGCG	GAAGTACCACGCGTACATAATAGC
Fragment 7	TGAAAGGGTGGGCGCG	GGTGGAGCGCCCATAAACT
Fragment 8	TGAAAGGGTGGGCGCG	GCCCGTATTAACATCGAGCAA
Fragment 9	CGTGGATCGAAGTACGGGT	GGCGCTCACGTACGCG
Fragment 10	AATGACGTCTGGGCTATTATGTACG	GGCGCTCACGTACGCG
Fragment 11	AACCCCAAGGACTCGCCGAT	GGCGCTCACGTACGCG
Fragment 12	GGTCATCGGCATGACTGG	GGCGCTCACGTACGCG

Dataset S1 | The microarray dataset is available online: http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1410031111/-/DCSupplemental/pnas.1410031111.sd01.xls

Chapter

3

The receptor proteins RLP23 and SOBIR1 mediate NLP-triggered immunity in *Arabidopsis*

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An RLP23-SOBIR1-BAK1 complex mediates NLP-triggered immunity.
Nature Plants, **1**:15140

Abstract

Plants have evolved a sophisticated immune system to detect microbial invaders. Recognition of extracellular pathogen-derived patterns generally relies on receptor-like kinases (RLKs) and receptor-like proteins (RLPs). The pattern provided by necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) is unique because of the widespread taxonomic occurrence of NLPs. They are produced by multiple bacterial, fungal and oomycete species and can be cytotoxic or non-cytotoxic. A conserved NLP-derived peptide of 24 amino acids, called nlp24, is sufficient to activate NLP-triggered immunity (NTI) in *Arabidopsis*. However, how NLPs are recognized remains unknown. Here, we demonstrate, by screening natural accessions of *Arabidopsis* and *rlk* and *rlp* T-DNA insertion mutants for loss of NLP-responsiveness, that RLP23 is required for NTI. Known RLPs rely on an adaptor RLK of the SUPPRESSOR OF BIR1 1 (SOBIR1)-type for signaling to mount an immune response upon ligand binding. Indeed, the *Arabidopsis sobir1* mutant did not respond to nlp24 suggesting that SOBIR1 is the RLP23 adaptor protein mediating NTI. Besides local activation of immunity, nlp24 treatment induces a systemic immune response that is also mediated by RLP23 and SOBIR1. Mutants that are compromised in salicylic acid biosynthesis or signaling were unable to trigger a systemic immune response after nlp24 treatment, indicating that NTI of distant tissues is similar to systemic acquired resistance (SAR). Although RLP23-mediated immunity is strong, we did not observe that *rlp23* mutant plants are more susceptible to two *NLP*-expressing pathogens, i.e. *Hyaloperonospora arabidopsidis* and *Botrytis cinerea*. A possible virulence function of non-cytotoxic NLPs was tested by transgenic overexpression of *NLPs* in the *Arabidopsis rlp23* mutant background. However, this did not lead to changes in disease susceptibility. A putative role of non-cytotoxic NLPs in enhancing plant susceptibility, therefore, remains elusive.

Introduction

Plants have many receptor proteins that directly or indirectly perceive pathogen attack, both intra- and extracellularly (Cook et al., 2015). All known receptors that recognize pathogen-derived extracellular patterns, i.e. non-self molecules or pathogen-inflicted damage, belong to either the receptor-like kinase (RLK) or receptor-like protein (RLP) family (Cook et al., 2015; Boutrot and Zipfel, 2017; Ranf, 2017). When a pattern is detected by an RLK or RLP pattern recognition receptor (PRR), a plethora of immune responses can be triggered. Early responses include increased ethylene biosynthesis, an oxidative burst, activation of the MAP kinase signaling cascade and, consequently, activation of defense-related genes (Boller and Felix, 2009). Some well-known RLKs are FLAGELLIN-SENSING 2 (FLS2) and EF-TU RECEPTOR (EFR), which detect bacterial patterns flagellin and bacterial elongation factor Tu, respectively (Couto and Zipfel, 2016; Fliegmann and Felix, 2016). The first *RLP* gene required for the recognition of a pathogenic microbe, the tomato *Cf-9* gene, was cloned over 20 years ago (Jones et al., 1994), and in recent years several other RLPs have been identified that detect patterns. For example, in *Arabidopsis thaliana*, RLP1, also known as ReMAX, for receptor of eMAX (enigmatic microbe-associated molecular pattern of *Xanthomonas*), mediates the recognition of an unknown bacterial pattern (Jehle et al., 2013b). Furthermore, RLP30 and RLP42 recognize endopolygalacturonases from the fungus *Botrytis cinerea* and a partially purified proteinaceous pattern called SCLEROTINIA CULTURE FILTRATE ELICITOR1 from the fungal pathogen *Sclerotinia sclerotiorum*, respectively (Zhang et al., 2013, 2014). In wild potato, the oomycete elicitin receptor ELICITIN RESPONSE (ELR) was identified (Du et al., 2015). The first PRR that recognizes a pattern of the parasitic plant *Cuscuta reflexa* CUSCUTA RECEPTOR 1 (CuRe1), was identified in tomato (Hegenauer et al., 2016). Finally, the *Nicotiana benthamiana* RECEPTOR-LIKE PROTEIN REQUIRED FOR CSP22 RESPONSIVENESS (NbCSPR) was described to confer recognition of csp22, a peptide derived from a bacterial cold-shock protein (Saur et al., 2016). However, the role of NbCSPR in csp22 recognition is disputed by Wang and colleagues. They were unable to corroborate that NbCSPR is required for csp22 recognition and instead demonstrated that the tomato RLK COLD SHOCK PROTEIN RECEPTOR (CORE) is a true csp22 PRR (Wang et al., 2016).

RLPs lack an intracellular kinase domain and therefore need to interact with adaptor proteins of the SUPPRESSOR OF BRASSINOSTEROID INSENSITIVE 1 (BRI1)-ASSOCIATED RECEPTOR KINASE (BAK1)-INTERACTING RECEPTOR-LIKE KINASE 1 (SOBIR1)-type to form a functional bimolecular receptor kinase (Liebrand et al., 2013, 2014; Gust and Felix, 2014). Furthermore, many RLKs and RLPs rely on SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES (SERKs) to be able to initiate a defense response. *Arabidopsis* encodes 5 *SERK* genes of which *SERK3/BAK1* and *SERK4/BAK1-LIKE1* (*BKK1*) are particularly important in RLK- and RLP-mediated immunity (Heese et al., 2007; Roux et al., 2011; Ma et al., 2016).

These SERKs are recruited by the immune receptors upon ligand perception after which a defense signal can be transduced (Albert et al., 2015; Couto and Zipfel, 2016; Postma et al., 2016). Similarly, the RLK CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) functions as a co-receptor, e.g., in *Arabidopsis* in the perception of bacterial peptidoglycan (together with LYM1 or LYM3) and fungal chitin (with LYK5), but also in rice (with CEBiP; Couto and Zipfel, 2016; Zipfel and Oldroyd, 2017).

Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) are apoplastic proteins secreted by bacteria, fungi, and oomycetes (Oome and Van den Ackerveken, 2014). As their name suggests, the first identified members of this family are cytotoxic (Bailey, 1995; Ottmann et al., 2009). Intriguingly, the genome sequences of the biotrophic *Arabidopsis* downy mildew *Hyaloperonospora arabidopsidis* revealed an expansion of its *NLP* family (Baxter et al., 2010). At first, this seemed odd: this obligate biotroph would kill its host cells with these cytotoxic proteins. However, all 10 *H. arabidopsidis* NLPs (HaNLPs) were found to be non-cytotoxic (Cabral et al., 2012; Oome and Van den Ackerveken, 2014). To find a function of the non-cytotoxic HaNLPs, *Arabidopsis* plants that expressed these *HaNLPs* were produced. Interestingly, most of the *HaNLP*-expressing plants displayed severe growth reduction, which was associated with the activation of the plant immune system. A highly conserved peptide fragment of 24 amino acids, derived from HaNLP3, named nlp24, was sufficient to elicit a defense response in *Arabidopsis* (Oome et al., 2014). Plants treated with nlp24 showed increased ethylene production, *PR-1* expression and high resistance to *H. arabidopsidis*. Peptides corresponding to the same region in a fungal and bacterial NLP activated NLP-triggered immunity (NTI) as well. Therefore, it was concluded that NLPs from three kingdoms of life act as a microbial pattern in *Arabidopsis* (Oome et al., 2014). Simultaneously, it was shown that a highly similar, 20 amino acid fragment (nlp20) of the cytotoxic *Pp*NLP of *Phytophthora parasitica* is also a potent pattern triggering immunity in *Arabidopsis* (Böhm et al., 2014).

In this chapter, we describe the finding that RECEPTOR-LIKE PROTEIN 23 (RLP23) and SOBIR1 are both required for NTI in Arabidopsis, suggesting they act as a bimolecular receptor. Furthermore, local application of nlp24 in *Arabidopsis* was found to induce systemic immunity to *H. arabidopsidis*. Finally, transgenic expression of *HaNLPs* in the *Arabidopsis rlp23* mutant did not alter disease susceptibility. The role of non-cytotoxic NLPs, besides their activity as patterns, therefore, remains enigmatic.

Results

RLP23 and SOBIR1 Are Required for NLP-triggered Immunity

To find the NLP PRR we made use of natural variation that occurs amongst *Arabidopsis* accessions and of *rlk* and *rlp* T-DNA insertion mutants (Wang et al., 2008; The 1001 Genomes Consortium, 2016). These lines and mutants were screened for nlp20-responsiveness (peptides used in this study are depicted in Table 1). Leaf pieces of each line were incubated in a buffered 1 µM nlp20 solution for 3 to 4 hours after which the ethylene accumulation was determined as a measure of immune activation. Out of 135 *Arabidopsis* accessions, of which 23 representative ones are depicted in Figure 1*A*, only three accessions, Kyoto, Bor-4 and JM-0 proved nlp20-unresponsive. Furthermore, nlp20-induced ethylene accumulation was observed in all tested RLK and RLP T-DNA insertion mutants except for *rlp23-1* (21 representative T-DNA insertion mutants for 18 RLPs are shown in Figure 1*B*). Strikingly, Kyoto, Bor-4 and JM-0 all had the same mutation in *RLP23*: a deletion of a guanine at position 1240 of the coding sequence leading to a frameshift and a premature stop codon in the part encoding leucine-rich repeat 13.

TABLE 1 | Peptides used in this study. Peptides were designed based on the conserved peptide sequence of HaNLP3 that is a potent trigger of immunity in *Arabidopsis* (Oome et al., 2014), except for nlp20 and flg22. Peptide nlp20 was based on the sequence of *Pp*NLP of *Phytophthora parasitica* and lacks the final 4 amino acids of the conserved heptapeptide motif (Böhm et al., 2014). The pattern flg22 corresponds to a conserved domain of flagellin from the bacterium *Pseudomonas aeruginosa* and elicits an immune response in *Arabidopsis* (Felix et al., 1999). Size indicates peptide length in number of amino acids.

Name	Organism of origin	Size	Amino acid sequence
nlp24 (HaNLP3)	*Hyaloperonospora arabidopsidis*	24	AIMYAWYFPKDSPMLLMGHRHDWE
nlp20 (*Pp*NLP)	*Phytophthora parasitica*	20	AIMYSWYFPKDSPVTGLGHR
nlp24 (HaNLP1)	*Hyaloperonospora arabidopsidis*	24	AIMFAYYFPKSQPRRSVSVRHSWE
nlp24 (HaNLP2)	*Hyaloperonospora arabidopsidis*	24	GIVYAWFFPKDSVRHGIGHRYDWE
nlp24 (HaNLP4)	*Hyaloperonospora arabidopsidis*	24	GIIFAWYFPKDSVRDGVGHRHDWE
nlp24 (HaNLP5)	*Hyaloperonospora arabidopsidis*	24	AIMFSWYFPKGFHDRKASRRHDWA
nlp24 (HaNLP6)	*Hyaloperonospora arabidopsidis*	24	GIVYAWYFPKDSVRDGIGHRYDWE
nlp24 (HaNLP7)	*Hyaloperonospora arabidopsidis*	24	AIAYAYYSPKAHPHQRVWIRHVWN
nlp24 (HaNLP8)	*Hyaloperonospora arabidopsidis*	24	AIMYALYFPKDMKVLNRGYRHAFE
nlp21 (HaNLP9)	*Hyaloperonospora arabidopsidis*	21	AIMYVWYFPKD---NRDDDRHDWE
nlp24 (HaNLP10)	*Hyaloperonospora arabidopsidis*	24	AIMYAWYFPKDAPDEESGQRHDWE
nlp24 (BcNEP2)	*Botrytis cinerea*	24	AIMYSWYMPKDEPSTGIGHRHDWE
nlp24 (BsNPP1)	*Bacillus subtilis*	24	AIMYSWYFPKDEPSPGLGHRHDWE
flg22	*Pseudomonas aeruginosa*	22	QRLSTGSRINSAKDDAAGLQIA

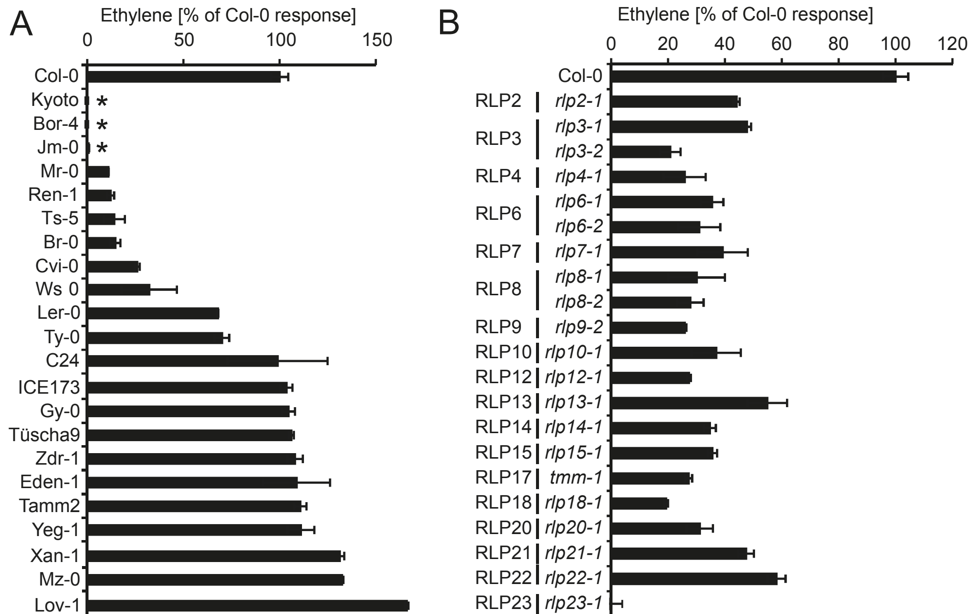

FIGURE 1 | Three *Arabidopsis* accessions and the T-DNA insertion mutant *rlp23-1* do not show an increased ethylene production after nlp20 treatment. (***A***) 135 *Arabidopsis* accessions, of which 23 are depicted here, were tested for increased ethylene accumulation upon 1 µM nlp20 treatment. Kyoto, Bor-4 and JM-0 showed no increased ethylene levels (marked by an asterisk). (***B***) Ethylene production in 21 *rlp* T-DNA insertion mutants with 1 µM nlp20. No increased ethylene accumulation was only observed in *rlp23-1* plants. Data are relative to nlp20-induced ethylene production in *Arabidopsis* Col-0 which was set at 100%. Error bars show standard deviation (SD) of 2 replicates.

Next, we tested if other NLP peptides also require RLP23 to induce increased ethylene accumulation. Indeed, all tested nlp24-based peptides of oomycete, bacterial and fungal origin (table 1), fail to increase ethylene production in *rlp23-1* plants (Figure 2). SOBIR1-type proteins are known to function as adaptor proteins for many RLPs to form a functional receptor kinase complex (Gust and Felix, 2014). Therefore, two *sobir1* T-DNA insertion mutants, *sobir1-12* (SALK_050715) and *sobir1-13* (SALK_009453; Alonso et al., 2003; Gao et al., 2009), were tested for ethylene accumulation in response to treatment with different nlp24 peptides (Table 1) and the well-known bacterial pattern flg22 (Figure 2). Compared to mock treatment, flg22 caused a similarly increased ethylene induction in Col-0, *rlp23-1,* and *sobir1* mutants demonstrating that FLS2-mediated immunity is not impaired in these lines. Interestingly, all nlp24 peptides, including those derived from HaNLPs, BcNEP2 from the fungus *Botrytis cinerea*, and BsNPP1 from the bacterium *Bacillus subtilis*, were unable to elicit increased ethylene production in *rlp23-1*, *sobir1-12* and *sobir1-13* showing similar ethylene levels as mock-treated Col-0 plants (Figure 2).

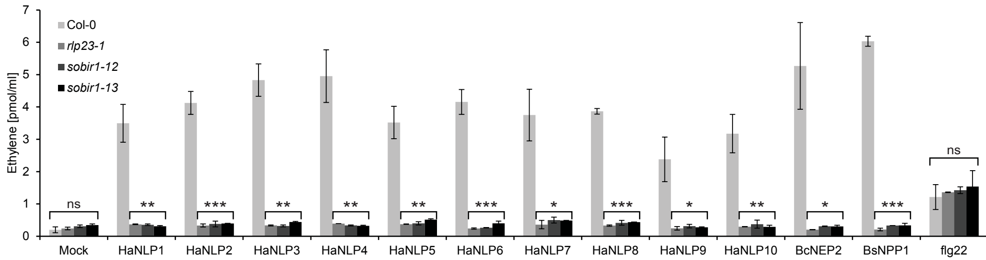

FIGURE 2 | Ethylene accumulation induced by nlp24-based peptides requires RLP23 and SOBIR1. *Arabidopsis* Col-0 and *rlp23* and *sobir1* T-DNA mutants (Col-0 background) leaf pieces were treated with 1 µM peptide solution. nlp24 peptides were based on the NLPs of the oomycete *Hyaloperonospora arabidopsidis* (HaNLPs), the fungal *Botrytis cinerea* NLP BcNEP2 and the bacterial *Bacillus subtilis* NLP BsNPP1 (Table 1). The bacterial pattern flg22 was used as a positive control. After 3 hours ethylene accumulation was determined by gas chromatography. Error bars show SD of 2 replicates. Asterisks indicate significant differences between Col-0 wildtype and *rlp23-1* and 2 *sobir1* T-DNA mutants in response to different peptides (one-way ANOVA, Tukey's honestly significant difference (HSD) post hoc test; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ns = not significant).

To see whether nlp24 treatment of the *rlp23* and *sobir1* mutants not only resulted in reduced ethylene production but also affected immunity, four leaves of Col-0, *rlp23* and *sobir1* plants were completely infiltrated with 100 nM nlp24 and the next day inoculated with *H. arabidopsidis* isolate Noco2. After six days, the number of conidiophores per nlp24-treated leaf was counted as a measure of susceptibility. Previously, Oome et al. observed that nlp24-treated leaves were fully resistant to Noco2, indicating that the peptide triggered a strong and effective immune response in *Arabidopsis* Col-0 plants. We observed a similar phenotype for nlp24-treated Col-0 leaves that became fully resistant to *H. arabidopsidis* (Figure 3). In contrast, two independent *rlp23* and *sobir1* mutants showed no significant decrease in susceptibility to *H. arabidopsidis* after nlp24 treatment. The sporulation levels were similar to mock-treated plants, indicating that the mutants were completely unresponsive to the nlp24 peptide. Interestingly, we did not observe a significant difference in susceptibility between mock-treated Col-0, *rlp23* and *sobir1* plants (Figure 3), suggesting that RLP23 and SOBIR1 do not reduce susceptibility of wild-type *Arabidopsis* to downy mildew.

Previously, it was demonstrated that *N. benthamiana* does not respond to nlp peptides (Böhm et al., 2014). Additionally, *N. benthamiana* encodes functional *SOBIR1* and *SOBIR1-like* genes that are required for RLP accumulation, stability and signaling (Liebrand et al., 2013; Gust and Felix, 2014). Therefore, we used this plant species to verify that RLP23 is required for nlp recognition. Forty hours after *Agrobacterium tumefaciens*-mediated transient expression of p35S::*RLP23*:*GFP* (green fluorescent protein) or p35S::*YFP* (yellow fluorescent protein) in *N. benthamiana*, leaf pieces were taken and treated with nlp24. Transient expression of *RLP23* resulted in a strong response to nlp24 as measured by the high production of ethylene. This response was absent in nlp24-treated *N. benthamiana* leaves expressing *YFP* and much weaker in mock-treated leaves of *RLP23*-expressing

plants (Figure 4). Taken together, these data strongly indicate that both RLP23 and SOBIR1 are required for NTI, and suggest that RLP23 is the PRR recognizing the NLP pattern.

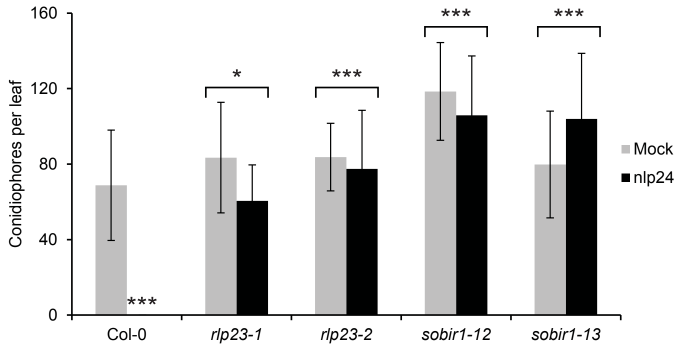

FIGURE 3 | Immunity against *H. arabidopsidis* induced by nlp24 is impaired in *rlp23* and *sobir1* mutants. Twenty-four hours prior to inoculation with *H. arabidopsidis* isolate Noco2 leaves were infiltrated with 100 nM nlp24 or 0.001 % DMSO (Mock). nlp24-treated leaves of Col-0 became highly resistant to downy mildew, whereas *rlp23-1*, *rlp23-2*, *sobir1-12,* and *sobir1-13*, were as susceptible as mock-treated leaves. Conidiophores were counted 6 days post-inoculation. Error bars show the standard deviation of 16-32 treatments. Asterisks indicate a significant effect of nlp24 compared with mock in Col-0 plants. Asterisks above brackets show a statistically significant difference between Col-0 and the mutant genotypes (two-way ANOVA, Tukey HSD post hoc test; $*P \leq 0.05$; $***P \leq 0.001$).

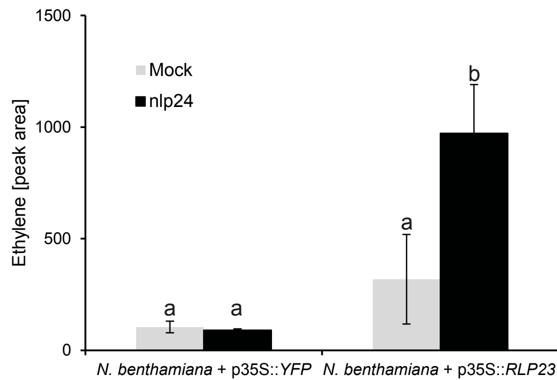

FIGURE 4 | Transient expression of *RLP23* confers nlp24 sensitivity in *Nicotiana benthamiana*. *N. benthamiana* plants were infiltrated with *A. tumefaciens* carrying either p35S::*RLP23*:*GFP* or p35S::*YFP*. Forty-eight hours later, leaf pieces were treated with 1 µM nlp24 or 0.01 % DMSO (Mock). Transformed leaves expressing *RLP23* gained nlp24 sensitivity, whereas *YFP*-expressing leaves did not. Error bars show SD of 5 replicates. Letters indicate a significant difference (two-way ANOVA, Tukey HSD post hoc test; $P < 0.001$).

nlp24 Induces Systemic Acquired Resistance in *Arabidopsis*

When scoring NTI to *H. arabidopsidis* in 5-week-old Col-0 we observed an interesting phenotype. Not only the local nlp24-treated leaves showed a strong immune reaction (Figure 3), also the untreated systemic leaves demonstrated similar levels of disease

resistance (Figure 5). This was reminiscent of systemic acquired resistance (SAR), an induced broad-spectrum defense mechanism in plants. Mobile signals and increased salicylic acid (SA) production lead to systemic immunity for a long duration, potentially even transgenerational (Fu and Dong, 2013). Pathogen recognition can serve as a trigger of SAR, however, the initial molecular signal after recognition is unknown (Fu and Dong, 2013).

We further investigated if RLP23 and SOBIR1 are also required for systemic NTI. Four leaves of Col-0, *rlp23* and *sobir1* plants were treated nlp24 and the next day, the whole plant was inoculated with *H. arabidopsidis* Noco2. Six days after inoculation the disease severity was scored in systemic tissue by measuring the presence of conidiophores. Similar to the locally nlp24-treated tissue (figure 3), *rlp23* and *sobir1* plants lost the ability to mount a systemic immune response after nlp24 treatment (Figure 5).

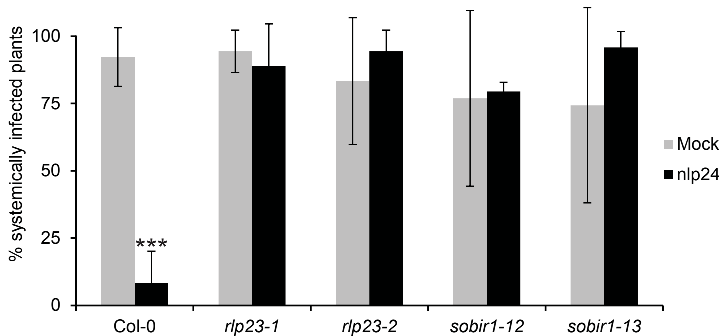

FIGURE 5 | Systemic resistance to *H. arabidopsidis* induced by nlp24 is lost in *rlp23* and *sobir1* mutants. Leaves were treated with nlp24 and 24 hours later infected with downy mildew isolate Noco2. Six days post-inoculation untreated systemic tissue was scored for the presence of sporulation 6 to 8 days after inoculation. Col-0 plants treated with nlp24 showed high levels of systemic resistance to *H. arabidopsidis*. *rlp23* and *sobir1* mutants were not able to activate nlp24-triggered systemic resistance. Error bars show the standard deviation of 3 independent experiments. Asterisks indicate a significant effect of nlp24 compared with mock in Col-0 plants (two-way ANOVA, Tukey HSD post hoc test; ***$P \leq 0.001$).

Next, we wanted to determine whether genes that are known to be required for SAR are involved in nlp24-induced resistance. Many key players have been identified that are required for SAR in *Arabidopsis*, e.g. NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS 1 (NPR1) which is a major player in SA signaling and ISOCHORISMATE SYNTHASE 1 (ICS1), a key enzyme needed for pathogen-induced SA biosynthesis (Fu and Dong, 2013). No clear role for ethylene in SAR has been reported, but to find out if ethylene is an important signal in nlp24-induced systemic resistance the ethylene signaling mutant *ethylene insensitive 2-1* (*ein2-1*; Guo and Ecker, 2004) was tested as well. The nlp24 (100 nM) peptide was infiltrated into 4 leaves and, the subsequent day, the whole plant was inoculated with *H. arabidopsidis* Noco2. Six to eight days after inoculation the presence of conidiophores was determined in untreated systemic tissue. As expected, Col-0 plants showed a strong, significant, decrease of infection in systemic leaves after nlp24 treatment when compared to mock-treated plants

(Figure 6). A similar decrease in infection levels of systemic leaves was observed in *ein2-1* plants. In contrast, the *salicylic acid induction deficient 2-1* (*sid2-1*) mutant, which carries a mutation in *ICS1*, as well as the *npr1-1* mutant did not show nlp24-induced systemic immunity; the infection levels in systemic leaves were similar to that of mock-treated plants (Figure 6).

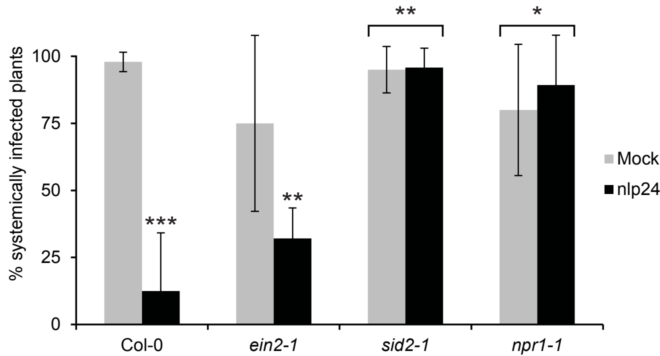

FIGURE 6 | nlp24-induced systemic resistance requires salicylic acid synthesis and signaling but not ethylene signaling. Downy mildew inoculation following local nlp24 treatment resulted in high resistance in systemic tissue in Col-0 and the ethylene signaling mutant *ein2-1*. This systemic resistance was not observed in a salicylic acid (SA) biosynthesis mutant *sid2-1* and an SA signaling mutant *npr1-1*. Error bars depict SD of four independent experiments. Asterisks indicate a significant treatment effect. Asterisks above brackets indicate a significant differential response of the mutant genotypes compared to Col-0 wildtype. (two-way ANOVA, Tukey HSD post hoc test; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

Another important player in SAR is the aminotransferase AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1), which is required for proper synthesis of pipecolic acid (Pip), a signal molecule that accumulates in local and systemic tissue upon pathogen infection. In turn, Pip is needed for the accumulation of SA in systemic tissue and *ald1* mutants are therefore SAR-compromised (Návarová et al., 2012). Furthermore, Pip also plays a critical role in local immune responses against bacteria (Návarová et al., 2012). In contrast to our previous SAR experiment, the infection levels of *ald1-1* mutant plants were determined by quantifying the number of spores per milligram of fresh weight for local and systemic tissue to offer a higher resolution of disease scoring (Figure 7). Comparable high levels of downy mildew resistance in Col-0 and *ald1-1* were observed in nlp24-treated tissue (figure 7*A*). Interestingly, mock-treated *ald1-1* plants showed significantly higher sporulation levels than mock-treated Col-0 plants in local and systemic tissue (Figure 7*A* & 7*B*), suggesting that ALD1 contributes to a basal level of resistance to *H. arabidopsidis*. Although nlp24 treatment led to a significant decrease in infection of *H. arabidopsidis* in *ald1-1* in systemic tissue when compared to mock-treated *ald1-1* plants, disease levels were similar to mock-treated Col-0 (Figure 7*B*). Again, local nlp24 treatment led to very low systemic infection levels in Col-0 plants (Figure 7*B*). We conclude that nlp24-triggered systemic immunity follows after recognition of nlp24 by RLP23 and SOBIR1 and relies on known SAR components (NPR1, ICS1, and ALD1).

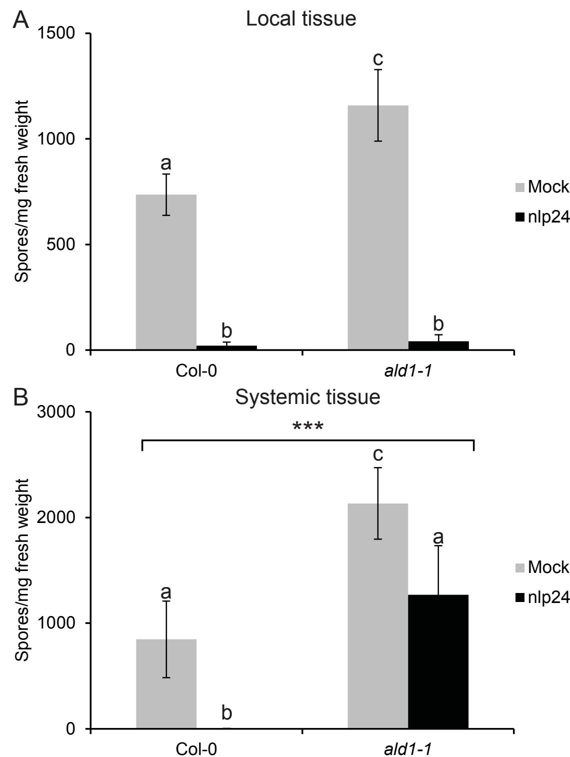

FIGURE 7 | ALD1 is required for systemic nlp24-induced resistance but not for local resistance to *H. arabidopsidis*. (***A***) Local nlp24-induced resistance to downy mildew *does* not require the aminotransferase ALD1. Leaves were infiltrated with 100 nM nlp24 and the following day treated with *H. arabidopsidis*. In both Col-0 and *ald1-1* plants this led to potent downy mildew resistance (***B***) Systemic nlp24-induced resistance requires ALD1. The systemic tissue of locally nlp24-treated Col-0 plants was highly resistant to the downy mildew pathogen. This systemic resistance was lost in the SAR mutant *ald1-1*. Error bars show the standard deviation of 5 replicates. Experiments were repeated three times with similar results. Letters indicate significant differences ($P \leq 0.05$). The asterisk shows a significant response of the *ald1-1* mutant in response to nlp24 when compared to Col-0 (***$P \leq 0.001$). For all statistical analyses, a two-way ANOVA with a Tukey HSD post hoc test was performed.

Overexpression of *HaNLPs* in *rlp23* Background Does Not Contribute to Immunity

Previously, we created 10 *HaNLP* overexpression lines in *Arabidopsis* (one for each HaNLP) to determine if this would render plants more susceptible to downy mildew infection. The transgenic lines showed a reduced growth phenotype that was found to be caused by the recognition of HaNLPs and subsequent strong activation of the immune system (Oome et al., 2014). We can now revisit the question if NLPs contribute to plant disease by using the *rlp23* mutant lines that do not trigger immunity in response to HaNLPs.

To do so, we generated estradiol-inducible *HaNLP3* (XVE:*NLP3*) lines in the *rlp23-1* mutant background by conventional genetic crosses. Two independent homozygous *rlp23-1* x XVE:*NLP3* F4 populations were inoculated with either *H. arabidopsidis* isolate Waco9

(seedlings) or the necrotrophic fungus *B. cinerea* (adult plants). One day prior to pathogen inoculation, plants were treated with estradiol to induce expression of *HaNLP3*. Downy mildew disease symptoms were measured by counting the spores per mg of fresh weight. *B. cinerea* disease levels were categorized into 4 levels of disease severity (I = lesion up to 2 mm, II = lesion up to 2 mm with chlorosis, III = lesion size 2-4 mm, IV – lesion bigger than 4 mm). Plants expressing *HaNLP3* in the wild-type Col-0 background showed increased resistance to both downy mildew and *B. cinerea* (Figure 8*A* & 8*B*). However, this resistance was lost in *rlp23-1* plants expressing *HaNLP3* as infection levels were not significantly different from control Col-0 and *rlp23-1* plants. A significant NLP-induced susceptibility was not observed in *rlp23-1* mutant plants as they were as susceptible to downy mildew and *B. cinerea* as Col-0 (Figure 8). So, even in the absence of NLP detection, the *rlp23* mutant does not show an altered disease susceptibility in response to HaNLP3.

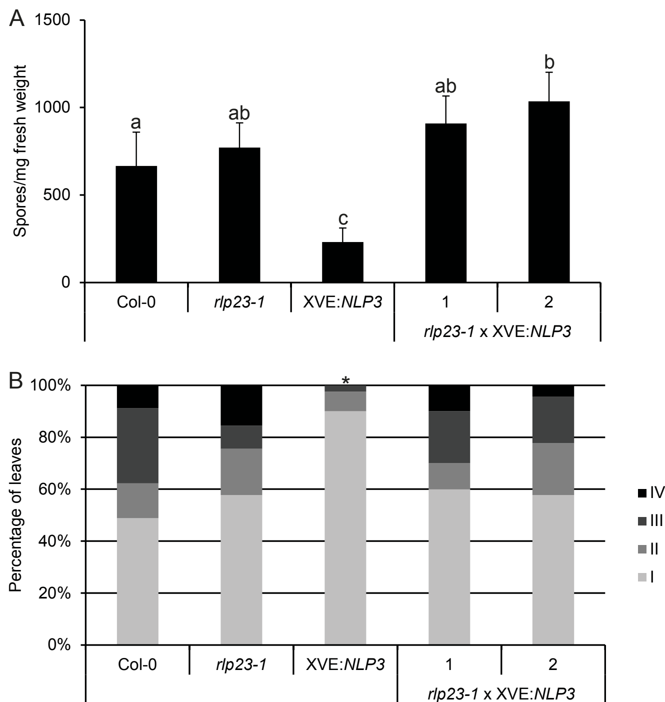

FIGURE 8 | Induced *HaNLP3* expression in *rlp23-1* background does not alter susceptibility to *H. arabidopsidis* and *B. cinerea*. *NLP3* expression was induced by estradiol treatment in XVE:*NLP3* and 2 independent *rlp23-1* x XVE:*NLP3* lines. Twenty-four hours after estradiol treatment plants were inoculated with either *H. arabidopsidis* (***A***) or *B. cinerea* (***B***). Infection levels were scored 3 and 6 days after inoculation for *B. cinerea* and *H. arabidopsidis*, respectively. In wildtype background, *HaNLP3* expression leads to increased resistance to *H. arabidopsidis* (***A***) or *B. cinerea* (***B***). The absence of the *RLP23* expression did not lead to increased susceptibility in whether *NLP3* was expressed or not. Error bars represent two times the standard error of the mean (***A***). Letters indicate statistically significant differences between genotypes (one-way ANOVA, Tukey HSD post hoc test; $P \leq 0.05$). An asterisk indicates a significantly different level of *B. cinerea* infection (χ^2 test; *$P \leq 0.05$ [***B***]).

In an independent experiment to assess a possible contribution of HaNLPs to disease susceptibility, *HaNLP* overexpression lines were created for each of the 10 HaNLPs in the *rlp23-2* mutant background. Although induced expression of *HaNLP3* did not lead to altered disease levels, constitutive overexpression during all developmental phases may. T3 generation seedlings expressing *HaNLPs*, or β-glucuronidase (*GUS*) as a control, in the *rlp23-2* background were tested in *H. arabidopsidis* disease assays, whereas adult plants were used in *B. cinerea* bioassays. As shown in Figure 9*A*, no significant differences in *H. arabidopsidis* sporulation were found when *HaNLP*-overexpressing *rlp23-2* plants were compared to the p35S:*GUS*, Col-0 and *rlp23-2* controls. Similarly, no NLP effect was found in the *B. cinerea* disease test: no significant differences were found between controls (Col-0, *rlp23-2,* and p35S:*GUS* in *rlp23-2* background) and *HaNLP* overexpression lines (Figure 9*B*). The observation that overexpression of *HaNLPs*, whether induced or stable, does not alter the level of susceptibility in *rlp23* mutants suggests that exposing plants to a single HaNLP protein does not significantly affect disease susceptibility.

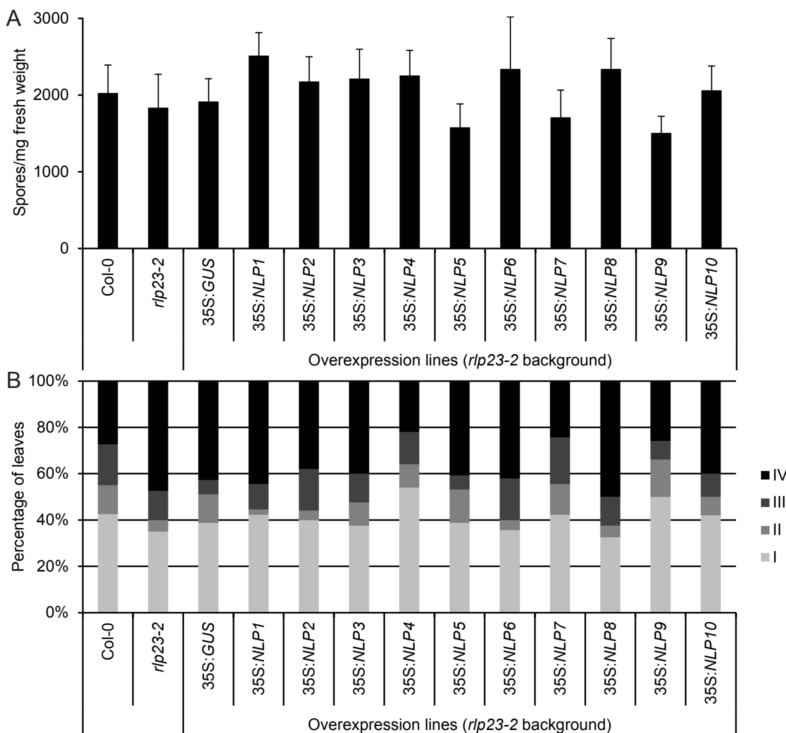

FIGURE 9 | Overexpression of *HaNLPs* does not significantly affect susceptibility to *H. arabidopsidis* and *B. cinerea*. *HaNLP* overexpression constructs in *rlp23-2* background did not show different infection levels of *H. arabidopsidis* (***A***) and *B. cinerea* (***B***) relative to Col-0, *rlp23-2* or *rlp23-2* 35S:*GUS*. Infection levels were measured 3 and 6 days after inoculation for *B. cinerea* and *H. arabidopsidis*, respectively. Error bars represent two times the standard error of the mean. For statistical analyses, a one-way ANOVA with Tukey HSD post hoc test (***A***) or a χ^2 test (***B***) was employed with an α of 0.05.

Discussion

RLP23 Is the NLP PRR

The finding that NLPs act as a molecular pattern was serendipitously made through ectopically expressing non-cytotoxic *HaNLPs* in *Arabidopsis*. Transgenic *HaNLP*-expressing plants showed a reduced growth phenotype that was found to be caused by the activation of immunity through the recognition of a small conserved fragment of NLPs, called nlp24 (Oome et al., 2014). However, the *Arabidopsis* proteins involved in nlp24 recognition were unknown. We used two approaches to pinpoint RLP23 as the putative NLP receptor. In one approach, we exploited natural variation in NLP sensitivity within *A. thaliana*. *Arabidopsis* accessions that did not respond with ethylene accumulation after nlp treatment all carried a defective *RLP23* gene that produces a non-functional PRR (Figure 1*B*). There was a high amount of variation in levels of nlp-induced ethylene accumulation in nlp-sensitive *Arabidopsis* accessions. This broad range of nlp-responsiveness could have several reasons. First, it may be due to different binding affinities of the nlp peptide to the receptor caused by amino acid polymorphisms that are found in RLP23 protein alleles (The 1001 Genomes Consortium, 2016). A second reason could be differences in *RLP23* mRNA and protein levels between accessions. A third, alternative, cause is that the different ethylene levels observed may have arisen through other, more generic, genetic differences in signal transduction components or the ethylene response pathway. In a second, reverse genetic approach, we found that nlp-induced immune activation was absent in *rlp23* T-DNA insertion mutants (Figure 1*A*). Ethylene accumulation induced by nlp24-based peptides from fungal, bacterial and oomycete origin was lost in these mutants (Figure 2). Moreover, *Arabidopsis* plants lacking RLP23 were unable to mount NTI against *H. arabidopsidis* (Figure 3). Intriguingly, transient expression of *RLP23* in the nlp24-insensitive plant species *N. benthamiana* conferred the ability to trigger nlp24-induced ethylene production (Figure 4).

The requirement of RLP23 for nlp24-responsiveness does not definitively prove that it is the PRR recognizing the peptide. However, binding studies from the collaborating Nürnberger group demonstrated that the RLP23 LRR domain physically interacts with nlp20 *in vitro* (Albert et al., 2015). In addition, nlp24, derived from *Pp*NLP, was shown to bind RLP23 *in planta*. This was demonstrated in *N. benthamiana* and *Arabidopsis* leaves expressing *p35S*::*RLP23-GFP* that were treated with nlp24 (*Pp*NLP) tagged with biotin. Subsequently, the leaves were treated with a chemical cross-linker and RLP23-GFP was immunoprecipitated. Binding of biotinylated nlp24 to RLP23-GFP was observed in both plant species. Interestingly, a large excess of non-biotinylated nlp24 (*Pp*NLP) was able to abolish binding whereas a biologically inactive nlp24 peptide lacking the first four amino acids (ΔAIMY) was not (Albert et al., 2015). Taken together, these data show that RLP23 is the *bona fide* nlp24 receptor.

SOBIRI Is Required for RLP23-mediated Immunity

RLPs only have a small intracellular domain and thus seem to require an adaptor protein to form a functional bimolecular receptor kinase that mediates further intracellular signaling (Gust and Felix, 2014). Indeed, for most RLP immune receptors there is evidence that the RLK SOBIR1 is required to trigger a defense response after pattern recognition (Liebrand et al., 2013; Boutrot and Zipfel, 2017; Ranf, 2017). An overview of immune receptors of the RLP family for which a genetic correlation and/or a protein interaction with SOBIR1 has been demonstrated is given in Table 2. We observed that *sobir1* mutants, like *rlp23* mutants, did not respond to nlp24 with an increased ethylene production (Figure 2) nor did they become immune to *H. arabidopsidis* in response to nlp24 treatment (Figure 3). In contrast, *sobir1* plants were not impaired in FLS2-mediated immunity: a similar flg22-induced increase in ethylene accumulation was observed in wild-type and *sobir1* mutants. Previously, it was demonstrated that RLP23 physically interacts with SOBIR1 (Bi et al., 2014). The Nürnberger group found that this interaction was not dependent on the nlp ligand (Albert et al., 2015). In this way, RLP23 seems to act similar to other RLP immune receptors in that it forms a bimolecular receptor complex with SOBIR1 (Gust and Felix, 2014).

Albert et al. also demonstrated that after nlp treatment SERK3/BAK1 is immunoprecipitated with the RLP23-SOBIR1 complex (Albert et al., 2015). Furthermore, it was demonstrated that, when *SERKs* were co-expressed in *N. benthamiana* with *RLP23* and *SOBIR1* in the presence of the nlp peptide, complexes were formed of RLP23-SOBIR1 with SERK3/BAK1, SERK1, SERK2, and SERK4/BKK1, but not SERK5 (Albert et al., 2015). The SERK5 protein is assumed to be non-functional in *Arabidopsis* Col-0 because of a polymorphism in its kinase domain (Ma et al., 2016). In the *Arabidopsis* accession Landsberg *erecta*, however, SERK5 seems to have an important role in brassinosteroid signaling and cell death control (Wu et al., 2015). One could predict that a functional SERK5 protein may, in contrast to the Col-0 protein allele, be recruited to RLP23-SOBIR1 in presence of nlp24. But this remains to be investigated. Interestingly, Albert et al. showed that BAK1 and BKK1 are important for NTI: nlp-induced ethylene accumulation, oxidative burst, and callose deposition were strongly reduced in the *bak1-5 bkk1-1* double mutant compared to wild-type. However, a small induction of ethylene production and callose deposition was still observed in *bak1-5 bkk1-1,* possibly by the redundant function of other SERKs. Similarly, it is known for FLS2-, EFR- and PEPR1/2-mediated immunity that *bak1-5 bkk1-1* is nearly, but not totally, insensitive to flg22, elf18, and Pep1, respectively (Monaghan and Zipfel, 2012). The recruitment of SERKs in the presence of a RLP-SOBIR1 ligand may be a common feature of RLP-mediated immunity. For example, the tomato RLPs Cf-4 and Cf-9 also recruit BAK/SERK3 in presence of their respective ligands (Postma et al., 2016). Whether other members of the SERK protein family play a role in NTI remains to be determined. Summarizing, in absence of nlp24, RLP23 interacts with SOBIR1. After

nlp24 recognition by RLP23, BAK1 is recruited to the RLP23-SOBIR1 complex, likely forming an active, tripartite signaling receptor complex (Figure 10; Albert et al., 2015; Shibuya and Desaki, 2015).

TABLE 2 | PRRs of the RLP family that associate with SOBIR1. RLPs that function in immunity for which there is either a genetic correlation or a protein interaction with SOBIR1 or both.

Gene name	Species	Function	Pattern origin	References
RLP1 (*ReMAX*)	*Arabidopsis*	PRR for eMax	Bacterial	Jehle et al., 2013a
RLP23	*Arabidopsis*	PRR for nlp24	Bacterial, fungal and oomycetal	Bi et al., 2014; Albert et al., 2015
RLP30	*Arabidopsis*	PRR for SCFE1	Fungal	Zhang et al., 2013
RLP42 (*RBPG1*)	*Arabidopsis*	PRR for endopolygalacturonases	Fungal	Zhang et al., 2014
Cf-2	Tomato	Guards tomato Rcr3	Fungal or nematodal	Liebrand et al., 2013
Cf-4	Tomato	Required for Avr4 recognition	Fungal	Liebrand et al., 2013
Cf-4E	Tomato	Required for Avr4E recognition	Fungal	Liebrand et al., 2013
Cf-9	Tomato	Required for Avr9 recognition	Fungal	Liebrand et al., 2013
Ve1	Tomato	PRR for Ave1	Fungal	Liebrand et al., 2013
Eix2	Tomato	PRR for xylanase	Fungal	Liebrand et al., 2013
I	Tomato	Required for Avr1 recognition	Fungal	Catanzariti et al., 2017
CuRe1	Tomato	PRR for a *Cuscuta* glycoprotein	Parasitic plant	Hegenauer et al., 2016
ELR	Potato	PRR for elicitins	Oomycete	Du et al., 2015; Peng et al., 2015

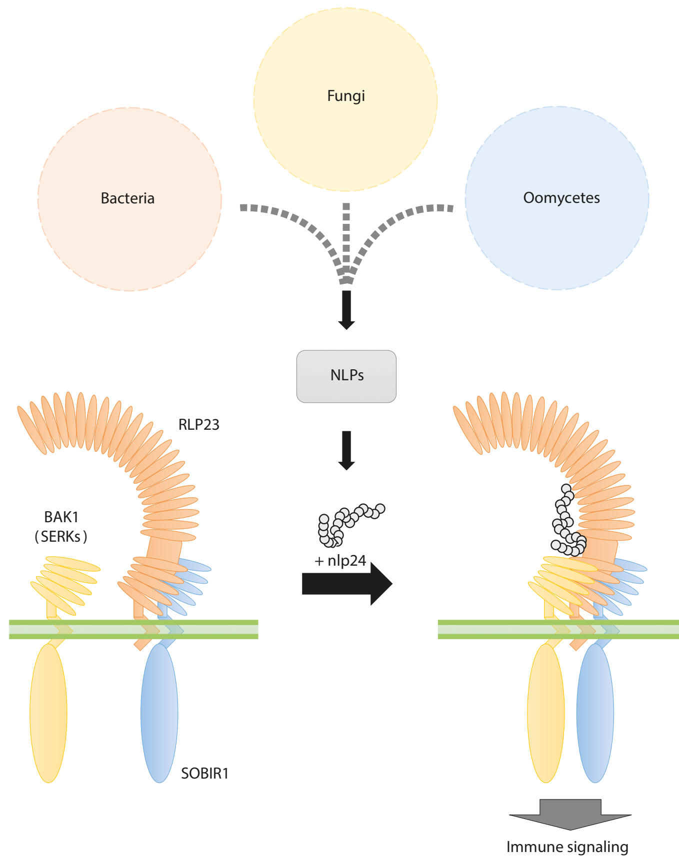

FIGURE 10 | Model for NLP perception and immune signaling. In its inactive state, RLP23 is bound to SOBIR1. When NLPs are recognized by RLP23, BAK1, and possibly other SERKs, are recruited to RLP23 and SOBIR1 forming an active receptor complex. Adapted from Shibuya and Desaki, 2015.

Pattern-triggered SAR

SAR is a process in which plants mount a systemic immune response after a local encounter with a pathogen (Fu and Dong, 2013). Groundbreaking papers in this field have made use of avirulent *Pseudomonas* bacteria that trigger a hypersensitive response in *Arabidopsis* that subsequently leads to SAR against a virulent *Pseudomonas* strain in distal tissues (Cao et al., 1994). More recently, it was shown that pattern recognition is sufficient to trigger SAR in *Arabidopsis* (Mishina and Zeier, 2007). We observed a similar response after local application of nlp24. Next to a strong local resistance against *H. arabidopsidis* (Figure 3), also systemic tissue became highly resistant to the downy mildew pathogen in wild-type *Arabidopsis* (Figure 5-7). Interestingly, nlp24-induced SAR was lost in *rlp23* and *sobir1* mutants (Figure 5), demonstrating that nlp24 needs to be recognized for it to induce SAR. The phytohormone SA plays a pivotal role in pattern- and microbe-induced

SAR and mutants that are impaired in either (pathogen-induced) SA biosynthesis or SA signaling are thus unable to trigger SAR (Mishina and Zeier, 2007; Fu and Dong, 2013). We determined that SAR triggered by the pattern nlp24 also requires SA. We made use of SAR mutants that are compromised in SA signaling (*npr1-1*), pathogen-induced SA biosynthesis (*sid2-1*) or systemic SA accumulation (*ald1-1*). All three mutants were unable to induce SAR after nlp24 treatment (Figure 6 and 7*B*). Interestingly, ALD1 also seems to contribute to basal resistance; mock-treated *ald1-1* plants had higher levels of systemic and local susceptibility (Figure 7*A* and 7*B*). Previously, the importance of ALD1 for local resistance and SAR to bacterial pathogens was demonstrated (Návarová et al., 2012). In conclusion, nlp24 is a potent trigger of SAR and the establishment of SAR in response to nlp24 relies on previously described SAR components.

Ectopic *HaNLP* Expression in *Arabidopsis* Does Not Affect Disease Susceptibility

For many NLPs, their role in pathogenicity is unclear. The function of cytotoxic NLPs for pathogens with a necrotrophic lifestyle seems obvious: killing plant cells so the pathogen can subsequently feed off the dead tissue. The bacterium *Pectobacterium carotovorum*, e.g., produces a cytotoxic NLP called NLP_{Pcc} that is important for its virulence: an NLP_{Pcc}-deficient *P. carotovorum* strain has reduced virulence on potato tubers (Mattinen et al., 2004; Pemberton et al., 2005). When this strain was complemented with NLP_{Pcc} or with cytotoxic NLPs from *P. parasitica* (PpNLP) and *Pythium aphanidermatum* (NLP_{Pya}) its virulence function was restored (Ottmann et al., 2009). However, the role of cytotoxic NLPs is not always that clear-cut. For example, *Vd*NLP1 and *Vd*NLP2 of the vascular wilt pathogen *Verticillium dahliae* strain JR2 are both needed for full virulence in *Arabidopsis* and tomato, but their homologs from strain V592 are dispensable for virulence on cotton (Zhou et al., 2012; Santhanam et al., 2013). More intriguingly, *Vd*NLP1 also affects conidiospore formation and vegetative growth of the pathogen *in vitro*, thus NLPs may have other functions, unrelated to pathogenicity (Santhanam et al., 2013). Interestingly, knocking out all four NLPs (three are cytotoxic) of the rice blast pathogen *Magnaporthe oryzae* did not lead to a significant change in disease levels in rice (Fang et al., 2017).

For non-cytotoxic NLPs, the story is even more obscure, as no reports on their presumed function have been published. They may play a role in pathogen development, like *Vd*NLP1 (Santhanam et al., 2013). However, non-cytotoxic NLPs have been reported to be mostly expressed early during infection. For example, 2 *NLP* genes of the fungal plant pathogen *Colletotrichum higginsianum*, *ChNLP3* and *ChNLP5*, are expressed during the biotrophic phase before appressorium formation and during host penetration, whereas the cytotoxic *ChNLP1* was specifically expressed at the switch to the necrotrophic phase (Kleemann et al., 2012). Similarly, Most *HaNLPs* are highly expressed during early infection, which makes their involvement in pathogenicity likely (Cabral et al., 2012). The fact that NLPs trigger plant immunity in *Arabidopsis* made it impossible to determine a putative

virulence function for the HaNLPs (Oome et al., 2014). The identification of the nlp24 PRR, RLP23, gave us the opportunity to examine the role of HaNLPs in more detail in the absence of immune activation. We generated inducible *HaNLP3* expression lines as well as overexpression lines of all *HaNLPs* in the *rlp23* mutant background. Overexpression of *HaNLPs*, induced or stable, did not alter the level of susceptibility to *H. arabidopsidis* and *B. cinerea* in these transgenic lines (Figure 8 and 9). Therefore, subjecting plants to a single HaNLP did not seem to affect disease susceptibility.

A role for NLPs in disease suppression thus seems unlikely, although this possibility cannot be fully dismissed. Perhaps several HaNLPs work in concert to suppress immunity. Unfortunately, no successful attempt has been reported on silencing or knocking-out genes in downy mildews. The fact that *H. arabidopsidis* encodes ten NLPs makes this even more challenging. It may be more convenient to study the functions of non-cytotoxic NLPs in pathosystems in which efficient pathogen transformation protocols have been well-established. A final function that can be considered for non-cytotoxic NLPs is a role in the recruitment of microbes to the pathogen-host interface. Highly specific microbial communities are formed when a plant is comes in contact with a given microbe (Rovenich et al., 2014). For example, enrichment for different bacteria is found when a plant is in contact with the obligate biotrophic oomycete pathogen *Albugo laibachii* or the basidiomycete yeast *Dioszegia* sp. (Agler et al., 2016). The exact influence of pathogens on the shaping of microbial communities is not yet clear, but secreted proteins, such as NLPs, may influence the composition of the microbiome.

Only a narrow range of plant species seem to perceive NLPs (Böhm et al., 2014). This makes RLP23 an interesting target to be used to engineer crops for higher resistance to microbial pathogens. A breakthrough paper demonstrated that transfer of *EFR* to *N. benthamiana* and tomato resulted in broad-spectrum disease resistance to bacterial pathogens (Lacombe et al., 2010) and since then the transfer of several PRRs to other plants to increase pathogen resistance to pathogens has been successfully demonstrated many times (Rodriguez-Moreno et al., 2017). Arguably, the widespread taxonomic distribution of NLPs may make RLP23 an even more attractive target. Transient expression of *RLP23* in *N. benthamiana* (Figure 4), but also stable expression in *N. benthamiana*, tomato, and potato conferred NLP-sensitivity, suggesting that the immune signaling pathways between these species, with the exception of RLP23, are similar (Albert et al., 2015). Strikingly, even though we observed no difference in resistance against *H. arabidopsidis* and *B. cinerea* between wild-type *Arabidopsis* and *rlp23* T-DNA insertion mutants (Figure 3, 5, 8 and 9), stable overexpression of RLP23 in potato resulted in significantly enhanced resistance against *Phytophthora infestans* and *Sclerotinia sclerotiorum*, both of which express *NLPs* (Albert et al., 2015). Presumably, *H. arabidopsidis* effector proteins are able to effectively suppress NTI as *Arabidopsis* is highly susceptible to this pathogen. *P. infestans* and *S. sclerotiorum* have evolved without the need to circumvent NTI and are impeded in their ability to

infect potatoes that express *RLP23*. This further demonstrates that the transfer of PRRs could constitute an incredibly powerful tool that could be applied to breeding durable resistance in crops.

Materials and Methods

Peptide Synthesis

Synthetic peptides were ordered at Genscript. nlp peptides were prepared as 10 mM stock in 100% DMSO. flg22 was dissolved in Milli-Q as a 1 mM stock solution.

Plant Growth Conditions

Plants were grown on potting soil (mix z2254, Primasta B.V., Asten, The Netherlands) at 21 °C, 75% relative humidity. Plants used for ethylene measurements and disease assays were grown under short day conditions (10 hours of light per day). *Arabidopsis* plants that were transformed using the floral dip method were grown under long day conditions (16 hours light per day). All seeds were stratified 3 days at 4 °C.

Ethylene Measurements

Ethylene accumulation induced by nlp peptides and flg22 was determined as previously described (Oome et al., 2014).

Generation of Transgenic Lines

Seven week old *Arabidopsis rlp23-1* (SALK_034225; Alonso et al., 2003) were crossed with XVE:*NLP3* plants (Oome et al., 2014). In subsequent generations plants were tested for homozygosity for the *rlp23-1* T-DNA insertion by PCR and for the XVE:*NLP3* insertion by qPCR. The primers used are depicted in Table 3. Plant genomic DNA was isolated according to the Sucrose Prep protocol (Berendzen et al., 2005) for PCR, and with the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich) for qPCR following the manufacturer's instructions.

The coding sequences of β-glucuronidase (*GUS*) from *Escherichia coli* (Karimi et al., 2002) and *HaNLPs* from *H. arabidopsidis* isolate Emoy2 were previously cloned into a pENTRY™/D-TOPO® vector (Thermo Fisher Scientific; Oome et al., 2014). Fusion 4 of *HaNLP3* was used that has the *HaNLP3* signal peptide exchanged for the *PsojNIP* signal peptide which results in more efficient secretion of the protein when produced *in planta* (Cabral et al., 2012).

Subsequently, pENTRY™/D-TOPO® vectors were recombined into the binary vector pFAST-G02 (Shimada et al., 2010). Resulting pFAST-G02 plasmids were transformed into *A. tumefaciens* GV3101 by electroporation. *A. tumefaciens* bacteria were grown on lysogeny broth (LB) agar plates supplemented with appropriate antibiotics. Bacteria were then resuspended in 30 ml LB, and mixed with 120 ml 50 g sucrose/l and 500 µl Silwet/l. *Arabidopsis rlp23-2* (GABI-Kat:738D01; Kleinboelting et al., 2012) floral tissues were dipped in *A. tumefaciens* suspension thrice per plant, with at least one day in between each dip. The first 24 hours past transfection, plants were kept at 100% relative humidity (Clough and Bent, 1998). Transgenic seeds were selected by fluorescence microscopy, and plants were genotyped by PCR. Stable T3 transformants which were homozygous for *rlp23-2* and the overexpression insertion were used in bioassays.

Transient Expression Assays

The *RLP23* coding sequence was amplified by PCR from *Arabidopsis* cDNA (primers in Table 3) and cloned into a pCR™8 TOPO® vector (Thermo Fisher Scientific) and recombined to pB7FWG2 (Karimi et al., 2002), a Gateway® vector with a GFP for C-terminal fusions. The plasmid was transformed into *A. tumefaciens* GV3101 by electroporation. The control *A. tumefaciens* C58C1 with the p35S::*YFP* plasmid was described previously (Cabral et al., 2012). Both *A. tumefaciens* strains carrying the *RLP23* and *YFP* transgene were grown overnight in LB with appropriate antibiotics in a shaking incubator (28 °C, 220 rpm). Overnight cultures were pelleted by centrifugation for 15 minutes at 1500 x *g* and resuspended in 3 mL 10 mM $MgCl_2$, 10 mM MES pH 5.6 and 100 µM acetosyringone. The final OD_{600} was set at 1.0 and bacterial suspensions were incubated at room temperature for 4 hours. Subsequently, leaves of 4-5 week old *N. benthamiana* plants were infiltrated using a needleless syringe. Twenty-four hours after inoculation with *A. tumefaciens*, leaf pieces of 3 x 3 mm were cut and kept overnight in deionized water at room temperature. Leaf pieces were tested for nlp24-sensitivity as described previously (Oome et al., 2014).

TABLE 3 | Primers used in this study.

Name	Forward	Reverse
pENTR	GTAAAACGACGGCCAG	CAGGAAACAGCTATGAC
pFAST	CAATCCCACTATCCTTCG	ATGCTCAACACATGAGCG
		TTTAAACTGAAGGCGGGAAA
GUS_RV		TGATAATCGGCTGATGCAGT
HaNLP1	CACCATGAGGACTGGCGCCTTC	CTCATTAAAAAGGCCAAGAAGCG
HaNLP2	CACCATGAAGACCAGTGCCTTC	TCAATAGTCATTGTCCTCGAC
HaNLP4	CACCATGAAGGCCAGCGCATTCCTG	TTAACTGTCGTAGCTATCTTGGC
HaNLP5	CACCATGAGCTTCCGGGCTCTAGTC	TCAGAATGCCCATGCCCAGGC
HaNLP6	CACCATGAAGGCCAGCGCATTC	TCAATCTTGCCTCGCTTAACCT
HaNLP7	CACCATGAGGATAGGTAAGTCCTTGTGC	CTATCCAGCCATTTCGTAAGG
HaNLP8	CACCATGAAGACTTTGTCTTGCTTGTAT	TCACTTCAGCGGTGCAAAG
HaNLP9	CACCATGAAGACCGGTCTCTTCTTGTA	TCAGCCTTCAACAAAGTCGTA
HaNLP10	CACCATGAAGGCCGTCGCCTTGTTG	CTAGCTAGCTGCGCTCACAT
RLP23	ATGTCAAAGGCGCTTTTGCATTTGC	ACGCTTTCTGCGTTTATTCAGACC
***rlp23-1*_INT**	ACCTGACCCGGTTAACTAAGT	TGGGGAACTCGTTGATGTCA
***rlp23-1*_TDNA**	TGGTTCACGTAGTGGGCCATCG	CCAGTTCACAAAGTAGTTTGGTGG
***rlp23-2*_INT**	ATTTTACTGGTATTGTGGCCTGTC	GTAGCTGGTGCAACTCAAAGAG
***rlp23-2*_TDNA**		ATATTGACCATCATACTCATTGC
PsojNIP_SP_FW	CACCATGAACCTCCGCCCTGCA	
HaNLP3_RV		TGCTCCATCTTTTTTCGTTTTAAACGG
ACT_qPCR	TCTTCCGCTCTTTCTTTCCA	TCCTTCTGGTTCATCCCAAC
NLP3_qPCR	CCAGTGGTGGTCTCCAAACT	AGCCCAGACGTCATTGTACC
M13	GTAAAACGACGGCCAG	CAGGAAACAGCTATGAC

Pathogenicity Assays

Downy mildew infection assays were performed with *H. arabidopsidis* isolate Noco2 (100 spores per µl) on adult plants and with isolate Waco9 (50 spores per µl) on seedlings. The downy mildew isolates were maintained on *Arabidopsis* Col-0 and transferred weekly to fresh 10-day old seedlings. Spores were collected from the highly susceptible Ws-*eds1* mutant to achieve the high level of inoculum used. To test local and systemic resistance, four leaves of each tested adult plant (4.5 weeks old) were infiltrated with 100 nM of nlp24 or mock (0.01% DMSO) one day before pathogen challenge. In the bioassays with *rlp23-1* XVE:*NLP3* seedlings, F4 plants were sprayed with 0.5 mM β-estradiol and 0.02% Silwet one day prior to inoculation with Waco9 to induce the expression of *NLP3*. The *H. arabidopsidis* spore suspension was applied with a spray gun and plants were subsequently left to dry to the air for ~ 30 min. Plants were incubated at 100% humidity at 16 °C with 10 hours of light per day. Spore counts in adult plants in the experiments with *ald1-1* were determined as follows. Six to eight days post inoculation the disease severity was assayed; for local

infection levels, the nlp24- or mock-treated leaves were cut and weighed and suspended in a known volume of water after which the number of spores per milligram of plant tissue was determined. For systemic disease severity, four untreated leaves were picked and infection levels were determined in the same way. Spore counts on seedlings and number of conidiophores per leaf for adult plants were determined as described by Oome et al., 2014. Systemic infection in *rlp23*, *sobir1*, *ein2-1*, *sid2-1* and *npr1-1* lines was scored by checking for signs of sporulation in untreated, systemic tissue.

B. cinerea strain B05.10 was grown, inoculated (100 conidia per µl) and scored as previously described (Van Wees et al., 2013).

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Chapter 4

Genetic dissection of NLP-triggered immunity in *Arabidopsis*

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Abstract

Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) form a superfamily of proteins secreted by many plant-associated bacteria, fungi and oomycetes. Previously, we showed that both cytotoxic and non-cytotoxic NLPs act as molecular patterns that trigger immunity in *Arabidopsis thaliana*. Specifically, a 24-amino-acid fragment, nlp24, derived from a conserved region of fungal, bacterial and oomycete type 1 NLPs induces defense in *Arabidopsis*. To elucidate the mechanism of NLP-triggered immunity (NTI), we performed a forward genetic screen in *Arabidopsis*. Here, we present our data on the genetic dissection of NTI, using a transgenic *Arabidopsis* line expressing the *NLP3* gene of the downy mildew *Hyaloperonospora arabidopsidis*, encoding a non-cytotoxic protein, from an estradiol-responsive promoter (XVE:*NLP3*). When treated with estradiol, immunity is strongly activated in XVE:*NLP3* plants, which is associated with severely stunted growth. M2 plants of an EMS-mutagenized XVE:*NLP3* population were screened for loss of NLP-triggered growth reduction. We selected 26 putative mutants that are nlp24-insensitive, and that, upon estradiol treatment, developed normally and remained susceptible to *H. arabidopsidis*. Among the obtained *decreased NTI* (*dni*) mutants we found 6 novel *rlp23* and 5 novel *sobir1* mutant alleles that affect the bimolecular NLP receptor. Four *dni* mutants, from 2 M1 pools, had wildtype *RLP23* and *SOBIR1* genes but were still impaired in NTI. Whole genome sequencing identified, on average, 101 genes with missense or nonsense mutations in these mutants. The genome sequences of the *dni* mutants originating from the same pool, share a large part of their EMS-induced mutations, and are likely to be allelic, having the same causal mutation responsible for the *dni* phenotype. Future research will unveil the identity of these *DNI* genes that are required for nlp24 perception and could play a role in general RLP-mediated immunity.

Introduction

Immune responses in plants generally start by receptor-mediated detection of nonself molecules that are found among different classes of microbes (Cook et al., 2015). Receptor-like proteins (RLPs) and receptor-like kinases (RLKs) trigger immunity upon recognition of an extracellular pattern (Boutrot and Zipfel, 2017). Initial pattern-induced responses include ion fluxes, a reactive oxygen species (ROS) burst, activation of mitogen-activated protein kinases (MAPKs) and the production of the phytohormone ethylene (Boller and Felix, 2009). Since the discovery of the first pattern recognition receptor (PRR), the RLK FLAGELLIN-SENSING 2 (FLS2), which detects flg22, the 22-amino acid epitope of bacterial flagellin, many other PRRs have been identified (Felix et al., 1999; Gómez-Gómez and Boller, 2000; Boutrot and Zipfel, 2017). For example, a 18-amino acid peptide, elf18, derived from bacterial elongation factor Tu (EF-Tu), is recognized by the RLK EF-TU RECEPTOR (ELR), and fungal chitin is perceived by LysM-CONTAINING RECEPTOR-LIKE KINASE 5 (LYK5) in *Arabidopsis thaliana* (Shibuya and Minami, 2001; Kunze et al., 2004; Zipfel et al., 2006; Cao et al., 2014). Upon recognition of their respective ligands, EFR and FLS2 associate with co-receptors of the SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) family, such as BRASSINOSTEROID INSENSITIVE 1 (BRI1)-ASSOCIATED RECEPTOR KINASE 1 (BAK1; SERK3) and BAK1-LIKE1 (BKK1; SERK4), whereas LYK5 forms a complex with CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) after chitin treatment (Chinchilla et al., 2007; Heese et al., 2007; Roux et al., 2011; Cao et al., 2014).

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PRRs of the RLP family lack an intracellular signaling domain and associate with SUPPRESSOR OF BAK1-INTERACTING RECEPTOR-LIKE KINASE 1 (BIR1) 1 (SOBIR1)-type RLKs to form a functional bipartite receptor kinase (Liebrand et al., 2013; Gust and Felix, 2014). Interestingly, when a pattern is detected by an RLP this leads to recruitment of BAK1, which in turn leads to activation of immunity (Albert et al., 2015; Postma et al., 2016). It seems, therefore, that RLK- and RLP-mediated responses converge early in defense signaling pathways. Nevertheless, there are distinct differences even between EFR- and FLS2-mediated immunity, which suggests that immune signaling diverges directly downstream of the PRR complex (Couto and Zipfel, 2016). For example, the receptor-like cytoplasmic kinase (RLCK), BRASSINOSTEROID-SIGNALING KINASE 1 (BSK1) associates with FLS2 and is required for flg22-induced but not for elf18-induced ROS production (Shi et al., 2013). In contrast, three genes that are potentially involved in endoplasmic reticulum quality control, are required for elf18-triggered immunity, but not for flg22-triggered immunity (Li et al., 2009).

Recently, Nep1-like proteins (NLPs) were described as molecular patterns that triggers immunity in *Arabidopsis thaliana* (Böhm et al., 2014; Oome et al., 2014). A conserved, 24-amino acid peptide derived from NLP3 of the downy mildew pathogen *Hyaloperonospora arabidopsidis* was found to be a potent elicitor of immunity. Also, nlp24

peptides from fungal and bacterial origin are able to trigger immunity (Oome et al., 2014). The nlp PRR, RLP23, was identified and it was shown to interact with SOBIR1 and to recruit BAK1 in presence of the NLP ligand (Albert et al., 2015). However, little is known about how the RLP23-SOBIR1-BAK1 complex activates NTI.

Here, we describe a forward genetic screen for *decreased NTI* (*dni*) mutants. Among the identified *dni* mutants we found novel *rlp23* and *sobir1* alleles that could aid in understanding the nlp24-RLP23-SOBIR1 interaction better, and help in dissecting early nlp24-induced signaling. Finally, 4 *dni* mutants were selected that had no mutations in *RLP23* and *SOBIR1*, were nlp24-insensitive, and showed no increased resistance to *H. arabidopsidis* after induction of the *NLP3* transgene. We genome sequenced these *dni* mutants that, ultimately, may illuminate NTI signaling components hitherto unknown.

Results and Discussion

We commenced a forward genetic screen in the *Arabidopsis* Col-0 XVE:*NLP3* background to identify novel *DNI* genes. In the XVE:*NLP3* line, treatment with estradiol leads to activation of the XVE construct (Zuo et al., 2000), resulting in expression of the *NLP3* transgene and subsequent secretion of NLP3. After 2 weeks of treatment with estradiol (reapplied every 2 days) this resulted in a reduced growth phenotype (Figure 1), caused by the activation of immunity (Oome et al., 2014). In this genetic screen, we initially screened for mutants with a restored growth phenotype after estradiol treatment, and further selection steps were performed to home in on a final selection of *dni* mutants. The screen was divided into 5 phases (Figure 2), which will be further discussed below.

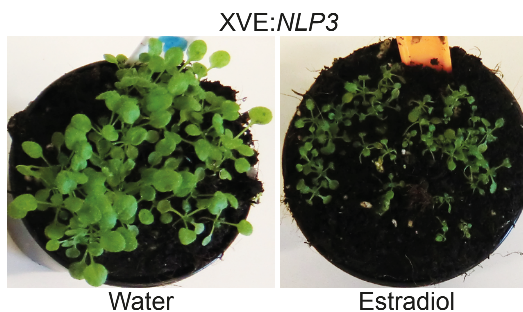

FIGURE 1 | *Arabidopsis* plants expressing *NLP3* show a severe growth reduction. Transgenic *Arabidopsis* XVE:*NLP3* plants were treated with 0.5 mM estradiol to activate the estradiol-responsive promoter or water as a control. After 2 weeks of estradiol treatment (reapplied every other day), *NLP3*-expressing plants show a strong growth reduction associated with the activation of immunity (Oome et al., 2014). In this genetic screen, M2 plants that developed like water-treated plants after estradiol treatment were selected. Adapted from Oome et al., 2014.

Phase I & 2: Selection of *dni* Mutants

Homozygous XVE:*NLP3* seeds (M0 parental generation) were treated with the mutagen ethyl methanesulfonate (EMS). The vast majority of mutations induced by EMS will result in cytosine/guanine to thymine/adenine transitions (Kim et al., 2006). Seeds of M1 plants were pooled in 390 batches derived from 16 M1 plants each (an overview of the *dni* screen is given in Figure 2). Seedlings of the M2 pools were treated with 0.5 mM estradiol for 2 weeks (reapplied every two days) and plants that developed normally, i.e. having a similar growth phenotype as the XVE:*YFP* control, were selected. Most M2 plants showed stunted growth, like the XVE:*NLP3* parental line, and were discarded. In total, 737 putative *dni* mutants were selected from 205 M2 pools (a cut-off of 10 plants maximum per pool was maintained). Because of an unfortunate minor contamination of the M0 seeds, all selected plants were checked for the presence of the *NLP3* transgene by PCR. Of the 737 selected plants, 127 did not carry the *NLP* transgene, leaving us with 610 plants selected from 183 pools. For a small subset of 124 plants, we tested whether *NLP3* was properly expressed after estradiol treatment, 38 did not express the transgene and were not taken into further consideration. We decided not to check *NLP* expression in all putative mutants, because a more efficient selection methods could be applied in following generations. Therefore, 572 M2 plants were left to set seed, of which 481 produced sufficient seeds to test in the M3 generation.

Phase 3: Selection of nlp24-insensitive and *H. arabidopsidis* Susceptible Mutants

In the previous phases, we could not rule out that mutants had restored growth because of a defective XVE:*NLP3* construct, e.g. as a result of decreased *NLP3* expression or a mutation in the nlp24 epitope that would result in a less potent molecular pattern. To mitigate this, M3 families were tested for nlp24-sensitivity by measuring ethylene accumulation in response to exogenously applied nlp24 peptide. Leaf pieces of M3 mutants, XVE:*NLP3* and *rlp23-1* (a T-DNA insertion mutant of the NLP PRR) plants were treated with 1 µM nlp24 or 0.1% DMSO (mock) and the levels of ethylene produced were determined by gas chromatography. Interestingly, out of 300 tested *M3* plants, from 112 pools, 243 (from 99 pools) showed a comparable increase in nlp24-induced ethylene production as the XVE:*NLP3* parental line. Results from 13 M3 plants were inconclusive, and only 44 putative mutants (from 22 pools) showed no nlp24-induced ethylene production, akin to the *rlp23-1* and mock-treated plants. Even though most M3s were nlp24-responsive, it does not necessarily mean that they are not of interest. One can envisage that NLPs need to be modified to expose the nlp24 pattern, e.g. to be unfolded or proteolytically cleaved. Supporting this idea was the finding that recombinant heat-denatured NLP3 protein induced higher ethylene production in *Arabidopsis* than non-denatured NLP3 (Oome et al., 2014). Mutations in NLP-modifying genes, e.g., secreted proteases, will be missed in our current screening setup. However, these mutants could be revisited at a later stage.

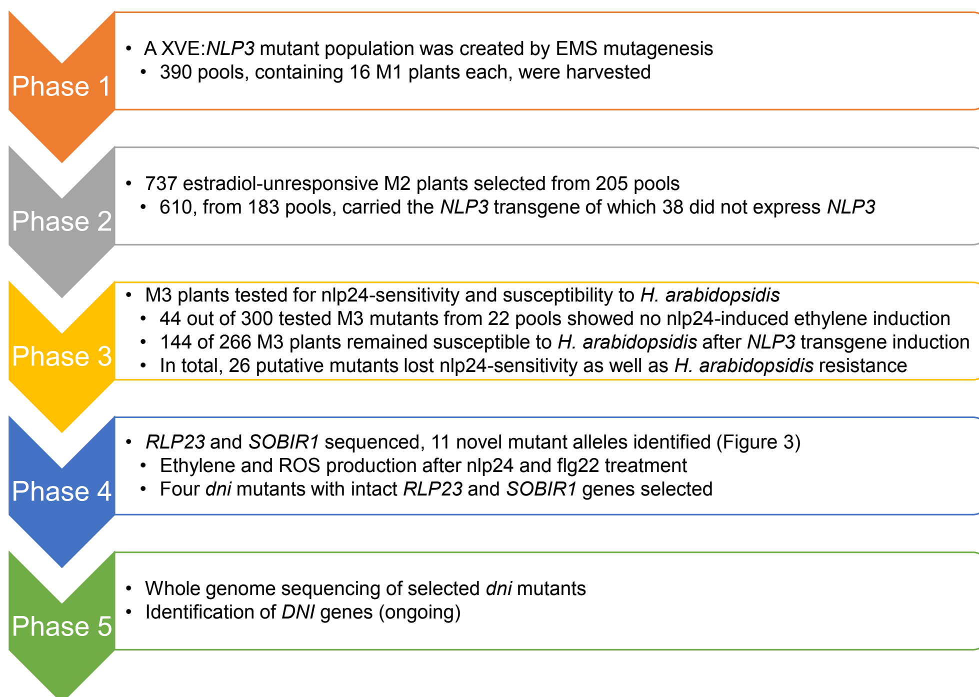

FIGURE 2. Overview of the forward genetic screen for *dni* mutants. Expression of *NLP3* in *Arabidopsis* activates immunity leading to resistance to *H. arabidopsidis* (Oome et al., 2014). To determine whether the selected mutants are impaired in NTI against *H. arabidopsidis*, M3 seedlings were treated with estradiol and the next day inoculated with *H. arabidopsidis* isolate Waco9. After 6-8 days we determined the level of sporulation of *H. arabidopsidis*. Remarkably, of the 266 M3 plants from 116 pools tested, 144 (from 72 pools) were as susceptible as the water-pretreated XVE:*NLP3* control, and are thus suggested to be impaired in NTI. Ninety-seven M3 plants (from 59 pools) demonstrated high levels of resistance to *H. arabidopsidis* comparable to the estradiol-pretreated XVE:*NLP3* parental line. For 25 mutant lines (from 23 pools) the results were inconclusive: disease tests were too variable to clearly distinguish a *dni* phenotype. Finally, we focused on the M3 mutants that were insensitive to exogenously applied nlp24 peptide, as well as susceptible to *H. arabidopsidis* after estradiol-induced *NLP3* expression. These 26 *dni* mutants, originating from 15 pools, were further analyzed in phase 4 (Figure 2).

Phase 4: Novel *rlp23* and *sobirl* Mutants

As *RLP23* and *SOBIR1* are both required for NTI, mutations in these genes are to be expected in a selection of *dni* mutants (Albert et al., 2015). To check for mutations in these genes, DNA was extracted from at least one mutant per pool (20 of the 26 remaining mutants from 15 pools) and *SOBIR1* and *RLP23* were sequenced (see Table S1 for an overview). Indeed, we found 7 *dni* plants from 7 pools that had a novel nonsynonymous mutation in *RLP23*, six of which were unique (Figure 3). Two independent mutants, 226-5 and 348-4, contained an identical mutant allele named *rlp23-3* with a nonsense mutation (W67STOP), leading to a premature stop codon in the N-terminal domain of RLP23, resulting in a nonfunctional protein. Mutant 226-1 originates from the same pool as 226-5, but we have not verified whether the *dni* phenotype observed in this mutant

is caused by the same mutation. Another premature stop codon was found in *rlp23-5* (line 268-1), W651STOP, in the island domain (ID) of RLP23. This truncated protein does not contain a transmembrane domain and therefore cannot anchor into the plasma membrane, rendering it non-functional.

Four missense mutations were identified, two in the leucine-rich repeats (LRRs) of the extracellular domain, *rlp23-4* (230-1) resulting in an aspartic acid to asparagine (D590N) substitution in LRR20 and *rlp23-6* (343-4) giving a glycine to arginine substitution in LRR25 (G762R; Figure 3). LRRs recognize protein motifs (Kobe and Kajava, 2001), and LRR20 and LRR25 may play a role in binding of the nlp24 pattern or are required for the interaction with SOBIR1. The bacterial pattern flg22 binds to 14 LRRs (LRR3 to LRR16) of FLS2 (Sun et al., 2013). The ID of FLS2 lies between LRR16 and LRR17 (Gómez-Gómez and Boller, 2000), and if binding of the nlp pattern occurs before the ID, the mutation in LRR20 of RLP23, which is located before the ID, could be detrimental to the interaction with the nlp24 ligand (Figure 3). The LRR25 region, in which the second mutation was identified, is located after the ID, and shares high sequence similarity with other RLPs and SOBIR1 and is probably part of the interaction interface between SOBIR1 and RLP23 (Gust and Felix, 2014). The tomato RLP Ve1, for example, only requires the 7 most C-terminal of its LRRs to interact with SOBIR1; a mutant lacking the first 30 out of 37 LRRs still co-immunoprecipitated with tomato SOBIR1 (Fradin et al., 2014).

We made an alignment of all *Arabidopsis* RLPs (*At*RLPs), to see if these mutated residues are conserved (Figure 4). Fifty-seven *At*RLPs were previously described (Wang et al., 2008), however, in a recent complete reannotation of the *Arabidopsis* Col-0 genome, Araport11, RLP8 is considered obsolete and RLP18 and RLP49 are now considered pseudogenes of RLP53 and RLP47, respectively (Cheng et al., 2017), making the total number of *At*RLPs analyzed here 54. Interestingly, the aspartic acid corresponding to position 590 of RLP23 and the glycine at position 762 are highly conserved, supporting the hypothesis that these residues are important for RLP23, or RLP function in general (Figure 4).

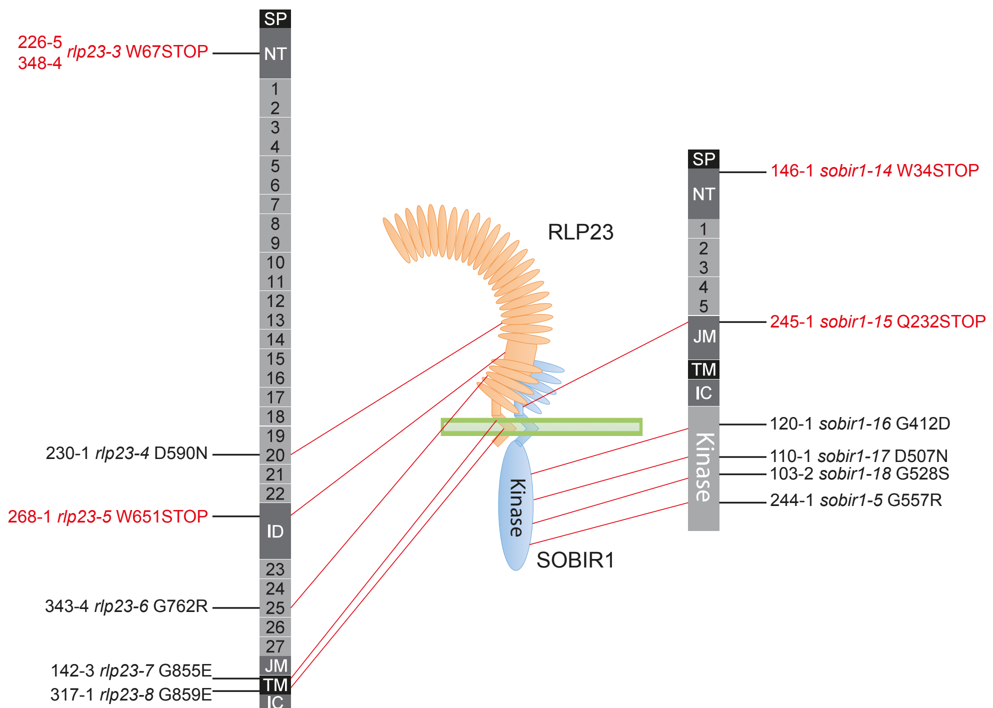

FIGURE 3 | Novel mutant alleles of RLP23 and SOBIR1 identified in the *dni* screen. Missense mutations are shown in black, nonsense mutations in red. SP: signal peptide; NT: N-terminal domain; ID: island domain; JM: juxtamembrane domain; TM: transmembrane domain; IC: intracellular domain; numbers depict leucine-rich repeat domains. If several *dni* mutants originating from the same pool with the same mutation in either *RLP23* or *SOBIR1* were identified, only one mutant is depicted in the figure. Adapted from Albert et al., 2015 & Shibuya and Desaki, 2015.

The two remaining mutations, *rlp23-7* (142-3) and *rlp23-8* (317-1), both lead to a glycine to glutamic acid substitution at position 855 and 859, respectively (G855E and G859E; Figure 3). Glutamic acid is negatively charged and may have a large impact in the protein structure. These substituted amino acids are located in the transmembrane domain of RLP23 and especially the glycine at position 859 is highly conserved (Figure 4). Transmembrane domains (TMs) often contain one or multiple GxxxG motifs, that are important for helix-helix interactions in the plasma membrane (Gust and Felix, 2014). RLP23 has a GxxxGxxxG motif, with the glycine residues corresponding to positions 855, 859 and 863. The substitutions at amino acids 855 and 859 are possibly responsible for a disruption of these helix-helix interactions and could impede RLP23 functioning. Indeed, analysis of the mutant proteins with TMHMM 2.0, a tool that predicts TMs, showed that these amino acid changes are detrimental to its function; no TMs were predicted when glycine was exchanged with glutamic acid at position 855 or 859, as opposed to one predicted transmembrane domain in wild-type RLP23 (Krogh et al., 2001).

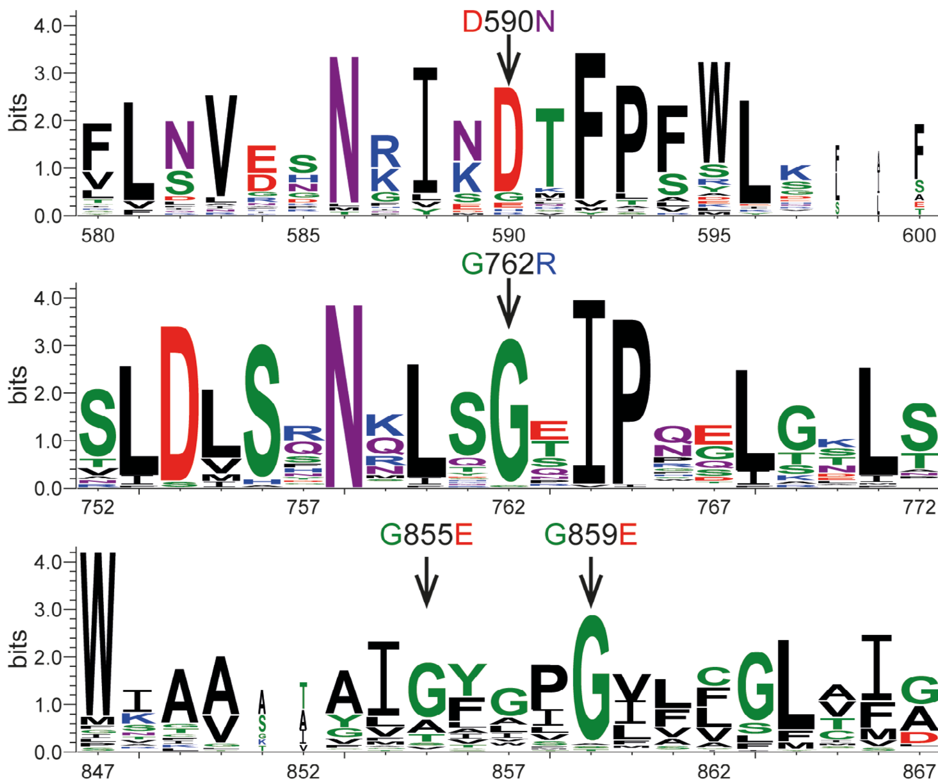

FIGURE 4 | Protein alignment of 54 *Arabidopsis* RLP sequences shows the relative conservation level of mutated residues. Sequences of *Arabidopsis* RLPs previously described were selected (Wang et al., 2008), with some exceptions. RLP18 and RLP49 are considered pseudogenes of RLP53 and RLP47, respectively, in the recent Araport11 genome reannotation. Furthermore, RLP8 is considered obsolete, making the total number of proteins used in this analysis 54 (Cheng et al., 2017). All 54 protein sequences were aligned using Clustal Omega (Sievers et al., 2011). Amino acids were categorized according to their chemical characteristics (Lewin, 1994). Mutated residues are indicated by arrows. Polar residues are depicted in green (glycine [G], serine [S], threonine [T], tyrosine [Y] and cysteine [C]), neutral residues in purple (glutamine [Q] and asparagine [N]), basic residues in blue (lysine [K], arginine [R] and histidine [H]), acidic amino acids in red (aspartic acid [D] and glutamic acid [E]) and hydrophobic amino acids in black (alanine [A], valine [V], leucine [L], isoleucine [I], proline [P], tryptophan [W], phenylalanine [F] and methionine [M]).

Next to the novel *rlp23* mutants, we also identified 6 *sobir1* mutant alleles in 9 *dni* mutants from 6 pools (Figure 3, table S1). Two nonsense mutations were found, *sobir1-14* (146-1; W34STOP) in the beginning of the coding sequence resulting in a premature stop codon in the N-terminal domain, just after the signal peptide, and a second *sobir1-15* (245-1, 245-6; Q232STOP) leading to a premature stop in the juxtamembrane domain. As these mutants produce a protein without transmembrane and kinase domain both mutations are considered null alleles. Four missense mutations were identified in the kinase domain of SOBIR1. *sobir1-5* (244-1), giving a glycine to arginine substitution at position 557

(G557R), was already described previously (Gao et al., 2009). It was identified in a forward genetic screen for suppressor of *bir1-1* mutants in the *bir1-1 pad4-1* background. Knocking out *BIR1* leads to extensive cell death and constitutive activation of immunity. *sobir1-1* (R162STOP) was the strongest suppressor of the *bir1-1* phenotype and analysis of F1 plants of a cross between *sobir1-1 bir1-1 pad4-1* and *sobir1-5 bir1-1 pad4-1* demonstrated that *sobir1-5* is allelic to *sobir1-1* (Gao et al., 2009).

Newly identified protein alleles have a glycine to aspartic acid substitution at position 412 (in 120-1 [*sobir1-16*]; G412D), an aspartic acid to asparagine substitution at position 507 (in 110-1, 110-3, 110-7 [*sobir1-17*]; D507N) and a glycine to serine substitution at position 528 (in 103-2 [*sobir1-18*]; G528S). To determine if these mutant residues are conserved in SOBIR1 orthologous proteins, a sequence alignment was made from the ORTHO04D004202 sub-family (Figure 5), which was collected from Dicots PLAZA 4.0 (Van Bel et al., 2017). This sub-family contains 94 SOBIR1 orthologs from 52 species (see Table S2 for an exhaustive description of the species and gene identifiers). All three amino acid positions in these novel alleles are highly conserved among SOBIR1 orthologs, and thus these residues are probably important for SOBIR1 kinase activity. Five *dni* mutants were not yet checked for their putative mutations in *SOBIR1*, because they originated from the same pool, i.e. 245-3, 245-4, 245-7, 245-8, 245-9 (Table S1). Verification by sequencing of *SOBIR1* in these mutants is ongoing.

Taken together, it is highly likely that these mutations in *RLP23* and *SOBIR1* cause the *dni* phenotype observed here, although, they still need to be confirmed. Crosses to *rlp23-1*, in the case of a mutation in *RLP23*, and *sobir1-12*, for *sobir1* lines, have been made and will be checked in the F1 generation for nlp24-sensitivity to test for allelism. If the mutations are causal, the F1 progeny should be insensitive to the nlp24 peptide, and unaffected in growth after estradiol-induced expression of *NLP3*.

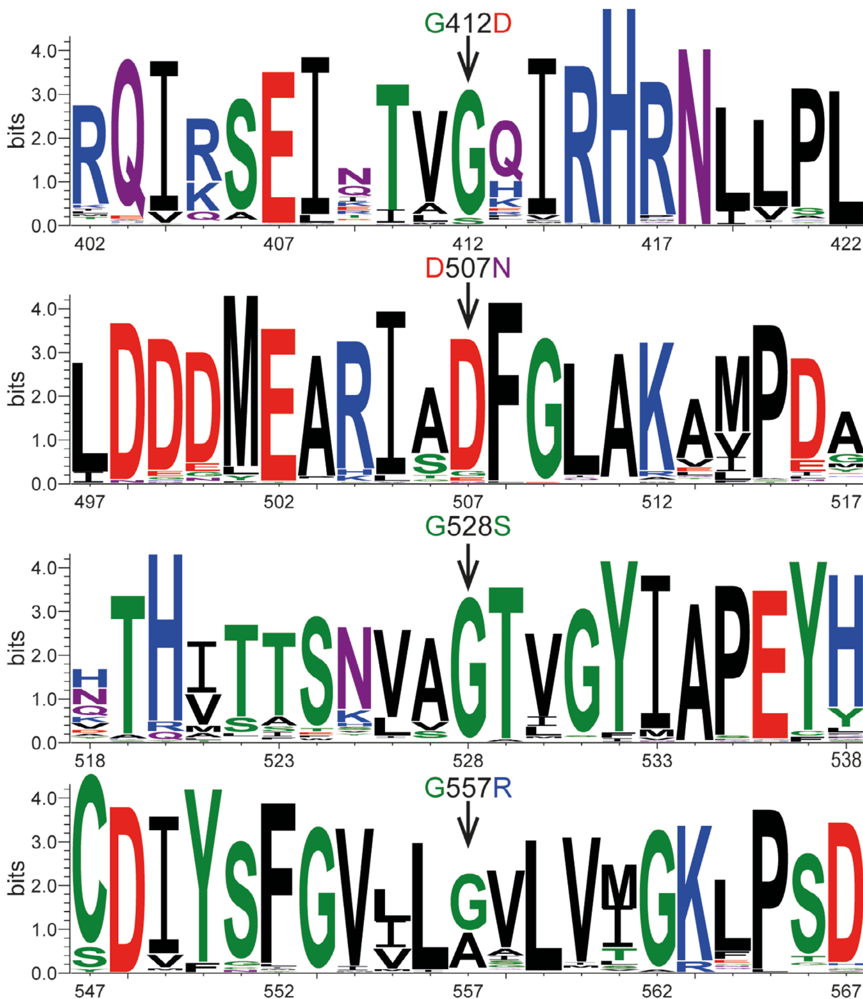

FIGURE 5 | Protein alignment of SOBIR1 orthologs reveals conserved residues in novel *sobir1* mutants. SOBIR1 sequences from the ORTHO04D004202 sub-family were acquired from Dicots PLAZA 4.0 (Van Bel et al., 2017) and aligned using Clustal Omega (Sievers et al., 2011). Amino acids were categorized according to their chemistry (Lewin, 1994). Mutated residues are indicated by arrows. Polar residues are depicted in green (G, S, T, Y and C), neutral residues in purple (Q and N), basic residues in blue (K, R and H), acidic amino acids in red (D and E) and hydrophobic amino acids in black (A, V, L, I, P, W, F and M).

The Final Stretch: Identification of Putative *DNI* Genes

Four *dni* mutants originating from 2 pools remained without a mutation in *RLP23* or *SOBIR1*: *dni1* and *dni2* from pool 335, and *dni3* and *dni4* from pool 354. We rechecked the absence of nlp24-induced ethylene induction in these mutants and tested the responsiveness to two other patterns, flg22 and BcPG3 (Figure 6 and Table 1), to see if these *dni* mutants are only impaired in NTI or that pattern-triggered immunity in general is decreased. The *Botrytis*

cinerea endopolygalacturonase BcPG3 is a potent trigger of immunity in *Arabidopsis* and its recognition is mediated by RLP42 (Zhang et al., 2014). The epitope of bacterial flagellin, flg22 is perceived by the FLS2 and this subsequently leads to the activation of immunity (Gómez-Gómez and Boller, 2000).

All patterns induced a significant defense response in the XVE:*NLP3* parental line (*P* ≤ 0.001) compared to mock (0.01% DMSO; Figure 6). No significant ethylene induction in response to the 3 tested patterns was observed in the *dni1* mutant. Similarly, *dni2* showed no significant increase in ethylene production in response to nlp24 and BcPG3, however, a small, albeit significant (P ≤ 0.05), response to flg22 was found when compared to mock. The negative controls for nlp24, *rlp23-1* and *sobir1-13* showed no nlp24-induced ethylene production, whereas the pattern flg22 that relies on the RLK FLS2 triggered a response similar to XVE:*NLP3*. BcPG3, whose perception is mediated by RLP42 and requires SOBIR1, failed to elicit a response in *sobir1-13* (Figure 6).

The phenotype of *dni3* and *dni4* was less clear, an intermediate, not significant, ethylene induction was observed in response to nlp24 and a high increase of ethylene production after flg22 treatment, comparable to the XVE:*NLP3* parental line. BcPG3 triggered no increase ethylene production in *dni4*, a BcPG3-induced response of *dni3* has not been tested yet (Table 1).

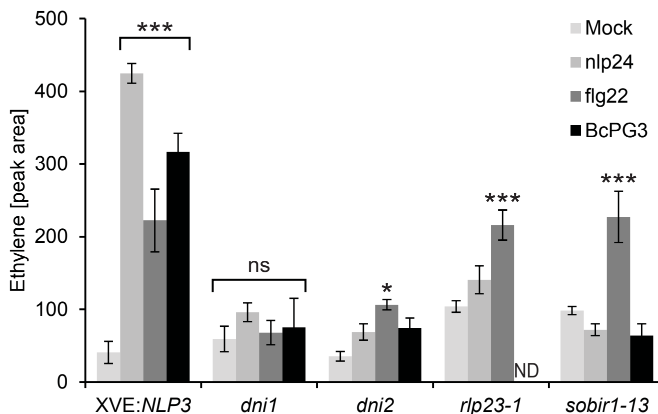

FIGURE 6 | *dni1* and *dni2* show no increased ethylene production after treatment with the patterns nlp24 and BcPG3. *Arabidopsis* XVE:*NLP3*, *dni1* (335-7), *dni2* (335-9), *rlp23-1* and *sobir1-13* leaf pieces were treated with 1 µM peptide (nlp24 and flg22) or protein (BcPG3) solution. After 4 hours ethylene accumulation was determined by gas chromatography. Error bars show standard deviation. Asterisks indicate a significant difference between the mock-treated and pattern-treated leaf pieces. (one-way ANOVA, Tukey HSD post hoc test; *$P \leq 0.05$; ***$P \leq 0.001$; ns = not significant). ND = not determined.

Next to ethylene, we determined the oxidative burst in response to the patterns nlp24 and flg22, to have another readout for NTI, next to growth phenotype and *H. arabidopsidis* resistance (Figure 7, Table 1). ROS production is a very early response and is activated through a different pathway than ethylene, i.e. their paths diverge early after pattern recognition (Boller and Felix, 2009; Zipfel and Robatzek, 2010). Two *dni1* plants were tested for nlp24-induced ROS production, *dni1* plant #1 had high ROS production after nlp24 treatment, even higher than the XVE:*NLP3* parental line. In contrast, in response to nlp24, *dni1* plant #2 showed a small increase in ROS production, similar to that of *rlp23-1* (Figure 7*A*). A similar pattern was observed for *dni4*, one plant had a strong ROS burst in response to nlp24, whereas the other tested plant did not (data not shown, summarized in Table 1). More repeats are needed to conclude whether these lines show an nlp24-induced ROS burst or not. For example, there could have been a mix-up that caused these, unexpected, ROS bursts in *dni1* and *dni4*. Mutants *dni2* and *dni3* showed a much weaker ROS burst than XVE:*NLP3* after nlp24 treatment, comparable to *rlp23-1* (Figure 7*A*; Table 1). Strikingly, an increased ROS production was observed in response to flg22 in all *dni* mutants, but not in the *fls2* control (Figure 7*B*; Table 1). This is in contrast to the flg22-induced ethylene production in *dni1* and 2, where no significant, or only a weak induction of ethylene was found (Figure 6). Presumably, the *DNI* genes responsible for the *dni1* and *dni2* phenotypes function downstream of or in parallel with ROS, but are still indispensable for NTI.

Interestingly, the ROS burst in response to nlp24 is longer lasting and it peaks later in comparison to flg22 (Figure 7). This was observed previously for nlp24, and for a pattern derived from the parasitic plant *Cuscuta reflexa* (Albert et al., 2015; Hegenauer et al., 2016; Albert and Fürst, 2017). Recognition of both patterns is mediated by RLPs, so there may be a temporal difference and a difference in amount of ROS-produced between RLP- and RLK-mediated immunity.

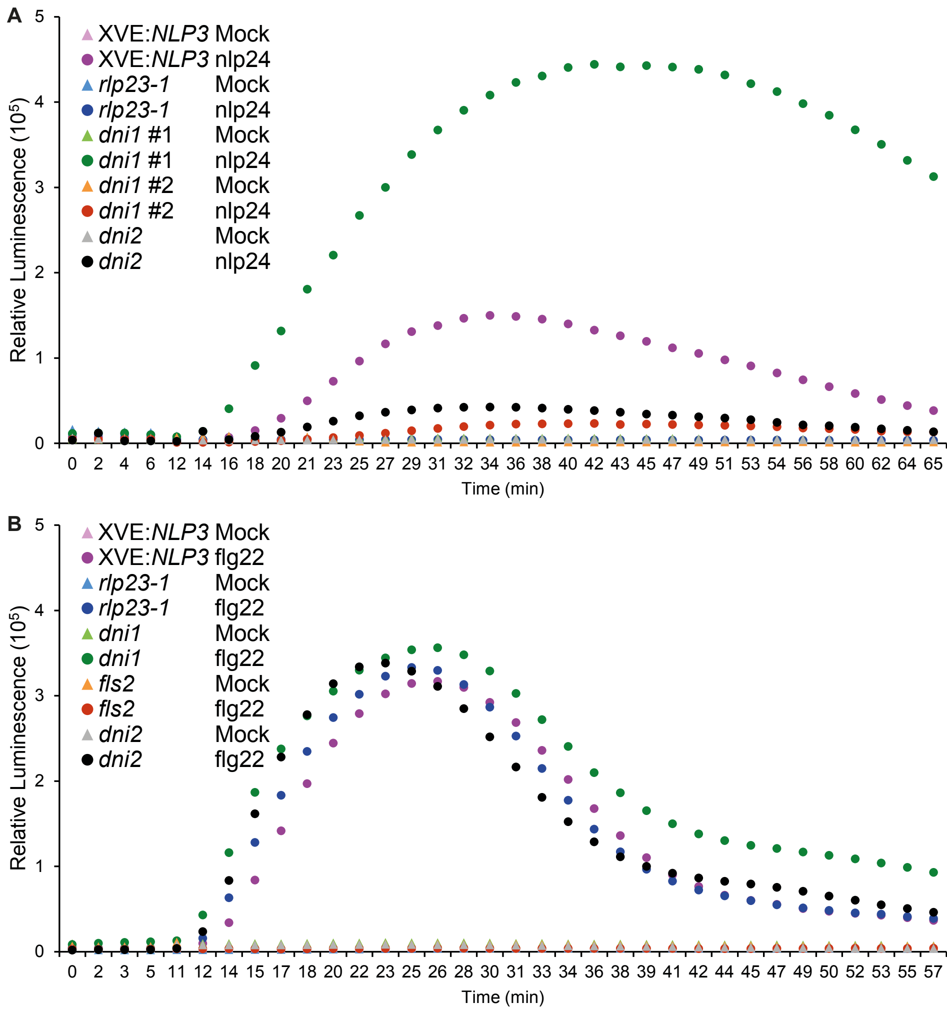

FIGURE 7 | *dni1* and *dni2* are not impaired in flg22-induced ROS production but seem to be impaired in nlp24-triggered ROS induction. Leaf pieces of *dni1*, *dni2*, *rlp23-1* and *fls2* were treated with nlp24 (A) or flg22 (B) and the luminescence was measured for approximately one hour as a proxy of ROS production.

TABLE 1 | Selected *dni* mutant phenotypes compared to the XVE:*NLP3* parental line. I = inconclusive; +/- = intermediate responsive; - = not responsive to nlp24, susceptible to *H. arabidopsidis* after induction of the *NLP3* transgene by estradiol, or no mutations in *RLP23* and *SOBIR1* coding sequence; ND = not determined. The mutant numbers before the hyphen refer to the pool in which they were found.

	Ethylene			ROS		*H. arabidopsidis* resistance	*RLP23/SOBIR1* affected?
	nlp24	flg22	BcPG3	nlp24	flg22		
XVE:*NLP3*	+	+	+	+	+	+	-
dni1 (335-7)	-	-	-	I	+	-	-
dni2 (335-9)	-	+/-	-	-	+	-	-
dni3 (354-1)	+/-	+	ND	-	+	-	-
dni4 (354-2)	+/-	+	-	I	+	-	-
rlp23-1	-	+	ND	-	+	-	+
sobir1-13	-	+	-	ND	ND	-	+

Based on the presented data (Figure 6 and 7, Table 1), we consider these *dni* mutants promising as they could reveal novel *DNI* genes, and help us to gain a better understanding of NTI. Genomic DNA was isolated from *dni1*, *dni2*, *dni3* and *dni4* (all in the M4 generation) and XVE:*NLP3* (M0) plants and subsequently sequenced using the Illumina NextSeq 500 platform to a depth of about 30x coverage. The sequences of the *dni* lines were compared to the M0 parent (XVE:*NLP3*) using the needle in the *k*-stack (NIKS) algorithm to detect homozygous mutations (Nordström et al., 2013). Only the most common EMS mutations (G/C to A/T) were selected. Subsequently, the NIKS output was aligned to the *Arabidopsis* Col-0 TAIR10 genome assembly (Lamesch et al., 2012) to identify the exact location of the mutations. Next, we filtered out all mutations that were not located within protein coding sequences (CDS). Lastly, the total number of EMS mutations in CDS was called and divided into synonymous and nonsynonymous ones (missense and nonsense mutations; Table 2). Similar numbers of mutations in CDSs were found in all *dni* plants, ranging from 132 to 156 in total. Sixty-four to 68 percent of these mutations resulted in an amino acid change (89-106 missense mutations; Table 2). Finally, 3-8 mutations leading to a premature stop codon were identified in each of the 4 *dni* mutants.

TABLE 2 | Number and type of EMS mutations found in protein coding sequences of selected *dni* mutants. Whole genome sequence data of *dni* mutants was compared to the XVE:*NLP3* parental line with the NIKS algorithm. Comparable numbers of EMS mutations were found in all *dni* mutants, varying between 132 and 156. The mutant numbers before the hyphen refer to the pool in which they were found.

Mutant	Total # of EMS mutations in CDS	Missense mutations	Nonsense mutations
dni1 (335-7)	132	89	4
dni2 (335-9)	150	96	3
dni3 (354-1)	134	91	6
dni4 (354-2)	156	106	8

To test if the mutants from the same pool, *dni1/dni2*, and *dni3/dni4*, originating from pool 335 and 354, respectively, share the same M1 parent, we compared the identified mutations. Interestingly, there was a big overlap between *dni* mutants that came from the same pool (Figure 8). Mutants from pool 335, *dni1* and *dni2*, shared 54 mutations, approximately 58 and 54 percent of their respective mutations (Figure 8*A*). Similarly, *dni3* and *dni4* from pool 354, shared 56 mutations, about 58 and 49 percent of the mutations in these respective *dni* mutants (Figure 8*B*). The causal mutations responsible for the *dni* phenotype are likely confined to *DNI* gene(s) found in the overlapping regions of the Venn diagrams shown in figure 8. Crosses have been made between *dni* mutants from the same pool to verify if they are allelic and thus share the same causal mutation. Notably, no overlap was found between the 54 candidate genes from pool 335 and 56 candidate genes from pool 354, meaning that two different *DNI* genes are responsible for the observed phenotypes summarized in Table 1. Interestingly, none of the candidate *DNI* genes have previously been described to have a role in plant immunity (data not shown).

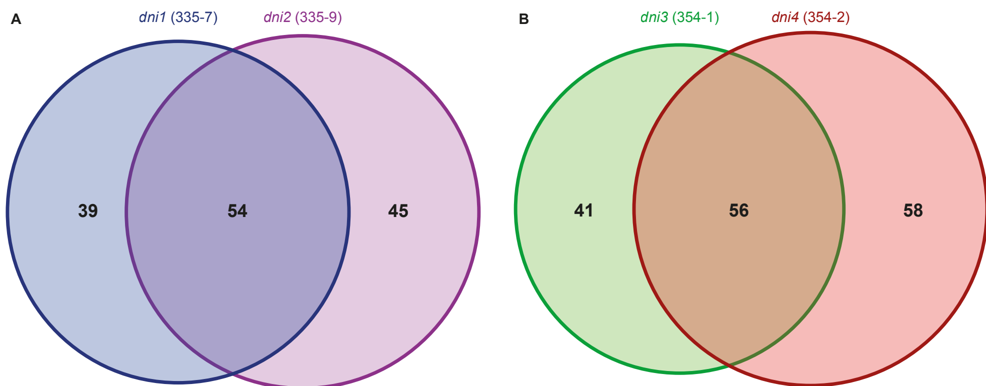

FIGURE 8 | Overlap between *dni1* and *dni2* and *dni3* and *dni4* reveal identical parental lineage. Similar mutations were found in *dni* mutants originating from the same pool. Numbers refer to the nonsynonymous substitutions found in these lines. Circles and overlap are proportional in size to the number of nonsynonymous mutations revealed by the NIKS pipeline.

Conclusions and Perspectives

In this study, we aimed to genetically dissect NTI in *Arabidopsis* by a forward genetic screen for NLP-insensitive mutants. We identified several *rlp23* and *sobir1* mutants unbeknownst to science that could help us to better understand the interactions that take place between the nlp24 ligand, RLP23, and SOBIR1. Furthermore, mutations in the SOBIR1 kinase domain could aid in dissecting early SOBIR1-mediated signaling, e.g. by measuring the kinase activity of the novel *sobir1* mutants. Finally, the candidate *DNI* genes that came forward through the genome sequencing of *dni1*, *dni2*, *dni3* and *dni4* could

yield new insight into early and late signaling events after NLP perception. Candidate genes could, e.g., encode for proteins that associate with the RLP23 receptor complex and subsequently activate the downstream signaling cascade. Also, mutations in downstream signaling components could be responsible for the phenotypes observed in the *dni* mutants. T-DNA mutants of candidate *DNI* genes can be tested for nlp24-responsiveness, e.g., by measuring ethylene and ROS production as well as *H. arabidopsidis* susceptibility after nlp24 treatment. If one of these mutants is confirmed to be less sensitive to nlp24, the respective *dni* mutants can be complemented to verify the role of the putative *DNI* gene in NTI. In this way we, ultimately, hope to gain a better understanding of signaling events that occur after nlp24 recognition.

Materials and Methods

Plant Materials and Growth Conditions

All plants were grown on potting soil (mix z2254, Primasta B.V.). After sowing, the seeds were stratified to break dormancy at 4 °C for 2-3 days in complete darkness. After the stratification period they were moved to a growth chamber at 21 °C, 75% relative humidity at long day conditions (16 hours light per day). The XVE:*NLP3* and XVE:*YFP* lines in *Arabidopsis* Col-0 background were generated and described previously (Oome et al., 2014). T-DNA mutants used in this study were *fls2* (SALK_141277), *sobir1-13* (SALK_009453) and *rlp23-1* (SALK_034225; Alonso et al., 2003; Shan et al., 2008; Gao et al., 2009; Albert et al., 2015).

EMS Mutagenesis

XVE:*NLP3* seeds were incubated overnight in 100 mM phosphate buffer pH 7.5. EMS was added to a final concentration of 0.4%, and the seeds were incubated for 8 hours at RT on a rotating table. Next, the seeds were washed several times in 1 M NaOH to remove residual EMS. M1 plants were propagated in pools of 16 plants. M2 seedlings were induced by spraying with an estradiol solution (0.5 mM β-estradiol 0.02% Silwet), three times per week (Monday, Wednesday Friday) for 2 weeks until a clear difference was visible.

Genotyping of Putative Mutants

To check the presence of the *NLP3* transgene in the M2 generation, DNA was extracted from adult plants using the Sucrose Prep protocol (Berendzen et al., 2005). The PCR primers that were used in this study are shown in Table 3. *NLP3* (PsojNIP_SP_FW and HaNLP3_RV primer pair) and a 370 base pair fragment of *ACTIN 2* (ACT2 primers) were PCR amplified to verify to integrity of the DNA (*ACTIN 2*) and confirm the presence of *NLP3*.

To see if *NLP3* was expressed, RNA was isolated from plants, 24 hours after induction of the transgene with estradiol, using the Spectrum Plant Total RNA Kit (Sigma-Aldrich). RNA was treated with DNase I to remove genomic DNA (Fermentas). RNA quantity and quality was assessed by NanoDrop 2000. DNA-free total RNA was converted in cDNA using RevertAid H minus Reverse Transciptase (Fermentas). A RT-PCR was performed on the cDNA with PsojNIP_SP_FW and HaNLP3_RV primers to check for *NLP3* expression and ACT2_Intron primers to confirm gDNA-free cDNA was created (product size gDNA = 631 base pairs, 192 base pairs on cDNA).

DNA was isolated from adult *M3* plants using the DNeasy Plant Mini Kit (Qiagen) to check for possible mutations in *RL23* or *SOBIR1*. *RLP23* and *SOBIR1* were PCR amplified using rlp23_FULL1 and SOBIR1_FULL1 primers, respectively. Amplified DNA was purified with the Agencourt AMPure XP system (Beckman and Coulter). Five µl DNA was used per sequencing reaction together with 5 µl primer (10 pmol per µl). Six primers were used for *RLP23* sequencing (rlp23_FULL2, rlp23_INT1 and rlp23_INT2, forward and reverse), and 4 for *SOBIR1* (SOBIR1_FULL2 and SOBIR1_INT, forward and reverse). Sequencing was performed by Macrogen Europe.

TABLE 3 | Primers used in this study.

Name	Forward	Reverse
rlp23_FULL1	CCCTTAGCTGCTTGACCATAC	TTTATCCTCATTTGCCCGCC
rlp23_FULL2	ACCATACTTTTGTTCTAGCCCG	TGAATGAAACCATGATCCCTCTG
rlp23_INT1	ACCTGACCCGGTTAACTAAGT	TGGGGAACTCGTTGATGTCA
rlp23_INT2	ATGGTGCCTCTTTACGGACA	GTCCCCGAGAGTTGGTTTCT
SOBIR1_FULL1	CTCAATAGATTAGTACCAGTTGCTG	TCGCCATTACAAATTTCTGCCA
SOBIR1_FULL2	TGTAGACTCCGACAACATCCAC	TCAAGTGTTCTTTTCCGTTATGT
SOBIR1_INT	TCCAACTTCAAGTCCCACGA	ACACCGAGAAGACAAACCCA
PsojNIP_SP_FW	CACCATGAACCTCCGCCCTGCA	
HaNLP3_RV		TGCTCCATCTTTTTTCGTTTTAAACGG
ACT2_Intron	TCTTCCGCTCTTTCTTTCCA	TCCTTCTGGTTCATCCCAAC
ACT2	GCTCTCCTTTGTTGCTGTTGACTA	CACTGTACTTCCTTTCAGGTGGTG

Peptide Synthesis

Peptide patterns were ordered at Genscript. Bacterial flg22 was dissolved in water and nlp24 in 100% DMSO. The pattern nlp24, AIMYAWYFPKDSPMLLMGHRHDWE, was derived from NLP3 of *H. arabidopsidis* and flg22, QRLSTGSRINSAKDDAAGLQIA, was based on the flagellin protein sequence of *Pseudomonas aeruginosa* (Felix et al., 1999; Oome et al., 2014).

H. arabidopsidis Disease Assays

Ten-day old M3, XVE:*NLP3* and XVE:*YFP* seedlings were sprayed with 0.5 mM β-estradiol in 0.02% Silwet or with 0.02% Silwet as a control. The next day, the seedlings were inoculated with 50 spores per µl *H. arabidopsidis* Waco9 spores. Plants were left to dry for approximately 30 minutes and subsequently kept at 100% humidity at 16 °C with 10 hours of light per day. The level of sporulation was assessed 6-8 days after pathogen challenge by counting the number of conidiophores.

Ethylene Measurements

Levels of ethylene accumulation were determined by gas chromatography as described previously (Felix et al., 1999; Oome et al., 2014).

ROS Measurements

Leaves of 4- to 5-week old *Arabidopsis* plants were cut into 2 x 4 mm rectangles and kept floating overnight in deionized water at room temperature in a closed petri dish. The next day, two leaf pieces were placed in a well of a white, flat-bottom 96-well plate (Greiner LUMITRAC™ 200) with 200 µl of a 20 µM L-012 and 1 µg/ml horseradish peroxidase solution. First, the background signal was measured for 15 minutes. Subsequently, the microbial patterns (final concentrations 1 µM flg22 or 10 µM nlp24) or mock (Milli-Q or 0.1% DMSO, respectively) were added and luminescence was measured for approximately one hour with a luminometer, each cycle taking approximately 100 seconds (Albert and Fürst, 2017).

Whole Genome Sequencing of *dni* Mutants

DNA of *dni1*, *dni2*, *dni3* and *dni4* and the XVE:*NLP3* parental line was isolated using the DNeasy Plant Mini Kit (Qiagen) including an RNase A treatment according to the manufacturer's protocol. Genomic DNA was quantified with the Qubit dsDNA HS Assay Kit and measured with the Qubit Fluorometer. Subsequently, 300 ng gDNA was suspended in 55 µl Low TE buffer (10x diluted). Next, gDNA was sheared in 350 base pair fragments using the Covaris S2 system (Covaris). Fragmented gDNA was prepared with the NeoPrep Library Prep System (Illumina). The libraries' quality was assessed by the Qubit Fluorometer and library concentration with the 2100 Bioanalyzer (Agilent). Libraries were equimolarly pooled and sequenced with the NextSeq 500 System (Illumina) on high output producing 75 base pair reads by the Utrecht Sequencing Facility (www.useq.nl).

EMS Mutation Calling

The genomes of sequenced *dni* mutants and the parental line were sequenced at ~30x coverage. Fastq files were processed and XVE:*NLP3* and *dni* mutants were compared to the *Arabidopsis* Col-0 TAIR10 genome assembly using the NIKS pipeline with standard settings (Lamesch et al., 2012; Nordström et al., 2013). Only EMS mutations were selected from the NIKS output file for further analysis. Subsequently, this file, containing the called EMS mutations, was processed by HTSeq-count (Anders et al., 2015) to filter out mutations that are not in the coding sequence of a gene, and call the genes with a EMS mutation using these settings: -m intersection, -strict, -t CDS, -stranded no, -I, gene_id. Next, the relative location of the EMS mutations within each coding sequence of a gene was determined using the Col-0 TAIR10 GTF annotation file. The output file generated reported all EMS mutations within gene coding sequences and the resulting change in amino acid (if any).

Creation of WebLogos

For SOBIR1 alignments, the ORTHO04D004202 sub-family was downloaded from Dicots PLAZA 4.0 (Table S2; Van Bel et al., 2017). The 54 selected *At*RLPs used for the analysis in this study were based on the 57 *At*RLPs described by Wang et al., 2008. However, RLP18 and RLP53 were removed as they are considered pseudogenes and RLP8 is obsolete (Cheng et al., 2017). SOBIR1 and *At*RLP protein sequences were aligned using Clustal Omega using the standard settings (Sievers et al., 2011). Sequence logos were generated using WebLogo 3, 10 amino acids upstream and downstream of the described mutations, except for *rlp23-7* (G855E) and *rlp23-8* (G859E) where the mutations were generated 8 downstream and 13 upstream of the substitution, and vice versa (Crooks et al., 2004).

Generation of Venn diagrams

Proportional Venn diagrams were generated from the comparison of all nonsynonymous substitutions found in the *dni* mutants using Biovenn (Hulsen et al., 2008; Jol, 2015).

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Supplemental Data

TABLE S1 | Overview of 26 *dni* mutants from 15 pools. Mutations found in RLP23 are depicted. X = no mutation found; ND = not determined.

No.	Mutant id	RLP23	SOBIR1
1	103-2	X	G528S
2	110-1	X	D507N
3	110-3	X	D507N
4	110-7	X	D507N
5	120-1	X	G412D
6	142-3	G855E	X
7	146-1	X	W34STOP
8	226-1	ND	ND
9	226-5	W67STOP	X
10	230-1	D590N	X
11	244-1	X	G557R
12	245-1	X	Q232STOP
13	245-3	ND	ND
14	245-4	ND	ND
15	245-6	X	Q232STOP
16	245-7	ND	ND
17	245-8	ND	ND
18	245-9	ND	ND
19	268-1	W651STOP	X
20	317-1	G859E	X
21	335-7	X	X
22	335-9	X	X
23	343-4	G762R	X
24	348-4	W67STOP	X
25	354-1	X	X
26	354-2	X	X

TABLE S2 | The 94 SOBIR1 orthologous proteins from 52 species used for the creation of the WebLogo in Figure 5.

Species	# of genes	Gene identifier(s)
Actinidia chinensis	2	Achn113931, Achn334801
Amaranthus hypochondriacus	1	AH022375
Arachis ipaensis	1	Araip.09PMN
Arabidopsis lyrata	1	AL4G27170
Arabidopsis thaliana	1	AT2G31880
Amborella trichopoda	1	ATR0199G026
Brassica oleracea	3	Bo3g026470, Bo4g177660, Bo4g177670
Brassica rapa	2	Brara.C01630, Brara.D01934
Beta vulgaris	1	Bv7_177070_myzy
Capsicum annuum	2	CAN.G126.94, CAN.G993.16
Cicer arietinum	1	Ca_07427.g
Cajanus cajan	1	C.cajan_31630.g
Coffea canephora	2	Cc02_g32040, Cc04_g10890
Citrus clementina	12	Ciclev10019280m.g, Ciclev10019677m.g, Ciclev10019853m.g, Ciclev10019897m.g, Ciclev10020580m.g, Ciclev10021254m.g, Ciclev10021753m.g, Ciclev10022278m.g, Ciclev10023696m.g, Ciclev10023722m.g, Ciclev10023893m.g, Ciclev10023971m.g
Citrullus lanatus	1	Cla019242.g
Cucumis melo	1	MELO3C026243
Corchorus olitorius	2	COL.COLO4_05418, COL.COLO4_19805
Carica papaya	1	Cpa.g.sc4.22
Chenopodium quinoa	1	AUR62001233
Capsella rubella	1	Carubv10025406m.g
Cucumis sativus	1	Cucsa.196310
Daucus carota	1	DCAR_007458
Eucalyptus grandis	4	Eucgr.B00174, Eucgr.K02774, Eucgr.K02775, Eucgr.L02485
Erythranthe guttata	2	Migut.D02032, Migut.O00198
Fragaria vesca	1	FVE10001
Glycine max	2	Glyma.04G190400, Glyma.06G175100
Gossypium raimondii	2	Gorai.003G108000, Gorai.008G203300
Hevea brasiliensis	2	HBR2879G010, HBR3293G005
Malus domestica	1	MDO.mRNA.g.815.30
Manihot esculenta	2	Manes.06G112200, Manes.14G057400
Marchantia polymorpha	1	Mapoly0115s0067
Medicago truncatula	2	Medtr3g075440, Medtr8g092950
Nelumbo nucifera	6	NNU_00100, NNU_02211, NNU_11249, NNU_21150, NNU_22007, NNU_25764
Oryza sativa ssp. *japonica*	1	LOC_Os06g18000
Picea abies	1	PAB00050400
Petunia axillaris	2	Peaxi162Scf00089g00111, Peaxi162Scf00763g00048
Pyrus bretschneideri	4	Pbr007767.1.g, Pbr007769.1.g, Pbr024019.1.g, Pbr036979.1.g
Physcomitrella patens	2	Pp3c10_16000, Pp3c14_17190
Prunus persica	1	Prupe.5G161700
Populus trichocarpa	2	Potri.012G090500, Potri.015G086800
Ricinus communis	1	RCO.g.30147.000103
Solanum lycopersicum	2	Solyc03g111800.2, Solyc06g071810.1
Selaginella moellendorffii	1	SMO134G0167
Schrenkiella parvula	1	Tp4g14230
Solanum tuberosum	2	PGSC0003DMG400015157, PGSC0003DMG400027071
Theobroma cacao	1	TCA.TCM_014517
Tarenaya hassleriana	1	THA.LOC104801545
Trifolium pratense	2	TPR.G24763, TPR.G35171
Utricularia gibba	1	UGI.Scf01513.23510
Vigna radiata var. *radiata*	1	Vradi05g05930
Ziziphus jujuba	2	ZJU.LOC107418206, ZJU.LOC107418229
Zea mays	1	Zm00001d045785

4

Chapter 5

NLP pattern recognition in lettuce is genetically complex and distinctly different from *Arabidopsis*

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Abstract

Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) are secreted by many plant-associated microorganisms. Recently, it was demonstrated that a short peptide sequence of NLPs (nlp24) is recognized in *Arabidopsis thaliana* and several other plant species. In *A. thaliana*, the receptor-like protein 23 (RLP23) is the nlp24 pattern recognition receptor (PRR). It detects the nlp pattern leading to the activation of pattern-triggered immunity. Similarly, nlp24 triggers immune responses in lettuce (*Lactuca sativa*). However, the mechanism of nlp recognition in lettuce is unknown. Here, we show that lettuce recognizes a broader range of nlp peptides than *Arabidopsis*. Furthermore, the lettuce genome does not encode an RLP23 ortholog, implying that NLPs are recognized by another receptor that has arisen by convergent evolution. In an attempt to find the NLP receptor gene in lettuce we first screened wild lettuce species for nlp24-induced ethylene accumulation. Surprisingly, most wild *Lactuca* species did not respond with increased ethylene production after nlp24 treatment, suggesting that wild lettuce cannot recognize NLPs. The nlp24-insensitive *Lactuca saligna* accession CGN05271 was selected to further study nlp24 recognition in lettuce. A backcross inbred line (BIL) population of cultivated lettuce and wild lettuce (*L. sativa* cv. Olof X *L. saligna* CGN05271) that covers 96% of the *L. saligna* genome was tested for nlp24-induced ethylene accumulation. All 29 BILs were nlp24-responsive, indicating that the *L. sativa* locus or loci required for nlp24 recognition are present in all BILs. In an F2 population of *L. saligna* CGN05271 X *L. sativa* cv. Olof only 4 out of 93 F2 progeny were nlp24-unresponsive, as they accumulated similar ethylene levels as *L. saligna* CGN05271, suggesting that recognition is mediated by two unlinked redundant loci. Unfortunately, further marker analysis failed to identify genomic regions linked to nlp24 recognition. Additional genetic crosses involving other nlp24-insensitive *Lactuca* lines could reveal if the recognition of the microbial NLP pattern is mediated by a PRR, as in *A. thaliana*.

Introduction

In the arms race with pathogens, plants have evolved a sophisticated immune system to detect microbial invaders in- and outside the cell and to deter them (Cook et al., 2015). Extracellular recognition relies on the detection of microbial patterns or damage-associated patterns through receptor-like kinases (RLKs) or receptor-like proteins (RLPs), collectively called pattern recognition receptors (PRRs; Gust et al., 2017; Ranf, 2017). When a molecular pattern is recognized, RLPs (and the adaptor protein SUPPRESSOR OF BRASSINOSTEROID INSENSITIVE 1 (BRI1)-ASSOCIATED RECEPTOR KINASE (BAK1)-INTERACTING RECEPTOR-LIKE KINASE 1 [SOBIR1]) and RLKs recruit co-receptors of the SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES (SERK) protein family, notably SERK3/BAK1 and SERK4/BAK1-LIKE1 (BKK1; Albert et al., 2015; Postma et al., 2016). A downstream signaling cascade is then initiated, leading to activation of immunity (Couto and Zipfel, 2016; Ranf, 2017). Early responses associated with pattern recognition are the accumulation of the phytohormone ethylene and an increased production of reactive oxygen species (ROS), and can ultimately contribute to a higher resistance to pathogens (Boller and Felix, 2009).

Recently, Nep1-like proteins (NLPs) were found to act as a molecular pattern in *Arabidopsis thaliana* (Böhm et al., 2014; Oome et al., 2014). NLPs are apoplastic proteins that are secreted by many plant-associated fungi, oomycetes, and bacteria (Meijer et al., 2014; Oome and Van den Ackerveken, 2014). The first described members of this family are cytotoxic (Bailey, 1995; Oome and Van den Ackerveken, 2014). However, in recent years, many non-cytotoxic NLPs have been described that are expressed by pathogens with a (hemi-)biotrophic lifestyle (Cabral et al., 2012; Dong et al., 2012; Kleemann et al., 2012; Stassen et al., 2012; Zhou et al., 2012).

To study the function of the non-cytotoxic NLPs of the obligate biotrophic downy mildew pathogen *Hyaloperonospora arabidopsidis* (HaNLPs), transgenic *A. thaliana* plants were generated that ectopically expressed these *HaNLPs*. Strikingly, for most *HaNLP* expressing *A. thaliana* plants, this resulted in a severe growth reduction that was associated with the activation of immunity (Oome et al., 2014). Subsequently, it was determined that *A. thaliana* recognizes a small region of the central part of NLPs that contains two conserved domains, the heptapeptide motif, which is a hallmark of all NLPs and the AIMY motif, which is highly conserved in type 1 NLPs (Oome and Van den Ackerveken, 2014; Oome et al., 2014). A synthetic peptide of 24 amino acids, called nlp24, derived from HaNLPs, but also from a fungal and a bacterial NLP, was sufficient to trigger a strong immune response. Interestingly, a 26-amino-acid peptide derived from a type 2 NLP of *Pectobacterium carotovorum* (NLP_{Pcc}) that lacks the AIMY motif was unable to induce plant defense responses in *A. thaliana* (Oome et al., 2014).

Downy mildews are well known for their narrow host range. *H. arabidopsidis* grows exclusively on *A. thaliana*, *Plasmopara viticola* thrives on grape, *Peronospora effusa* on spinach, and the economically most important pathogen of lettuce is the downy mildew *Bremia lactucae* (Slusarenko and Schlaich, 2003; Michelmore et al., 2009; Gessler et al., 2011; Feng et al., 2017).

B. lactucae, like other downy mildews, has undergone a large expansion of its NLP repertoire. Interestingly, the predicted *B. lactucae* NLPs (*Bl*NLPs) mostly group in *B. lactucae*-specific clades (Stassen et al., 2012). Similarly, NLPs of *H. arabidopsidis* mostly group in species-specific clades (Oome and Van den Ackerveken, 2014).

The first identified NLP, Nep1, was isolated from culture filtrates of *Fusarium oxysporum* f. sp. *erythroxyli*, a fungal pathogen of coca (*Erythroxylum coca*; Bailey, 1995; Bailey et al., 2002). NLPs were also identified in *F. oxysporum* f. sp. *lycopersici* and related *F. graminearum* and *F. verticillioides* (Ma et al., 2010). Therefore, it is likely that another economically important pathogen of lettuce, *F. oxysporum* f. sp. *lactucae*, produces NLPs, although this has not been demonstrated. Since its first report in the 1960s in Japan, *F. oxysporum* f. sp. *lactucae* has spread to the Unites States of America, Europe and Western Asia (Mbofung et al., 2007).

Another major treath to lettuce production is the fungal pathogen *Verticillium dahliae* (Vallad et al., 2006). *V. dahliae* encodes several *NLPs* and there is litte variation in this gene family between different isolates of the pathogen (Santhanam et al., 2013). Taken together, lettuce is threatened by several *NLP*-expressing pathogens.

Here, we demonstrate that cultivated lettuce recognizes nlp24, leading to activation of immunity and resistance to *B. lactucae*. Furthermore, we determined that cultivated lettuce, in contrast to *A. thaliana*, recognizes nlp26 derived from the type 2 NLP, NLP_{Pcc}. This, and the apparent lack of an RLP23 ortholog in lettuce suggests the existence of a different PRR in lettuce. This prompted us to search for the encoding locus in lettuce. A backcross inbred line (BIL) population and an F2 population of *L. sativa* cv. Olof X *L. saligna* CGN05271 was tested for nlp24-responsiveness. All BILs were nlp24-responsive, as were all but four F2 plants. Unfortunately, further marker analysis did not lead to the identification of the NLP receptor. However, we think that a more comprehensive genetic study of the *L. sativa* cv. Olof X *L. saligna* CGN05271 F2 population should allow for the identification of the elusive NLP receptor in lettuce.

Results

nlp24 Triggers Ethylene Production and Resistance to Downy Mildew in Lettuce

To find out whether NLPs are recognized in other species than *A. thaliana,* we, together with our collaborators Böhm et al., (2014), performed a nlp sensitivity screen with a range of different plant species. For this, the pattern nlp20 was used that is derived from the type 1 NLP *Pp*NLP of *Phytophthora parasitica* and is highly similar to nlp24: it only lacks the final four amino acids of the heptapeptide motif. It was found that nlp20 is perceived by several other Brassicaceae too. Furthermore, parsley (*Petroselinum crispum*), *Nicotiana benthamiana*, *Solanum* spp., and the more closely related *Arabidopsis lyrata* did not recognize nlp20. Interestingly, the distantly-related species *Lactuca sativa* (lettuce) did respond to nlp20 (Böhm et al., 2014).

The increase in ethylene production in lettuce was also observed after treatment with the nlp24 peptide (figure 1*A*). To see whether this response was linked to increased immunity, 1 µM nlp24 or 0.01% dimethyl sulfoxide (DMSO, mock treatment) was vacuum infiltrated into *L. sativa* cv. Olof leaf discs. The next day, the leaf discs were inoculated with spores of the downy mildew *B. lactucae* and the level of sporulation was assessed after 8 days (Figure 1*B*). Indeed, nlp24 treatment led to a significant decline in sporulation levels compared to mock-treated leaf discs (Figure 1*B*), confirming that nlp24 activates immunity and induces resistance to *B. lactucae*. Based on the sequence of the nlp24 motif in *Bl*NLPs it is expected that they are functional as triggers of immunity in *A. thaliana* and *L. sativa* too (Figure 2).

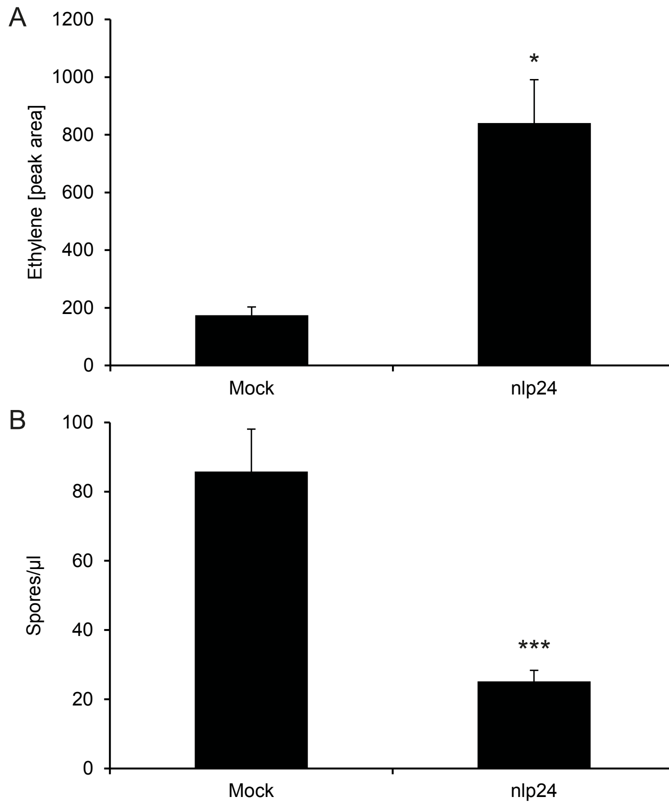

FIGURE 1 | Treatment with nlp24 induces ethylene production and *Lactuca sativa* resistance to *B. lactucae*. (***A***) Ethylene production in lettuce is induced in response to nlp24. Leaf pieces were incubated for 4 hours in a buffered solution containing 1 µM nlp24 or 0.01% DMSO (mock) before ethylene concentrations were determined by gas chromatography. (***B***) Resistance to *B. lactucae* in *L. sativa* cv. Olof is induced by nlp24. *L. sativa* leaf discs were vacuum-infiltrated with 1 µM nlp24 or 0.01% DMSO (mock) 24 hours prior to inoculation with 20 µl *B. lactucae* isolate Bl:24 spore suspension (120 spores/µl). Sporulation was assessed 8 days post inoculation. Error bars show standard deviation (SD). For ethylene measurements $n = 3$, for sporulation assessment $n = 5$. Experiments were repeated three times with similar results. Asterisks mark statistically significant difference to mock treatments as determined by Student's *t*-test, *P≤0.05, ***P≤0.001.

FIGURE 2 | Sequence alignment of nlp24 with 5 *Bl*NLPs reveals sequence similarity. The peptide regions of five previously described *Bl*NLPs (Stassen et al., 2012) matching to nlp24 were aligned to that of HaNLP3 with Clustal Omega, manually adjusted, and subsequently visualized in MView (Brown et al., 1998; Sievers et al., 2011). Residues are colored by identity to the nlp24 query and by chemical properties, with other residues in grey. Dots above the query indicate the tenth and twentieth residue, respectively.

Lettuce Recognizes a Broader Range of nlp Peptides than *Arabidopsis* and Lacks an *RLP23* Ortholog

In *A. thaliana*, RLP23 was identified as the NLP receptor (Albert et al., 2015). However, not all nlp peptides corresponding to the conserved nlp24 region are recognized in *A. thaliana*. A 26-amino-acid peptide (nlp26) derived from a type 2 NLP, NLP_{Pcc}, from *P. carotovorum* that lacks the conserved AIMY motif did not trigger immunity in *A. thaliana* (Oome et al., 2014). We compared the levels of ethylene production in lettuce and *A. thaliana* in response to nlp24 and nlp26. Strikingly, unlike *A. thaliana*, lettuce accumulated similar levels of ethylene after nlp24 and nlp26 treatment (Figure 3). Lettuce, thus, has a broader recognition specificity of nlp peptides than *A. thaliana*.

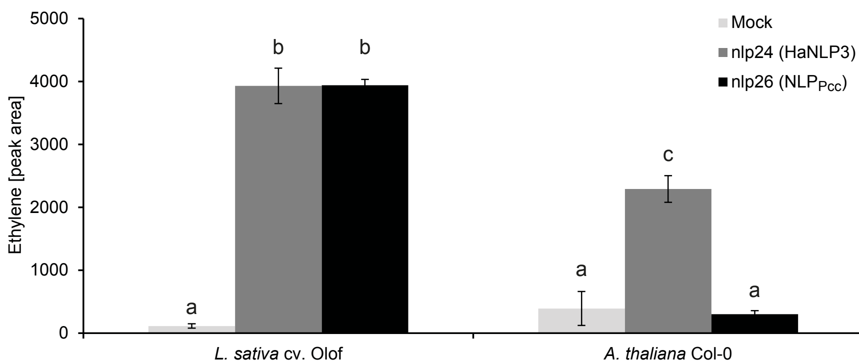

FIGURE 3 | Lettuce has broader nlp recognition than *A. thaliana*. Ethylene production in *L. sativa* cv. Olof is induced in response to the nlp26 fragment of the type 2 NLP_{Pcc}, but not in *A. thaliana*. Leaf pieces were incubated for 4 hours in a buffered solution containing 1 µM nlp26 (NLP_{Pcc}), 1 µM nlp24 (HaNLP3) as a positive control, or 0.01% DMSO (mock) before ethylene concentrations were determined by gas chromatography. Letters indicate a significant difference between plants and/or treatments (error bars depict SD of three measurements, two-way ANOVA, Tukey HSD post hoc test; $*P \leq 0.05$).

The distant relationship between *A. thaliana* (Brassicaceae) and lettuce (Asteraceae) and the broader nlp recognition in lettuce suggest that the perception of nlp patterns has arisen by convergent evolution. However, to rule out the presence of an RLP23 ortholog in lettuce we compared the phylogeny of RLPs in lettuce and *A. thaliana*. One commonly used approach to identify orthologs is that of reciprocal best hit (RBH): proteins are considered orthologous to each other when both come up as the best hit in the other genome (Ward and Moreno-Hagelsieb, 2014). To see whether the recently published lettuce genome (Reyes-Chin-Wo et al., 2017) encodes a predicted RLP23 ortholog we performed a reciprocal BLAST search with the protein sequences of RLP23 and several other *A. thaliana* RLKs and RLPs.

The RLKs SOBIR1 and BAK1 are expected to be present in most plant species because of their pivotal role in pattern-triggered immunity (Gust and Felix, 2014; Liebrand et

al., 2014). Similarly, the RLPs CLAVATA2 (CLV2; RLP10) and TOO MANY MOUTHS (TMM; RLP17) play important roles in plant development and are therefore likely conserved (Yang and Sack, 1995; Kayes and Clark, 1998). Finally, we selected 5 PRRs to be checked for orthologs in lettuce, two members of the RLK family and three of the RLP family. The RLKs FLAGELLIN-SENSING 2 (FLS2) and EF-TU RECEPTOR (EFR) are the flg22 and elf18 PRR, respectively (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006). RLP1 (ReMAX) is involved in the detection of a bacterial pattern called eMax (Jehle et al., 2013a; Jehle et al., 2013b) and RLP30 and RLP42 both detect fungal patterns: SCFE1 and polygalacturonases, respectively (Zhang et al., 2013; Zhang et al., 2014). As expected, RBHs were found in the predicted lettuce proteome for SOBIR1, BAK1, CLV2 and TMM (Table 1). The only PRR, of 5 tested, for which a RBH was found in lettuce was FLS2. Pattern recognition receptors are, thus, not necessarily highly conserved. Most interestingly, the reciprocal BLAST approach did not yield an RLP23 orthologue (Table 1). Furthermore, phylogenetic analysis revealed that *RLP23* is part of a Brassicaceae-specific clade, and the *A. thaliana* genome contains 13 genes paralogous to *RLP23* (Figure 4*A*). In contrast, *FLS2* is the only *A. thaliana* RLK amongst all other plant species (Figure 4*B*). The lack of an apparent *RLP23* RBH in the lettuce proteome and the fact that *RLP23* is part of Brassicaceae-specific clade, together with the broader recognition of nlp peptides in lettuce, makes it highly likely that a PRR different from RLP23 is responsible for the perception of NLPs in lettuce.

TABLE 1 | The lettuce genome does not encode an *RLP23* ortholog. Protein sequences of RLKs and RLPs that have a role in immunity and/or development in *Arabidopsis* were blasted against the *L. sativa* cv. Salinas genome (Reyes-Chin-Wo et al., 2017) and top hits were subsequently blasted back to the *Arabidopsis* genome to identify possible orthologous proteins. Numbers in the remarks column refer to the position of the proteins in the reciprocal BLAST search.

Name	Family	AGI code	Lettuce top hit	Reciprocal top BLAST hit	Ortholog	Remarks
SOBIR1	RLK	AT2G31880	Lsat_1_v5_gn_4_78640.1	SOBIR1	Yes	
BAK1	RLK	AT4G33430	Lsat_1_v5_gn_7_79861.1	BAK1	Yes	
EFR	RLK	AT5G20480	Lsat_1_v5_gn_2_121160.1	AT3G47570 (LRR-RLK)	No	EFR #2, similar score
FLS2	RLK	AT5G46330	Lsat_1_v5_gn_7_32801.1	FLS2	Yes	
ReMAX (RLP1)	RLP	AT1G07390	Lsat_1_v5_gn_1_29681.1	AT1G74190 (RLP15)	No	ReMAX is #3
CLV2 (RLP10)	RLP	AT1G65380	Lsat_1_v5_gn_1_58361.1	CLV2 (RLP10)	Yes	
TMM (RLP17)	RLP	AT1G80080	Lsat_1_v5_gn_1_34341.1	TMM (RLP17)	Yes	
RLP23	RLP	AT2G32680	Lsat_1_v5_gn_1_60601.1	AT1G45616 (RLP6)	No	RLP23 is #13
RLP30	RLP	AT3G05360	Lsat_1_v5_gn_9_10221.1	AT5G25910 (RLP52)	No	RLP30 is #27
RLP42	RLP	AT3G25020	Lsat_1_v5_gn_7_97821.1	AT1G47890 (RLP7)	No	RLP42 is #7

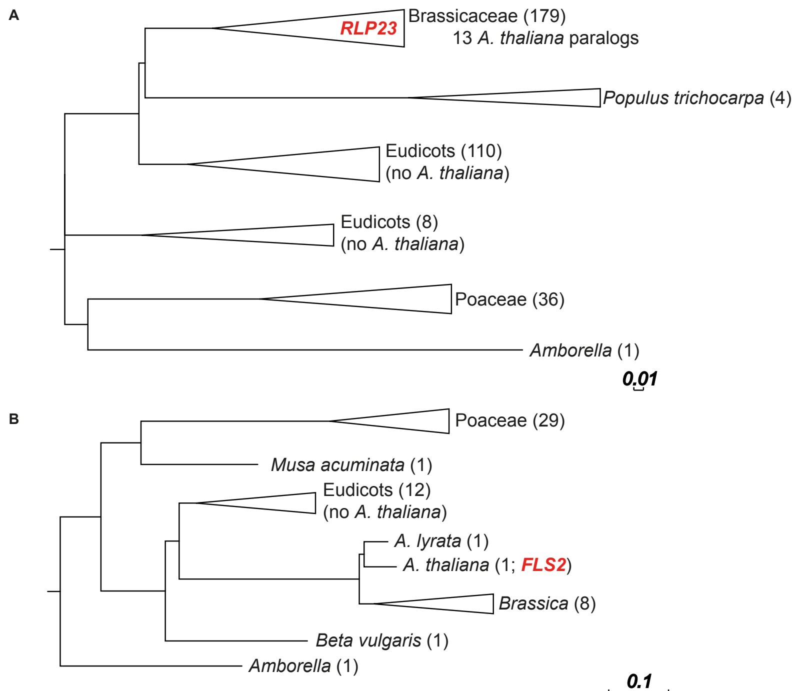

FIGURE 4 | Phylogenetic comparison of *RLP23* (*A*) and *FLS2* (*B*). Data was taken from Ensembl plants and visualized in Evolview using *Amborella* as an outlier. Number of homologous genes are depicted between parentheses.

Most Wild *Lactuca* Species Do Not Accumulate Ethylene after nlp24 Treatment

Genetic approaches have been instrumental in the identification of PRR genes from different plant species. For example, two receptor genes in tomato were identified by making use of a mapping population of a cross between cultivated and wild plants (Hegenauer et al., 2016; Wang et al., 2016). A similar approach was taken here by making use of wild lettuce germplasm. We tested 12 wild lettuce accessions, belonging to 4 species, for nlp24-responsiveness and used flg22 as a positive control. Strikingly, 11 out of 12 accessions did not respond with an increased ethylene production after treatment with 1 µM nlp24 (Figure 5), whereas *Lactuca saligna* CGN15705 was the only responding accession. Unfortunately, a significant flg22-induced ethylene production was only observed in *Lactuca aculeata* CGN09357 and *Lactuca altaica* CGN04664 and not in cultivated lettuce (Figure 4). The non-responsiveness of some many accessions makes it unclear if the wild lettuce lines are truly nlp24-unresponsive, or if pattern-triggered ethylene accumulation does not occur in these species, or if our detection methods are not sensitive enough.

We decided to continue our research with *L. saligna* CGN05271 because it showed no ethylene accumulation in response to nlp24 and because there is a well described *L. sativa* cv. Olof x *L. saligna* CGN05271 backcross inbred line (BIL) population available (Jeuken and Lindhout, 2004). However, as stated above, based on the data in figure 5 we could not exclude the possibility that *L. saligna* CGN05271 lacks a measureable pattern-triggered ethylene response. Therefore, an alternative pattern-triggered response, ROS production upon treatment with nlp24 and flg22 peptides was measured in *L. saligna* CGN05271 and *L. sativa* cv. Olof to verify if *L. saligna* is indeed nlp24-unresponsive, but can still respond to other patterns (Figure 6).

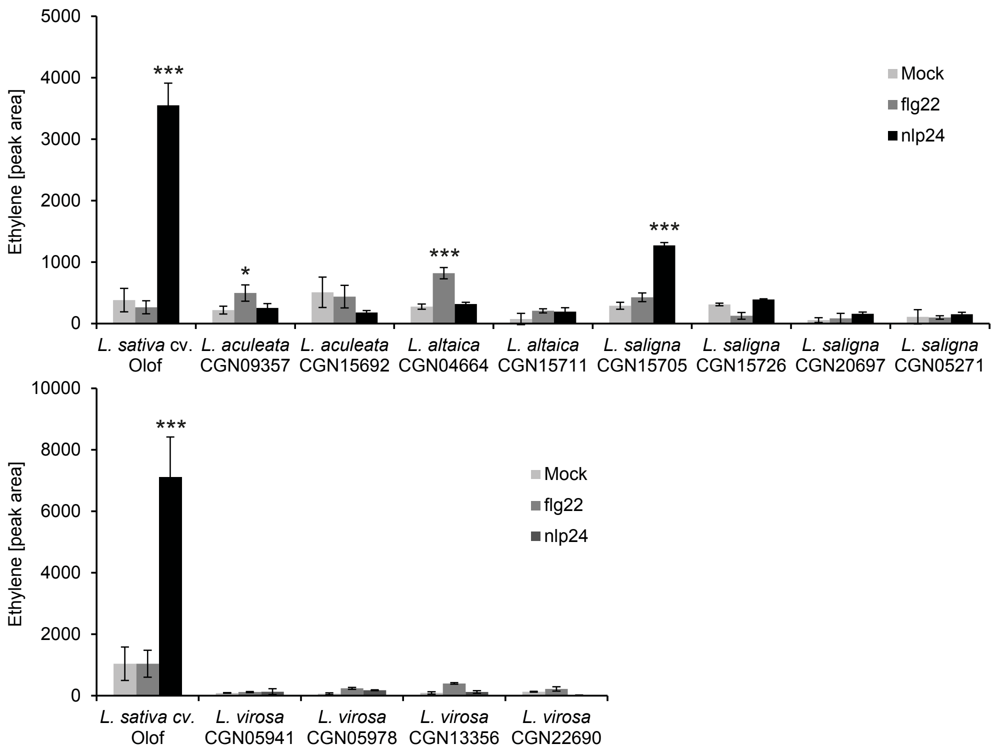

FIGURE 5 | Most wild lettuce species do not show increased ethylene production after nlp24 treatment. An increased ethylene production was observed in cultivated lettuce (*L. sativa* cv. Olof) and in *L. saligna* CGN15705 (wild lettuce) in response to nlp24, but not in *L. aculeata*, *L. altaica*, *L. virosa*, and other tested *L. saligna* accessions. Lettuce leaf pieces were incubated for 4 hours in a buffered solution containing 1 μM nlp24, 1 μM flg22, or 0.01% DMSO (mock) before ethylene concentrations were determined by gas chromatography. Asterisks indicate a statistically significant difference between mock-treated and flg22-treated or nlp24-treated plants, error bars depict SD (two-way ANOVA, Tukey HSD post hoc test; *P ≤ 0.05; ***P ≤ 0.001).

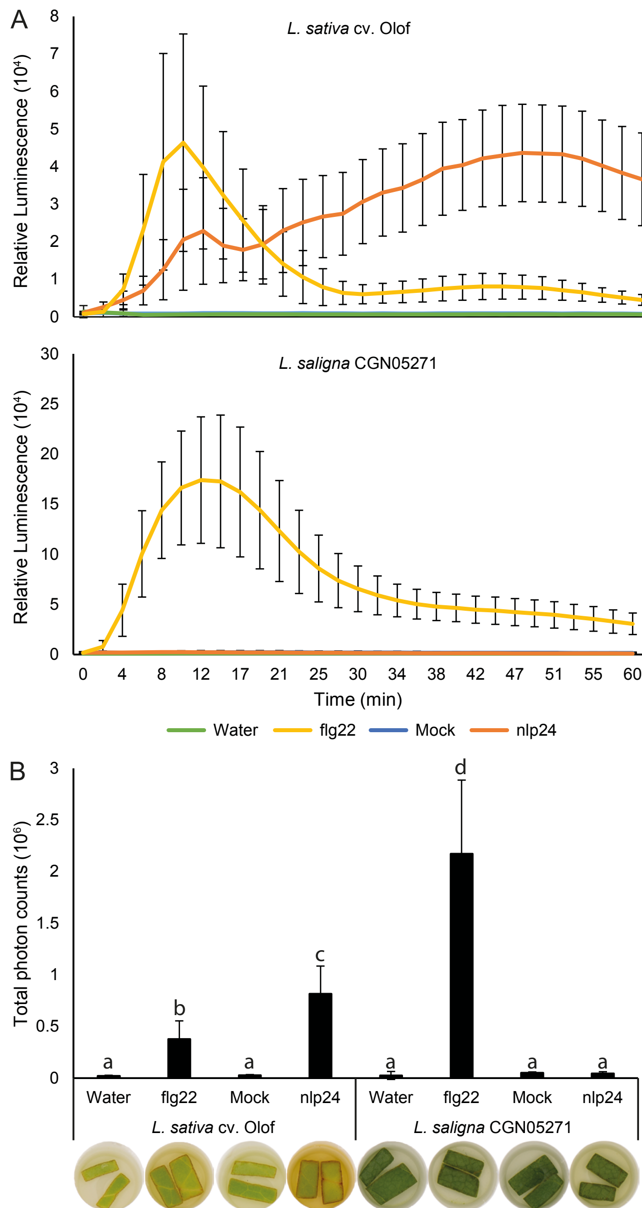

FIGURE 6 | Treatment with nlp24 does not lead to increased ROS production in *Lactuca saligna* CGN05271. (***A***) A rapid ROS burst is induced after flg22 (1 µM) treatment in *L. sativa.* cv. Olof and *L. saligna* CGN05271. In response to nlp24 (10 µM), ROS production is increased *L. sativa* cv. Olof, albeit delayed and more persistent when compared to flg22-induced ROS. In contrast, ROS production in *Lactuca saligna* CGN05271 after nlp24 treatment is similar to mock-treated plants. (***B***) Cumulative photon counts taken from ***A*** depict total ROS production over 1 hour in response to flg22 and nlp24 in *L. sativa.* cv. Olof and *L. saligna* CGN05271. After 24 hours, a more severe response to the nlp24 treatment is observed in nlp24-treated *L. sativa.* cv. Olof by apparent anthocyanin production and yellowing of the reaction mixture. Error bars in ***A*** and ***B*** show standard deviation.

Letters indicate a significant difference between plants and/or treatments, error bars show SD (N=6 for mock-treated and untreated [water] plants, N=12 for flg22- and nlp24-treated plants, two-way ANOVA, Tukey HSD post hoc test; $P \leq 0.05$).

Leaf pieces of both lettuce species were treated with 1 µM flg22 or 10 µM nlp24 after which the ROS production was measured. For this, luminol L-012 was used as substrate. Luminol is oxidized by peroxidases in the presence of ROS, which leads to the emission of light that can be recorded with a luminometer (Albert and Fürst, 2017). The flg22 pattern induced a rapid ROS burst in both lettuce species. In contrast, nlp24-triggered ROS production was only observed in *L. sativa* cv. Olof (Figure 6*A*). Interestingly, the nlp24-induced ROS burst was delayed, but continued longer than the flg22-triggered response (Figure 6*A*). Previously, a similar response was observed in *A. thaliana* (Chapter 4; Albert et al., 2015). Another response of *L. sativa* cv. Olof was observed 24 hours after the measurements: nlp24-treated leaf pieces had turned red at the edges, indicative of anthocyanin production, and the reaction mixture had turned yellow/orange (Figure 6*B*). This nlp24-induced response was stronger than the flg22-induced response in *L. sativa* cv. Olof. This could be due to a difference in the peptide concentrations used, 1 µM flg22 or 10 µM for nlp24, respectively. More likely though, there is a difference between flg22- and nlp24-triggered immunity in *L. sativa* cv. Olof. Dose response curves with flg22 and nlp24 are needed to corroborate this. The cumulative photon counts over the one hour measuring period further revealed that the flg22-induced response is stronger in *L. saligna* CGN05271 when compared to *L. sativa* cv. Olof (Figure 6*B*). Most importantly, there was no significant difference between, water-, mock- (0.1 % DMSO), and nlp24-treated *L. saligna* CGN05271 leaf pieces, indicating that *L. saligna* CGN05271 is nlp24-insensitive, but can respond to another pattern, flg22. In conclusion, a *L. sativa* cv. Olof x *L. saligna* CGN05271 cross is suitable to genetically map nlp24-responsiveness.

L. sativa cv. Olof x *L. saligna* CGN05271 Backcross Inbred Lines All Respond to nlp24

To find out if a genomic region that is linked to nlp24 recognition could be identified, we made use of a BIL population that was previously generated from a cross between *L. sativa* cv. Olof and *L. saligna* CGN05271. This population of *L. sativa* cv. Olof with mostly single introgression segments of *L. saligna* CGN05271 consists of 29 individual BILs and covers approximately 96% of the *L. saligna* CGN05271 genome (Jeuken and Lindhout, 2004). Leaf pieces of the BILs, together with the parental *L. sativa* cv. Olof and *L. saligna* CGN05271 lines were treated with 1 µM nlp24 and four hours later the ethylene accumulation was determined. Interestingly, all 29 BILs were responsive to nlp24, i.e. they produced similar levels of ethylene as *L. sativa* cv. Olof (Figure 7). This suggests that nlp24 recognition is conferred by a region of the genome not covered by this BIL population or that *Lactuca sativa* cv. Olof has multiple genes that encode redundant NLP PRRs (duplicate gene action).

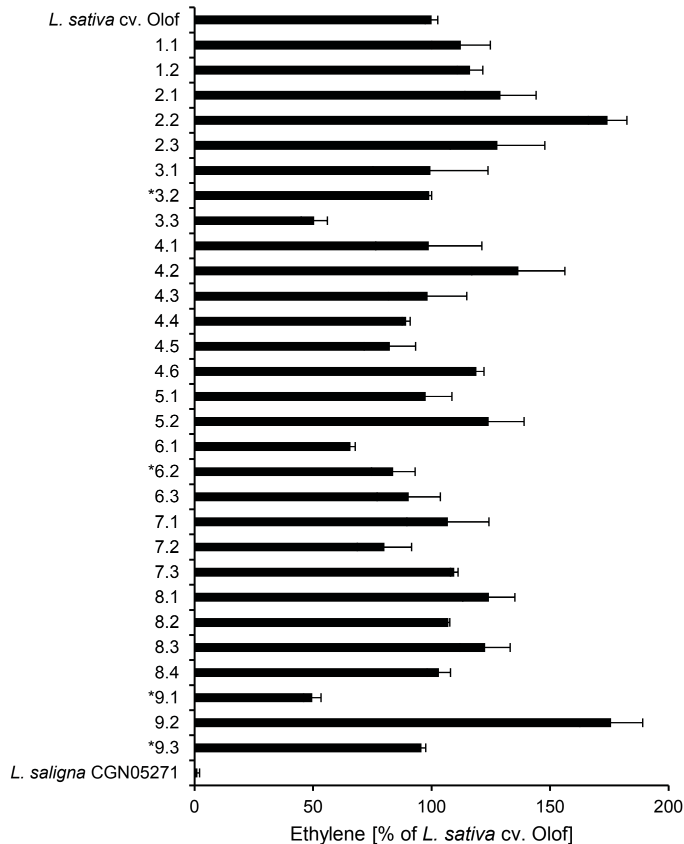

FIGURE 7 | *L. sativa* cv. Olof x *L. saligna* CGN05271 backcross inbred lines (BILs) show a similar level of ethylene production after nlp24 treatment as *L. sativa* cv. Olof. A *L. sativa* cv. Olof x *L. saligna* CGN05271 BIL population consisting of 29 members were tested for increased ethylene production after 1 μM nlp24 treatment. Leaf pieces were treated with 1 μM nlp24, and subsequently, the ethylene production was determined. All BILs responded similarly to nlp24 as the *L. sativa* cv. Olof parental line. For BILs marked by an asterisk the BILs's motherplant has a heterozygous introgression of *L. saligna* CGN05271, therefore, the progeny will segregate. At least 10 individual plants were tested of these lines. Data are relative to nlp24-induced ethylene production in *L. sativa* cv. Olof which was set at 100%. Error bars show SD of 3-10 replicates.

Most *L. sativa* cv. Olof x *L. saligna* CGN05271 F2 Plants Are nlp24-responsive

To further study the genetics underlying nlp24 recognition we tested an F2 population of *L. sativa* cv. Olof x *L. saligna* CGN05271. In total, 244 F2 seeds were sown of which only 110 germinated. Of those, 93 survived into adulthood and were usable to test for nlp24-responsiveness (see Table 2 for overview). Eighty-nine proved responsive to nlp24 (similar to *L. sativa* cv. Olof) and only 4 were unresponsive (#35, #80, #152 and #239, similar to *L. saligna* CGN05271). An example of a sensitive and an insensitive F2 plant is shown in Figure 8. The low number of unresponsive plants suggest a genetic model in which two

unlinked dominant genes encode for redundant NLP PRRs. In this case, one would expect a 15:1 ratio for nlp24-responsive and unresponsive F2 plants, respectively. Based on this assumption, the two-gene hypothesis cannot be rejected (χ^2-test; $P > 0.6$).

TABLE 2 | Overview of the tested F2 population.

Sown	Germinated	Tested	Responsive	Unresponsive	Sampled
244	110	93	89	4	91

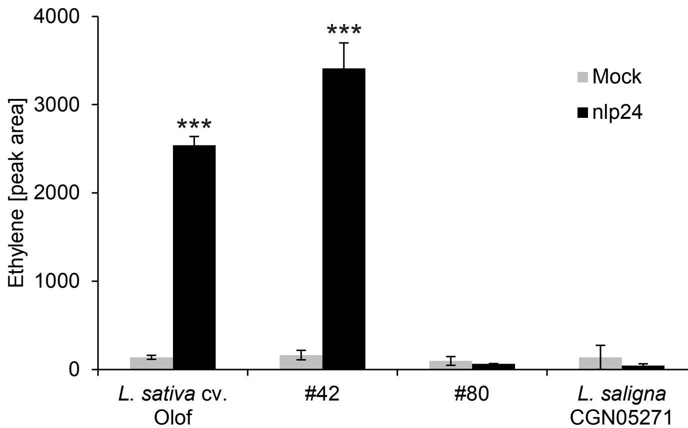

FIGURE 8 | Ethylene production is induced in *L. sativa* cv. Olof and F2 plant #42 but not in #80 and *L. saligna* CGN05271 after nlp24 treatment. Lettuce leaf pieces of *L. sativa* cv. Olof, *L. saligna* CGN05271 and two plants of a *L. sativa* cv. Olof x *L. saligna* CGN05271 F2 population were treated with 1 µM nlp24 or mock (0.01% DMSO) and incubated for 4 hours before the ethylene accumulation was determined. *L. sativa* cv. Olof and F2 plant #42 show a significantly increased ethylene production after nlp24 treatment, whereas #80 and *L. saligna* CGN05271 (Error bars show SD of 2 measurements, two-way ANOVA, Tukey HSD post hoc test; ***$P \leq 0.001$).

Subsequently, samples were taken from both parental lines and 91 out of 93 tested F2 plants (two died before sampling). DNA was isolated from all samples and genotyped for three markers raised against regions that were not represented in the BIL population. Marker CLS_S3_7724 is located at the top of chromosome 7 at position ~2 megabase (Mb).The exact location of marker CLX12996 is unknown, but it is located between marker QGC23M07 (at 166 Mb) and CLSM5902 (173 Mb) on chromosome 9. Finally, RIN4 is located at the top of chromosome 9 at about 15 Mb (Reyes-Chin-Wo et al., 2017). Unfortunately, these regions were not linked nlp24-responsiveness in the tested F2 plants (Table 3): The nlp24-insensitive F2 plants #35 and #80 were heterozygous for all three markers. Plants #152 and #239 were homozygous for *L. sativa* cv. Olof at marker CLX12996. #152 is homozygous *L. saligna* CGN05271 for marker CLS_S3_7724 and heterozygous for RIN4. In contrast, #239 is homozygous *L. sativa* cv. Olof for RIN4 and heterozygous for CLS_S3_7724 (Table 3).

TABLE 3 | Marker analysis of nlp24-insensitive *L. sativa* cv. Olof x *L. saligna* CGN05271 F2 plants.

	Marker name		
F2	**CLS_S3_7724**	**CLX12996**	**RIN4**
#35	Heterozygous	Heterozygous	Heterozygous
#80	Heterozygous	Heterozygous	Heterozygous
#152	*L. saligna* CGN05271	*L. sativa* cv. Olof	Heterozygous
#239	Heterozygous	*L. sativa* cv. Olof	*L. sativa* cv. Olof

All nlp24-unresponsive plants were grown to set seed. Only one of four nlp24-unresponsive plants was fertile, #152, and generated approximately 50 seeds. Unfortunately, this is often the case with interspecies hybrids. The F3 offspring of this line remains to be tested for nlp24-responsiveness. If this F3 population is unresponsive, it can be employed to make backcrosses to *L. sativa* cv. Olof which can be very helpful in further dissecting NLP-triggered immunity in lettuce.

Discussion

The Pattern nlp24 Triggers Immunity in Cultivated Lettuce

Responsiveness to nlp peptides is restricted to a select number of taxonomic groups that were tested. For example, all solanaceous species that were tested previously (i.e., potato, tomato and *N. benthamiana*) are insensitive (Böhm et al., 2014). In contrast, *A. thaliana* and several other Brassicaceae recognize nlp24 (Böhm et al., 2014; Oome et al., 2014). This limited representation of pattern-recognition is not unheard of, e.g., the bacterial pattern elongation factor-Tu is recognized in *A. thaliana*, but not in Solanaceous spp., *Medicago* spp. or the monocotyledonous cereal crops wheat (*Triticum aestivum*) and rice (*Oryza sativa*; Kunze et al., 2004; Lacombe et al., 2010; Lu et al., 2015; Schoonbeek et al., 2015; Schwessinger et al., 2015; Pfeilmeier et al., 2017). In contrast, recognition of bacterial pattern flg22 is present in a large number of plant taxa; flg22-responsiveness is found across most major groups of higher plants, but is lacking in the moss *Physcomitrella patens* (Boller and Felix, 2009; Bressendorff et al., 2016).

Here, we showed that nlp24 is a potent trigger of immunity in *L. sativa* too (Figure 1). Interestingly, cultivated lettuce has a broader NLP recognition spectrum than *A. thaliana*: nlp26, derived from a type 2 NLP of *P. carotovorum* (NLP_{Pcc}) does not trigger immunity in *A. thaliana*, but does in *L. sativa* cv. Olof (Figure 3). There is evidence for different recognition specificities amongst orthologous PRRs. FLS2 from *A. thaliana*, e.g., recognizes flg22 variants that do not trigger immunity in tomato (*Solanum lycopersicon*) in the presence of the tomato FLS2 ortholog (Chinchilla et al., 2006). Although the same may be true for cultivated lettuce and *A. thaliana*, we were unable to find a RLP23 ortholog in the *L. sativa*

5

genome (Table 1). Therefore, it is likely that a different PRR has evolved in lettuce that recognizes NLPs.

Furthermore, a recent report demonstrated that a *Colletotrichum orbiculare* NLP triggers immunity in several members of the cucurbit family (Azmi et al., 2018). Interestingly, not nlp24 is recognized, but rather a 32-amino-acid conserved region located at the C-terminus was responsible for triggering immunity in cucumber (*Cucumis sativus*) and melon (*Cucumis melo*), but not in *N. benthamiana* (Azmi et al., 2018). Thus, multiple, independent regions of NLPs can be recognized by different plants. This is reminiscent of flagellin; not only flg22 functions as a pattern but several other epitopes have been described that are recognized by different PRRs in plants and animals (Fliegmann and Felix, 2016).

L. saligna CGN05271 is a Valuable Resource to Dissect NLP-triggered Immunity in *L. sativa* cv. Olof

We screened wild *Lactuca* species for nlp24-induced ethylene production and found that most species showed no increased accumulation (Figure 5). Therefore, within the genus *Lactuca*, nlp24 recognition is mostly restricted to *L. sativa*. Similarly, in the *Arabidopsis* genus, most *A. thaliana* accessions are nlp24-responsive (Albert et al., 2015). However, within the same genus *Arabidopsis lyrata* is not (Böhm et al., 2014). To genetically dissect NLP recognition the *Lactuca* species, *L. saligna* CGN05271, was particularly interesting because of the wealth of crosses available: amongst others a BIL population of *L. sativa* cv. Olof and *L. saligna* CGN05271 with mostly single introgressions of *L. saligna* CGN05271 (Jeuken and Lindhout, 2004). Furthermore, seeds of a F2 population from this wide cross were available as well. However, based on nlp24-triggered ethylene production we could not conclude whether *L. saligna* CGN05271 is insensitive to nlp24; both nlp24 and the positive flg22 control failed to induce an increase in ethylene production compared to mock (Figure 5).

Therefore, we measured nlp24- and flg22-induced ROS production in *L. saligna* CGN05271 and *L. sativa* cv. Olof (Figure 6). A strong, rapidly induced, ROS burst was detected in response to flg22 in *L. sativa* cv. Olof and *L. saligna* CGN05271. However, no increase in ROS production in response to nlp24 was observed in *L. saligna* CGN05271, demonstrating that this line is nlp24-insensitive. Interestingly, the dynamics of ROS production in *L. sativa* cv. Olof induced by nlp24 and flg22 differed: flg22 induced a rapid ROS burst, whereas nlp24-induced ROS was delayed (Figure 6*A*). Similarly, ROS production mediated by RLP23 and the RLP CuRe1 is delayed compared to FLS2 in *A. thaliana* and tomato, respectively (Albert et al., 2015; Hegenauer et al., 2016; Albert and Fürst, 2017). This longer lag phase and lower peak production of ROS may be something that RLP-mediated responses have in common, possibly related to SOBIR1 signalling. If so, the NLP PRR in lettuce is likely to be an RLP too.

NLP Pattern Recognition is Genetically Complex in *L. sativa* cv. Olof

The screen of the *L. sativa* cv. Olof x *L. saligna* CGN05271 BIL population for nlp24-sensitivity yielded no clear leads as to which genomic regions confer recognition (Figure 7). This could have several reasons. Firstly, the NLP PRR locus may not be covered by this BIL population, as it spans 96%, but not all, of the *L. saligna* CGN05271 genome (Jeuken and Lindhout, 2004). Unfortunately, marker analysis of genomic regions not covered by the BIL population did not yield a locus involved in nlp24 recognition (Table 3). The recent genome publication of *L. sativa* cv. Salinas has revealed that a part of chromosome 6 is also not represented in the BIL population (Reyes-Chin-Wo et al., 2017). Markers that cover this genomic region could be tested on nlp24-unresponsive plants to see whether nlp24 recognition is linked to this chromosome.

Secondly, there could be multiple NLP receptors present; lettuce has undergone a whole-genome triplication and there could be high levels of redundancy (Reyes-Chin-Wo et al., 2017). This becomes apparent when looking at the top three RBHs of *A. thaliana* FLS2 against the lettuce genome, all three return putative FLS2 orthologs, although there functionality has not been validated.

Recently, a similar method as used in this research, has proven to be successful in the identification of two PRRs in tomato. By mapping the responsiveness in recombinant inbred lines to a pattern from the parasitic plant *Cuscuta reflexa* and the bacterial cold shock protein pattern the PRRs CuRe1 and CORE were identified (Hegenauer et al., 2016; Wang et al., 2016). Unfortunately, recognition of NLPs is more complex and more in-depth screens need to be employed to elucidate NLP detection in lettuce.

We tried to resolve these problems by analysis of an F2 population of *L. sativa* cv. Olof x *L. saligna* CGN05271. Strikingly, only 4 out of 93 tested F2 plants were nlp24-unresponsive (Table 2 and Figure 8). The low number of F2 individuals that are nlp24-insensitive are congruent with the hypothesis of two redundant dominant PRRs that recognize nlp24, where a 15:1 ratio of nlp24-sensitive : insensitive plants is expected. Therefore, two loci, homozygous for the *L. saligna* CGN05271 alleles, would be expected in the non-responsive F2 lines.

Based on the assertion that an RLP is the nlp24 PRR, a 'brute force' approach could be taken to identify the NLP PRR in lettuce. All *L. sativa* RLPs could be cloned and transiently expressed in *N. benthamiana* and checked for nlp24-sensitivity. Similar to *RLP23* expression, the putative lettuce NLP PRR is expected to confer the ability to recognize nlp24 to *N. benthamiana* (Albert et al., 2015). Alternatively, a comparative genomics approach could be taken. Known PRRs belong to the RLK or RLP family of proteins (Boutrot and Zipfel, 2017). The elusive NLP PRR in lettuce possibly belongs to the same category. Lettuce encodes 696 RLKs, comparable to the numbers that were identified in *A. thaliana* (Shiu and Bleecker, 2001; Dardick and Ronald, 2006; Reyes-Chin-Wo et al.,

2017). With the advent of more affordable sequencing techniques and the availability of an excellent reference genome (Reyes-Chin-Wo et al., 2017), whole-genome sequencing of the nlp24-insensitive F2 population and comparing these genomes to the parental line could provide receptor candidates of the RLK or RLP family, or an as yet unknown receptor type. Finally, a biochemical method may prove effective in receptor identification. The rice chitin receptor, CEBiP, e.g., was isolated from the plasma membrane of rice cells by chitin high-affinity binding (Kaku et al., 2006). It could be feasible to identify high-affinity binding proteins in the lettuce plasma membrane that interact with nlp24 and this could be instrumental in identifying the NLP PRR.

If the comparative genomics or biochemical approach is successful in identifying receptor candidates, heterologous expression of these candidates in *N. benthamiana* should clarify if these genes are sufficient to transfer nlp24-responsiveness (Albert et al., 2015).

Materials and Methods

Peptide Synthesis

Peptides were synthesized by Genscript. All peptides were dissolved in 100% DMSO (nlp peptides) or Milli-Q (flg22) as a 10 mM or 1 mM stock, respectively. All peptide patterns used in this study are shown in Table 4.

TABLE 4 | Peptides used in this study. The nlp peptides were described previously (Oome et al., 2014). Peptide nlp26 was based on the sequence of NLP_{Pcc} of *P. carotovorum* and lacks the conserved AIMY motif. The bacterial pattern flg22 corresponds to a flagellin domain from *Pseudomonas aeruginosa* and elicits an immune response in *A. thaliana* and lettuce (Felix et al., 1999; Roy et al., 2013). Size indicates the length of the peptides in amino acids.

Name	Organism of origin	Size	Amino acid sequence
nlp24 (HaNLP3)	*Hyaloperonospora arabidopsidis*	24	AIMYAWYFPKDSPM--LLMGHRHDWE
nlp26 (NLP_{Pcc})	*Pectobacterium carotovorum*	26	GSFYALYFLKDQILNGVNSGHRHDWE
flg22	*Pseudomonas aeruginosa*	22	QRLSTGSRINSAKDDAAGLQIA

Plant Growth Conditions

Plants were grown on potting soil (mix z2254, Primasta B.V., Asten, The Netherlands) at 21 °C, 75% relative humidity. Plants used for ethylene measurements and disease assays were grown under long day conditions (16 hours of light per day). Lettuce plants used for ROS measurements were grown under short day conditions (10 hours of light per day). All seeds were stratified for 2-3 days at 4 °C before they were moved to the growth chamber.

Ethylene Measurements

Lettuce plants of 4-8 weeks were used for ethylene assays. The ethylene accumulation induced by nlp peptides and flg22 was assessed by the previously described method (Oome et al., 2014)

ROS Measurements

A slightly adapted version of a recently described method to quantitatively measure the oxidative burst was used (Albert and Fürst, 2017). Leaf pieces of 2 by 4 mm were cut from 4-8 week old lettuce plants and were kept overnight floating on deionized water in a closed petri dish. The following day, two leaf pieces were placed in one well of a white, flat-bottom 96-well plate (Greiner LUMITRAC™ 200) containing 200 µl of a 20 µM L-012 and 1 µg/ml horseradish peroxidase solution. First, the background signal was measured for 15 minutes. Subsequently, the microbial patterns (final concentrations 1 µM flg22 or 10 µM nlp24) or control (Milli-Q or 0.1% DMSO) were added and luminescence was measured for one hour with a luminometer, each cycle taking approximately 100 seconds (± 1 second per well).

Pathogenicity Assays

L. sativa cv. Olof leaf discs with a diameter of 1.5 cm were stamped out and vacuum infiltrated with water or 1 µM nlp24. Twenty-four hours later, the leaf discs were inoculated with a 20 µl droplet of *Bremia lactucae* isolate Bl:24 spore suspension (120 spores/µl). The leaf discs were kept on water-saturated filter paper at all times and were kept at 100% humidity at 16 °C with 10 hours of light per day. Eight days after inoculation the level of sporulation was assessed by suspending the leaf discs in a known volume of water after which the number of spores per µl were counted (Böhm et al., 2014).

Creation of Gene Trees

Gene trees were generated using Ensembl Plants release 37 (Bolser et al., 2016) by selecting A. thaliana *FLS2* (EPlGT00890000127170) and *RLP23* (EPlGT00890000127175; Bolser et al., 2016). Closest homologs are displayed from maximally 49 species. Sub-trees that include the *Amborella* ortholog were selected and exported in the Newick format. In Evolview the tree was further adjusted by collapsing branches with clear taxonomic groups and by adding taxonomic information and the number of homologs (Zhang et al., 2012).

Marker Analysis

One small leaf disc (0.5 centimeter diameter) per plant was collected in a 1.5 ml microcentrifuge tube. Subsequently, the plant material was flash frozen in liquid nitrogen

and stored at -80 °C until further processing. Plant tissue was centrifuged at 4500 RPM to get all material to the bottom of the tube. Next, 20 µl 0.5 M NaOH was added and the samples were ground using a tissue striker for 5 minutes. Twenty µl 100 mM Tris was added immediately after the grinding step and the samples were centrifuged briefly to collect the sample in the bottom of the tube. Five µl of the leaf sample was transferred to a tube containing 200 µl 100 mM Tris and was thoroughly mixed by pipetting up and down. For the PCR reaction, 1 µl of the plant tissue mix was added to the reaction volume (Wang et al., 1993). Primers are depicted in table 5.

Table 5 | PCR markers used in this study.

Name	Forward	Reverse
CLS_S3_7724	TTCCCTCACTGGATGGAAAG	GCCTGTTTTGCTGCTTTTTC
RIN4	ATAGACCAAATTGCCGTCCA	CCCCTTTCAATTTTGATCGT
CLX12996	TCTTGGCCTCTCATTGATCC	CCAACGGGGAACACAAATAC

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Chapter

General discussion

Evolving Views on Pathogen Recognition: a Brief Historical Perspective

For centuries, it has been acknowledged that certain plant varieties are more susceptible to disease-causing microbes than others. As early as 1815, Thomas Andrew Knight, in a letter to Sir Joseph Banks, proposed that wheat varieties resistant to mildew or rust should be bred (Banks, 1806; Knight, 1815). Several attempts at this were made in the 19th century, a prime example being William Farrer, an Australian wheat breeder, who stated that susceptibility to rust is hereditary in wheat (Biffen, 1905). This was corroborated by Sir Rowland H. Biffen: he identified, by analysis of a cross between wheat varieties that are resistant or susceptible to yellow rust (*Puccinia striiformis* f.sp. *tritici*, previously *Puccinia glumarum*), that disease resistance follows Mendel's laws of inheritance (Biffen, 1905, 1907). The F2 population segregated in a 3:1 ratio of susceptible to resistant plants, showing that rust resistance, in this cross, was caused by a single recessive gene (Biffen, 1905).

A leap forward in our understanding of plant-pathogen interactions came from the hands of Harold H. Flor, one of the pioneers of phytopathology. In the 1930s and 1940s he laid the foundation of what later became known as the gene-for-gene hypothesis (Flor, 1971; Loegering and Ellingboe, 1987). He postulated, by studying the flax (*Linum usitatissimum*)-flax rust (*Melampsora lini*) pathosystem, that the ability of a pathogen to cause disease and of a host to resist is determined by single gene pairs, pathogen-derived avirulence (*Avr*) genes and plant resistance (*R*) genes (Flor, 1942; Lawrence et al., 2007). Or in the words of Flor, from his 1942 paper:

> "...*the pathogenic range of each physiological race of the pathogen is conditioned by pairs of factors that are specific for each different resistant or immune factor possessed by the host variety.*"

It took until the early nineties before the first *R* genes that conform this definition were identified (Johal and Briggs, 1992; Martin et al., 1993; Bent et al., 1994; Jones et al., 1994; Mindrinos et al., 1994; Whitham et al., 1994; Lawrence et al., 1995). Most *R* genes belong to the nucleotide-binding oligomerization domain [NOD]-like receptor (NLR) family of intracellular proteins that directly or indirectly perceive pathogen-derived effector proteins (Dodds and Rathjen, 2010; Cesari, 2017). Effectors are defined as all microbe-secreted molecules that alter host-cell structure and function to facilitate infection (Hogenhout et al., 2009). When an effector protein is detected by the host, it is also known as an Avr protein. *RPS2* from *Arabidopsis thaliana* and *N* from tobacco (*Nicotiana tabacum*) were the first *NLR* genes cloned required for resistance to *AvrRpt2*-carrying *Pseudomonas syringae* and tobacco mosaic virus, respectively (Bent et al., 1994; Mindrinos et al., 1994; Whitham et al., 1994). The third NLR protein that was found is encoded by the *L6* gene from flax (Lawrence et al., 1995). It was later demonstrated, in Flor's gene-for-gene system

flax-flax rust, that the L6 resistance protein directly interacts with AvrL567, an effector protein secreted by *M. lini* (Dodds et al., 2006).

This gene-for-gene model, however, could not explain all forms of plant defense, in particular not those that are less specific and provide resistance to a broader range of pathogens. The discovery of general elicitors, molecules that trigger immunity, started with the isolation of a polypeptide from the fungal pathogen *Monilinia fructicola*, which induced the production of the phytoalexin monilicolin A in bean (*Phaseolus vulgaris*; Cruickshank and Perrin, 1968). Phytoalexins are antimicrobial compounds that accumulate only under conditions of abiotic or biotic stress (Darvill and Albersheim, 1984). Interestingly, in contrast to Avr factors described by Flor, elicitors are not confined to a certain pathogen species and elicit responses in many plant species (Darvill and Albersheim, 1984). Many non-specific elicitors have been identified since then (Boller and Felix, 2009).

At the opening lecture of the annual Cold Spring Harbor Symposium on Quantitative Biology in 1989, the immunologist Charles A. Janeway Jr., one of the fathers of vertebrate innate immunity research, presented his pattern recognition hypothesis, which describes that pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs; Janeway, 1989). He wondered how primitive organisms were able to distinguish between self and non-self, and thusly theorized:

> *"The most likely possibility is that primitive effector cells bear receptors that allow recognition of certain pathogen-associated molecular patterns that are not found in the host. I term these receptors pattern recognition receptors."*

He further argued that these 'ancient' PRRs are still important in the vertebrate innate immune system (Janeway, 1989). Indeed, with the discovery of PRRs of the Toll-like receptor (TLR) family, it was found that PRRs play a key role in vertebrate innate immunity (Kawai and Akira, 2010). Mammalian TLR5, e.g., recognizes bacterial flagellin (Hayashi et al., 2001). Plant pathologists were inspired by this theory and readily applied it to their observations of elicitors (Nürnberger and Brunner, 2002; Nürnberger et al., 2004).

In 2006, the gene-for-gene and pattern recognition hypothesis were combined into a single, cohesive, model (Jones and Dangl, 2006). This 'zigzag' model divides the plant immune system in two branches. The first layer of defense concerns the recognition of PAMPs by PRRs, extracellular receptors belonging to the receptor-like kinases (RLKs) and receptor-like proteins (RLPs). Upon recognition, pattern-triggered immunity (PTI) is activated. However, pathogens have evolved effector proteins that can interfere with this immune response, leading to effector-triggered susceptibility (ETS). The second layer consists of intracellular receptors, the aforementioned *R* genes of the *NLR* protein family, that can directly or indirectly detect these effector proteins resulting in effector-triggered immunity (ETI). ETI is often associated with a hypersensitive response (HR), a form of

programmed cell death. This molecular arms race goes on indefinitely, pathogens can again evade recognition by losing or mutating the effector or by acquiring new effector proteins to suppress the plant immune system, resulting again in ETS. Finally, plants can evolve to recognize new or slightly altered effector proteins, leading to ETI (Jones and Dangl, 2006). PAMPs are not necessarily restricted to pathogens as many non-pathogenic microbes possess the same molecular patterns, so the term microbe-associated molecular pattern (MAMP) and subsequently MAMP-triggered immunity (MTI), were deemed more appropriate (Ausubel, 2005).

Although the zigzag model is very helpful in describing plant immunity, it fails to explain all plant-invader symbioses (Thomma et al., 2011; Cook et al., 2015). For example, the zigzag model cannot sufficiently explain the interaction with necrotrophic pathogens. Furthermore, the strict dichotomy between PRRs and R proteins, MAMPs and effectors, and MTI and ETI often does not hold up and could, potentially, stifle the ability of people who strictly try to adhere to this model to form new hypotheses. A clear example of the blurred MTI-ETI dichotomy are the Nep1-like proteins (NLPs), described in this thesis. Many NLPs comply with the definition of an effector protein mentioned above. However, they also act as MAMPs in *Arabidopsis* and lettuce (Chapter 2 and 5). Finally, the zigzag model does not incorporate endogenous molecules that are detected as damage-associated patterns (Cook et al., 2015).

Therefore, a new way of looking at all plant symbioses has been proposed with the Invasion Model. The Invasion Model is an inclusive model, that aims to describe all symbiotic interactions with plants (Cook et al., 2015). When an interaction is initiated, invasion patterns (IPs), are recognized by IP receptors (IPRs). An IP can be any immunogenic ligand, either exogenous or endogenous, that is recognized by plants. An IPR can entail any detection mechanism, including PRRs (RLKs and RLPs), R proteins (i.e. NLRs) or even Dicer-like proteins that are part of the antiviral immune system (Cook et al., 2015). Upon recognition by an IPR, an IP-triggered response (IPTR) can be activated, that results in either a cessation of the symbiosis or the symbiont utilizes IPTR to continue the interaction. For example, necrotrophic pathogens can hijack the plant-induced HR, in what is referred to as an inverse gene-gene interaction (Friesen et al., 2007; Lorang et al., 2007). Finally, effector proteins can suppress IPTR, which will also lead to a continuation of the symbiosis. However, effector proteins could be detected as IPs, leading to IPTR again (Cook et al., 2015).

Overall, the Invasion Model sufficiently encompasses all current knowledge on pathogen perception and thus offers an attractive alternative to the ubiquitous zigzag model. However, the all-inclusive description of plant symbioses defined by the Invasion Model can make it difficult to describe specific interactions in more detail. It is like broad strokes on a painting, it is a good way to start, i.e. to form your hypothesis, but to fill in the details

you will need a small paintbrush (i.e. a comprehensive understanding of the molecular players involved). The authors of the Invasion Model rightly claim that all models should be continually challenged via experimentation to advance scientific knowledge (Cook et al., 2015). As of now, the Invasion Model stands strong, but as history has shown, new discoveries will lead to new insights, which in turn, will result in future model refinements and adjustments to fit current data.

A Novel Molecular Pattern Triggers Immunity in *Arabidopsis*

In recent years, several new extracellular patterns and their cognate plant receptors have been described (Boutrot and Zipfel, 2017). In chapter 2, we describe the discovery of a novel microbial pattern, NLPs. NLPs are secreted by many plant-associated microorganisms, such as bacteria, fungi, and oomycetes (Oome and Van den Ackerveken, 2014). The first described NLPs are known for their ability to induce necrosis in dicotyledonous plants (Bailey, 1995; Gijzen and Nürnberger, 2006). It was, therefore, surprising that the genomes of the obligate biotrophic downy mildew pathogens revealed an expansion of the NLP family (Baxter et al., 2010; Seidl et al., 2011; Stassen et al., 2012; Sharma et al., 2015; McGowan and Fitzpatrick, 2017). In an analysis of NLPs of the downy mildew pathogen *Hyaloperonospora arabidopsidis* (HaNLPs), it was found that they are all non-cytotoxic. Even HaNLP3, which shares the highest similarity to cytotoxic NLPs was unable to trigger necrosis (Cabral et al., 2012). A chimera of HaNLP3 and the cytotoxic PsojNIP – an NLP from the pathogen *Phytophthora sojae* – revealed that HaNLP3 only differs in a small exposed domain. Swapping of two regions in HaNLP3 with those of PsojNIP changed to protein from non-cytotoxic to cytotoxic, i.e. it gained necrosis-inducing activity (Cabral et al., 2012).

To further study possible functions of HaNLPs, we generated transgenic overexpression lines for all HaNLPs in *Arabidopsis* (Oome et al., 2014). Intriguingly, we observed that expression of most HaNLPs resulted in severe growth reduction which correlated with high resistance to *H. arabidopsidis*. Indeed, we demonstrated that HaNLPs triggered immunity and that a small region of 24 amino acids (nlp24) was sufficient to activate NLP-triggered immunity (NTI). This peptide contains two conserved segments, the heptapeptide motif, present in most NLPs and a section starting with an AIMY motif that appears to be most conserved in type 1 NLPs. nlp24 peptides derived from fungal and bacterial type 1 NLPs triggered immunity, whereas a peptide derived from a type 2 NLP (from *Pectobacterium carotovorum,* NLP_{Pcc}), that lacks the AIMY motif did not (Oome et al., 2014). Similar activation of *Arabidopsis* immunity was observed with *Pp*NLP, a cytotoxic *Phytophthora parasitica* NLP (Böhm et al., 2014). The immunogenic activity of the full length *Pp*NLP was separated from its cytotoxic activity by denaturing the protein or mutating 2 residues that

are required for cytotoxicity. The inactivated protein still activated immunity, whereas the heat-denatured type 2 NLP, NLP_{Pcc} did not. A 20-amino acid peptide (nlp20, overlapping with the 20 first amino acids of nlp24) derived from *Pp*NLP was found to be sufficient to trigger immunity (Böhm et al., 2014).

In chapter 3, we describe the identification of the NLP PRR RLP23. Also, we show that the adaptor protein SOBIR1, is required for NTI. Furthermore, it was demonstrated that, upon recognition of the nlp24 ligand, BAK1 is recruited to the RLP23-SOBIR1 receptor complex (Albert et al., 2015). However, little is known about downstream signaling components required for NTI. To identify such components, we initiated a forward genetic screen for decreased NTI (*dni*) mutants, which is described in chapter 4. *DNI* candidate genes have been identified, and future research is expected to reveal the molecular players that play a pivotal role in NTI. Potentially, it could also help to identify key players in RLP-, SOBIR1, or RLK-mediated immunity in general.

The finding that NLPs act as a molecular pattern in *Arabidopsis* did not help us to understand the function of non-cytotoxic NLPs. The identification of the NLP PRR, RLP23 (Albert et al., 2015), gave us the possibility to study the effect of *HaNLP* expression in the receptor mutant background in which NTI is not triggered. However, we did not observe any effect of transgenic *HaNLP* expression on the susceptibility of *Arabidopsis* to the pathogens *H. arabidopsidis* and *Botrytis cinerea*, which both produce NLPs (Chapter 3).

The crystal structure of a cytotoxic NLP of *Pythium aphanidermatum*, NLP_{Pya}, could give clues to the putative function of non-cytotoxic NLPs. NLP_{Pya} shows similarity to actinoporins (Ottmann et al., 2009), cytolytic proteins of sea anemones (Rojko et al., 2016). These proteins target membrane lipids and after attaching to the sugar head groups of these lipids insert themselves into the membrane, forming a multimeric pore-like structure that causes the cell to lyse (Rojko et al., 2016). Cytotoxic NLPs may function in a similar fashion by associating with a membrane lipid and lysing the cell. No crystal structure of a non-toxic NLP has been solved, but based on sequence similarity it is likely that these NLPs form a comparable tertiary structure (Cabral et al., 2012; Oome and Van den Ackerveken, 2014). Our preliminary, unpublished, data supports this: HaNLP3 was shown to bind to a lipid fraction of the plant membrane. There it may play a role in accommodating pathogen infection. Identification of NLP-binding sites, therefore, could give us leads to how these proteins function. Altering or blocking these sites can then potentially reveal the role of HaNLPs.

One Pattern, Multiple Receptors

In chapter 5, we studied NLP perception in lettuce. Interestingly, we observed that not only nlp24, but also nlp26 derived from the type 2 NLP_{Pcc} activated NTI. Further analyses revealed that no *RLP23* ortholog is present in the lettuce genome. This broader recognition and the lack of an orthologous receptor was the start of a still ongoing quest for the elusive lettuce NLP PRR. nlp24 and nlp26 are not the only epitopes of NLPs that are recognized by plants. Recently, it was demonstrated that the 32-amino acid C-terminal conserved region of a *Colletotrichum orbiculare* NLP triggered immunity in cucumber (*Cucumis sativus*) and melon (*Cucumis melo*; Azmi et al., 2018). This seems to indicate that there are at least three PRRs that perceive NLPs: RLP23 in *Arabidopsis* and two distinct, unidentified, PRRs in lettuce and cucurbits.

Another example of different epitopes derived from one protein and multiple receptors that recognize these different domains is bacterial flagellin (Fliegmann and Felix, 2016). flg22, a 22-amino-acid flagellin-derived peptide is recognized by the RLK FLS2 in many plant species (Felix et al., 1999; Gómez-Gómez and Boller, 2000; Boller and Felix, 2009). C-terminal of flg22 lies another pattern, flgII-28, which triggers immunity in solanaceous species (Cai et al., 2011; Hind et al., 2016). Recently, its receptor FLS3 was identified in tomato (Hind et al., 2016). The final region of flagellin that is known to act as a pattern in plants is CD2-1, derived from the C-terminal region of the protein. This region is a potent trigger of immunity in rice (*Oryza sativa*) and acts independently of rice FLS2 (Katsuragi et al., 2015). Interestingly, vertebrates also perceive several distinct immunogenic regions of flagellin. Extracellular, TLR5 perceives conserved N- and C-terminal antiparallel strands of flagellin (Hayashi et al., 2001). Intracellularly, the NAIP5–NLRC4 inflammasome detects other conserved N- and C-terminal regions of flagellin than TLR5 (Zhao et al., 2011; Halff et al., 2012). Strikingly, plant NLRs share functional and structural similarities to animal intracellular immune receptors such as NAIP5 and NLRC4, although they likely evolved independently (Jones et al., 2016). Taken together, this demonstrates that organisms have evolved multiple receptors to detect distinct immunogenic regions of widespread microbe-associated molecules.

Perspective: Breeding for Durable Resistance

It is projected that by 2050 we need to approximately double the world's food production to feed an ever-growing population (Tilman et al., 2011). Pathogens pose a serious threat for agriculture and food security, and durable solutions to combat plant diseases need to be implemented (Fones et al., 2017). To emphasize this, it is estimated that filamentous pathogens, i.e. fungi and oomycetes, cause yield losses that could feed 500 to 4,000 million more mouths *per annum* (Fisher et al., 2012).

Improvement of disease resistance by plant breeders mostly relies on the introgression of *R* genes (i.e. NLRs) in crops (Dangl et al., 2013). However, introgressed *R* genes are often not very durable; pathogens quickly overcome these newly imposed barriers. One approach, the pyramiding of *R* genes, has been proposed to provide more durable resistance (Dangl et al., 2013). Indeed, preliminary data of field trials led by Jonathan Jones show that the incorporation of 3 *R* genes from wild potato in cultivated potato yields a *Phytophthora infestans*-resistant crop (The Sainsbury Laboratory, 2017). Furthermore, there is proof-of-concept that more distantly related species could be used as a source for *R* genes. For example, transfer of the NLR *CcRRP* of pigeonpea (*Cajunus cajan*) to soybean conferred full resistance to Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi*. This approach could prove to be incredibly important: ASR can only be controlled by fungicides since there are no commercial soybean (*Glycine max*) cultivars available that provide durable resistance (Kawashima et al., 2016). Similarly, breeders struggle to keep up with two other rapidly spreading diseases, wheat rust and wheat blast, caused by *Puccinia graminis* f. sp. *tritici* and *Magnaporthe oryzae* pathotype *Triticum*, respectively (Figueroa et al., 2016; Islam et al., 2016). New methods to rapidly identify novel resistance genes, such as MutRenSeq, can also help to quickly develop disease-resistant crops to halt these emergent diseases (Steuernagel et al., 2016).

An alternative, successful, breeding approach has been the usage of so-called susceptibility (*S*) genes. An *S* gene can be any plant gene that is required for successful infection by a pathogen, and loss-of-function mutations will, thus, lead to decreased susceptibility (Lapin and Van den Ackerveken, 2013; van Schie and Takken, 2014). A famous example is barley (*Hordeum vulgare*) Mildew Resistance Locus O (MLO). A loss-of-function mutation in the *Mlo* gene has provided durable, broad-spectrum resistance against the powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* for over four decades (Büschges et al., 1997; Kusch and Panstruga, 2017), Strikingly, *mlo*-based resistance has been proven to work in many plant species, including important crops (Kusch and Panstruga, 2017). Another *S* gene that shows promise is *DOWNY MILDEW RESISTANT 6* (*DMR6*). *Arabidopsis dmr6* mutants offer broad-spectrum resistance to *H. arabidopsidis*, *Pseudomonas syringae* pv. *tomato* DC3000 and *Phytophthora capsici* (Zeilmaker et al., 2015). *DMR6* genes are highly conserved (Zeilmaker et al., 2015) and application of DMR6 technology in lettuce and spinach in the combat against downy mildew disease has great potential (Van Damme and Van den Ackerveken, 2017).

Finally, transfer of PRRs of the RLK and RLP family of proteins could provide quantitative resistance against many pathogens, and, potentially, even parasitic plants (Hegenauer et al., 2016; Boutrot and Zipfel, 2017; Rodriguez-Moreno et al., 2017). A breakthrough paper demonstrated that transfer of *Arabidopsis* EFR to *N. benthamiana* and tomato (*Solanum lycopersicon*) results in broad-spectrum disease resistance to bacterial pathogens (Lacombe et al., 2010). Since then, different transgenic *EFR*-expressing crops have been generated

(see Table 1 for an overview of interspecies PRR transfer). Transfer of EFR to potato (*Solanum tuberosum*), the model legume *Medicago truncatula* and the monocotyledonous crops rice (*Oryza sativa*) and wheat (*Triticum aestivum*) has not only conferred the ability to recognize the bacterial pattern elf18, but also resulted in enhanced resistance to several bacterial pathogens (Lu et al., 2015; Schoonbeek et al., 2015; Schwessinger et al., 2015; Boschi et al., 2017; Pfeilmeier et al., 2017).

In Chapter 3, we described RLP23 as a novel PRR. Interestingly, transfer of RLP23 to potato conferred nlp20-sensitivity. Furthermore, it resulted in higher resistance against two NLP-producing pathogens: *P. infestans* and the fungus *Sclerotinia sclerotiorum* (Albert et al., 2015).

Also, lectin RLKs are thought to have roles in the perception of biotic and abiotic stress patterns (Wang et al., 2014). Their role in biotic stress is supported by the fact that expression of distinct *Arabidopsis* lectin RLKs in potato and *N. benthamiana* resulted in enhanced resistance to the oomycete pathogens *P. infestans*, *P. capsici* and the bacterial pathogens *P. carotovorum*, *Pseudomonas syringae* pv. *syringae* and *Pseudomonas syringae* pv. *tabaci* (Bouwmeester et al., 2014; Huang et al., 2014; Wang et al., 2015, 2016b).

Three PRRs of tomato have been successfully transferred to other species (Table 1). First, stable expression of the immune receptor Ve1, which is required for the recognition of the *Verticillium* effector protein Ave1, in *Arabidopsis*, tobacco and cotton (*Gossypium hirsutum*), resulted in enchanced resistance to several *Verticillium* spp. (Fradin et al., 2011; Song et al., 2017). Second, the RLK COLD SHOCK PROTEIN RECEPTOR (CORE), was found to be the PRR for csp22, a 22-amino acid peptide derived from bacterial cold shock proteins, and subsequent expression of *CORE* in *Arabidopsis*, resulted in increased resistance to *P. syringae* pv. *tomato* DC3000 (Wang et al., 2016a). Remarkably, a PRR that recognizes a glycopeptide pattern derived from the parasitic plant *Cuscuta reflexa*, CUSCUTA RECEPTOR 1 (CuRe1), conferred pattern sensitivity when stably expressed in the otherwise insensitive wild tomato species *Solanum pennellii* and *N. benthamiana* and exhibited increased resistance to the parasitic plant (Hegenauer et al., 2016).

The rice immune receptor Xa21 perceives the tyrosine-sulfated form of RaxX, a bacterial protein. A 21-amino acid tyrosine-sulfated synthetic peptide derived from RaxX, RaxX21-sY, is sufficient to trigger immunity (Pruitt et al., 2015). Expression of *Xa21* in sweet orange (*Citrus sinensis*) and banana (*Musa* sp.) resulted in higher resistance to *Xanthomonas axonopodis* pv. *citri* and *Xanthomonas campestris* pv. *musacearum*, respectively (Mendes et al., 2010; Tripathi et al., 2014). Interestingly, *Xa21* expression also led to higher resistance to *P. syringae* pv. *tomato* DC3000 and *Ralstonia solanacearum* in *Arabidopsis* and tomato, respectively (Afroz et al., 2011; Holton et al., 2015). However, it is unclear how significant these findings are as RaxX seems to be confined to the *Xanthomonas* genus (Pruitt et al., 2015).

A large family of secreted proteins in oomycetes are the elicitins (Derevnina et al., 2016) Elicitins are detected by the RLP ELICITIN RESPONSE (ELR) in the wild potato species *Solanum microdontum*. Transfer of ELR to cultivated potato led to enhanced resistance to *P. infestans*, an oomycete pathogen that encodes many elicitin and elicitin-like genes (Du et al., 2015). As a final example, transfer of the flagellin PRR FLS2 from *N. benthamiana* to sweet orange, resulted in higher disease resistance to bacterial pathogen *Xanthomonas citri* (Hao et al., 2016).

These examples of interspecies PRR transfer (Table 1) do not only show the viability of this approach in breeding, but also proof that PRR signaling is highly conserved. In rice, e.g., *Arabidopsis* EFR was shown to interact with two crucial components for XA21-mediated immunity: OsSERK2 and XA21 binding protein 24 (XB24). Silencing of OsSERK2 or overexpression of XB24 led to a loss of elf18-triggered immunity (Holton et al., 2015). Thus, even PRR transfer from dicotyledonous to monocotyledonous plants and vice versa confers recognition of patterns and increases resistance. The transfer of PRRs, individual or stacked, thus offers an alternative to breeding approaches that rely on the introgression of classical *R* genes (Dangl et al., 2013). One could fathom a breeding strategy wherein PRRs are combined with NLRs to potentially yield durable quantitative and qualitative extracellular and intracellular immune responses resulting in durable resistance in crops (Rodriguez-Moreno et al., 2017). As described in this thesis, *RLP23* could be a useful *PRR* gene for this combined approach as the protein recognizes a pattern that is produced by many plant-associated microbes and NLP-perception is not common in most species of flowering plants and important crops (Böhm et al., 2014). Such a breeding strategy has the potential to lead to more durable resistance and decreased pesticide usage, thereby contributing to more sustainable agriculture.

TABLE 1. Examples of interspecies PRR transfer.

Species of origin	Protein	Class	Pattern
Arabidopsis thaliana	RLP23	RLP	nlp24, derived from NLPs
	EFR	RLK	elf18, derived from bacterial elongation factor-Tu
	FLS2	RLK	flg22, derived from flagellin
	LORE	RLK	lipopolysaccharide (LPS)
	LecRK-I.9 (DORN1)	RLK	extracellular ATP
	LecRK-IX.1 & 2	RLK	unknown
	LecRK-VI.2[2]	RLK	unknown
Oryza longistaminata	XA21	RLK	RaxX21-sY, derived from bacterial RaxX
Solanum lycopersicon (tomato)	Ve1	RLP	Ave1
	CuRe1	RLP	Peptide of *Cuscuta reflexa*
	CORE	RLK	csp22, derived from bacterial cold-shock protein
Solanum microdentum (wild potato)	ELR	RLP	Elicitins
Nicotiana benthamiana	NbFLS2	RLK	flg22, derived from flagellin

[1] = Heterologous expression of *A. thaliana FLS2* (*AtFLS2*) in *S. lycopersicon* (tomato) conveys additional recognition specificity, i.e. flg22 variants that are not recognized by wildtype tomato LeFLS2, are by *AtFLS2*-expressing tomato plants (Chinchilla et al., 2006; Robatzek et al., 2007). [2] = Enhances immunity as part of the FLS2 receptor complex.

Transferred to	Sensitivity conveyed?	Enhanced resistance to	Reference
Solanum tuberosum (potato)	Yes	*Phytophthora infestans* and *Sclerotinia sclerotiorum*	Albert et al., 2015
Solanum lycopersicon (tomato)	Yes	*Xanthomonas perforans* and *Ralstonia solanacearum*	Lacombe et al., 2010
Nicotiana benthamiana	Yes	*Agrobacterium tumefaciens* and *Pseudomonas syringae* pv. *syringae* & pv. *tabaci*	Lacombe et al., 2010
Oryza sativa (rice)	Yes	*Xanthomonas oryzae* pv. *oryzae* and *Acidovorax avenae* subsp. *avenae*	Lu et al., 2015; Schwessinger et al., 2015
Triticum aestivum (wheat)	Yes	*Pseudomonas syringae* pv. *oryzae*	Schoonbeek et al., 2015
S. tuberosum (potato)	Yes	*R. solanacearum*	Boschi et al., 2017
Medicago truncatula	Yes	*R. solanacearum*	Pfeilmeier et al., 2017
S. lycopersicon (tomato)	Yes[1]	Not tested	Chinchilla et al., 2006
N. benthamiana	Yes	Not tested	Ranf et al., 2015
Nicotiana tabacum (tobacco)	Yes	Not tested	Ranf et al., 2015
S. tuberosum (potato)	Unknown	*P. infestans*	Bouwmeester et al., 2014
N. benthamiana	Unknown	*P. infestans* and *Phytophthora capsici*	Bouwmeester et al., 2014; Wang et al., 2016b
N. benthamiana	Unknown	*P. infestans* and *P. capsici*	Wang et al., 2015, 2016b
N. benthamiana	Unknown	*Pectobacterium carotovorum* and *P. syringae* pv. *syringae* & *pv. tabaci*	Huang et al., 2014
Citrus sinensis (sweet orange)	Yes	*Xanthomonas axonopodis* pv. *citri*	Mendes et al., 2010
S. lycopersicon (tomato)	Unknown	*R. solanacearum*	Afroz et al., 2011
Musa sp. (banana)	Yes	*Xanthomonas campestris* pv. *musacearum*	Tripathi et al., 2014
Arabidopsis thaliana	Unknown	*Pseudomonas syringae* pv. *tomato* DC3000	Holton et al., 2015
A. thaliana	Yes	*Verticillium dahliae* and *V. albo-atrum*	Fradin et al., 2011
N. tabacum (tobacco)	Yes	*Verticillium nonalfalfae*	Song et al., 2017
Gossypium hirsutum (cotton)	Yes	*V. dahliae*	Song et al., 2017
Solanum pennellii	Yes	*Cuscuta reflexa*	Hegenauer et al., 2016
N. benthamiana	Yes	*Cuscuta reflexa*	Hegenauer et al., 2016
A. thaliana	Yes	*Pseudomonas syringae* pv. *tomato* DC3000	Wang et al., 2016a
S. tuberosum (potato)	Yes	*P. infestans*	Du et al., 2015
C. sinensis (sweet orange)	Yes	*Xanthomonas citri*	Hao et al., 2016

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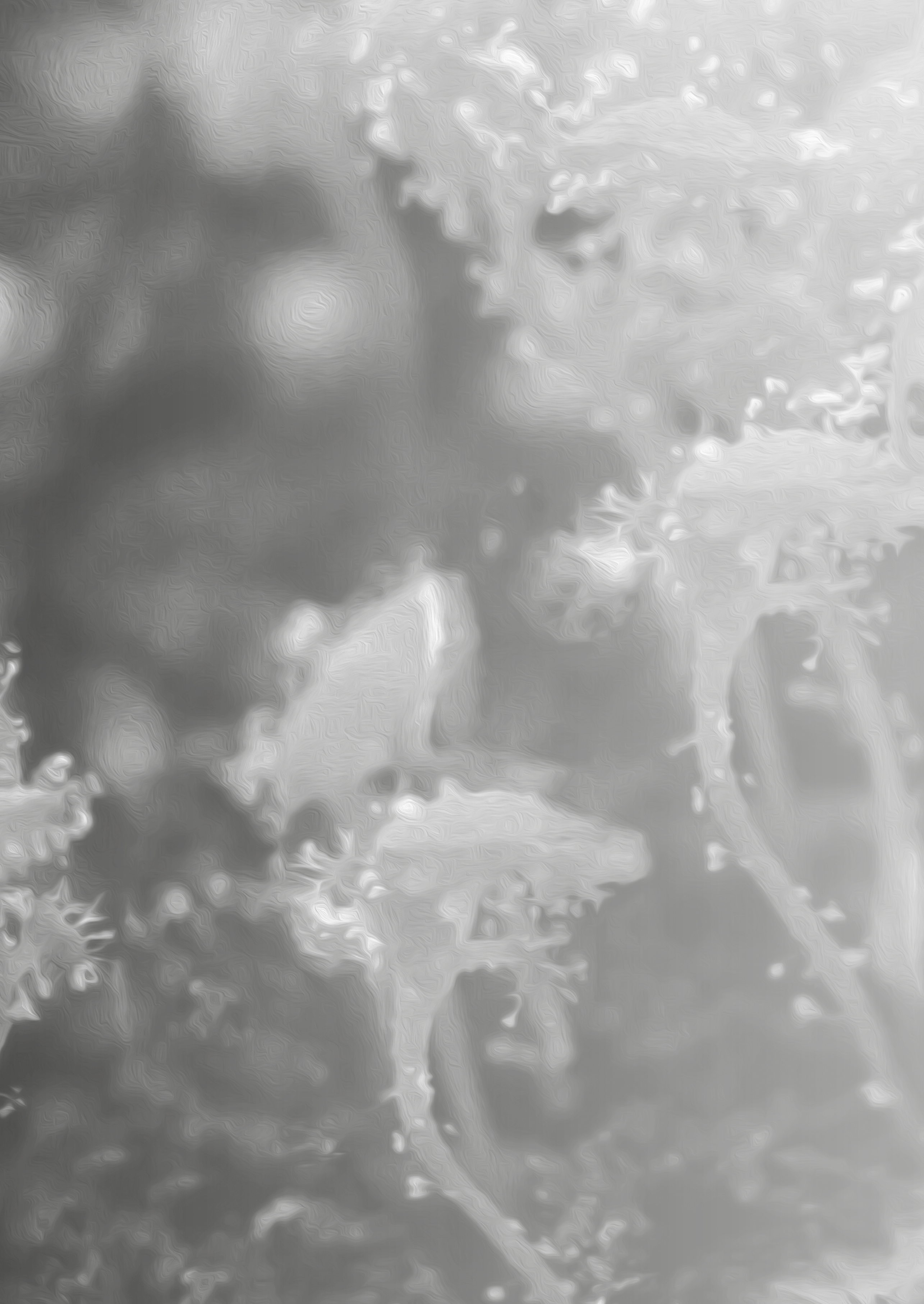

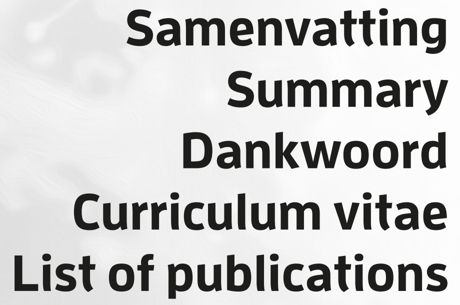

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Samenvatting

Planten kunnen ziekteverwekkers (pathogenen) aan de binnen- en buitenkant van hun cellen waarnemen. Voor extracellulaire herkenning maakt de plant gebruik van receptoren in het plasmamembraan die in twee typen voorkomen: receptorachtige kinases (receptor-like kinases, RLK's) en receptorachtige eiwitten (receptor-like proteins, RLP's). Deze eiwitten herkennen specifieke moleculen, patronen genaamd, afkomstig van ziekteverwekkers. Patroonherkenning leidt vervolgens tot de activatie van het immuunsysteem van de plant en in veel gevallen tot effectieve onderdrukking van het pathogeen en tot resistentie.

Een grote groep van pathogenen behoort tot de klasse van de oömyceten. Wereldwijd zorgen oömyceetziekteverwekkers zoals de valse-meeldauwpathogenen en *Phytophthora*-soorten voor grote gewasverliezen en daarom is het kweken van oömyceetresistente gewassen een belangrijke onderzoekstak binnen de plantenveredeling. De bekende extracellulaire patronen van oömyceten met een (hemi)biotrofe levensstijl, worden in **Hoofdstuk 1** uitvoerig besproken.

De ontdekking van een van deze patronen, de familie van necrose- en ethyleeninducerende peptide 1-achtige eiwitten (Nep1-like proteins, NLP's), wordt beschreven in **Hoofdstuk 2**. NLP's worden gesecreteerd door verscheidene, voornamelijk plant-geassocieerde, bacteriën, schimmels en oömyceten. NLP's staan, zoals de naam suggereert, vooral bekend om hun cytotoxiciteit: blootstelling van tweezaadlobbige planten aan deze eiwitten leidt tot celdood (necrose). Dit is nuttig voor necrotrofe ziekteverwekkers die zich kunnen voeden op dood plantenweefsel. In het laatste decennium echter zijn er ook vele NLP's geïdentificeerd in (hemi)biotrofe pathogenen die hun gastheerplant niet doden. Eerder onderzoek aan de NLP's van de valse meeldauw *Hyaloperonospora arabidopsidis* (HaNLP's), een obligaat biotrofe ziekteverwekker van de modelplant *Arabidopsis thaliana* (de zandraket), toonde aan dat deze NLP's niet cytotoxisch zijn. In een zoektocht naar de functie van deze eiwitten, zijn *Arabidopsis*-planten gegenereerd die deze *HaNLP's* tot expressie brengen. Vrij verwonderlijk leidde dit tot planten die dwerggroei vertoonden. Groeiremming wordt onder andere in verband gebracht met de activatie van het immuunsysteem: energie die normaal gesproken geïnvesteerd wordt in de ontwikkeling van de plant, wordt verbruikt in de afweerrespons. Ook hier bleek dit het geval te zijn: de planten die HaNLP's produceerden waren niet alleen kleiner maar ook zeer resistent tegen de valse meeldauw. Om dit verrassende effect onafhankelijk van het groeifenotype te bestuderen introduceerden we het HaNLP3-gen onder controle van een induceerbare promotor in *Arabidopsis*. Geïnduceerde *HaNLP3*-expressie zorgde ook voor hoge resistentie tegen valse meeldauw alsmede voor de activatie van afweergerelateerde genen.

Voor het herkennen van eiwitpatronen is niet altijd het hele eiwit vereist. In het geval van NLP's bleek een een centraal domein van deze eiwitten voldoende voor de activatie van het immuunsysteem. Dit peptide van 24 aminozuren, nlp24 genaamd, bevat twee sterk geconserveerde regionen: het heptapeptidemotief en het AIMY-motief. Dit laatste motief is enkel hoog geconserveerd in type 1 NLP's. De behandeling van planten met nlp24-peptiden, afgeleid van type 1 NLP's van één schimmel, één bacterie en één oömyceet (HaNLP3), leidde tot de productie van het afweergerelateerde plantenhormoon ethyleen en hoge resistentie tegen *H. arabidopsidis*. We namen deze reactie niet waar na behandeling met een nlp-peptide afgeleid van een type 2 NLP die het AIMY-motief ontbeerde. NLP's afkomstig van schimmels, bacteriën en oömyceten, worden dus herkend als moleculaire patronen in *Arabidopsis*.

Vervolgens rees de vraag hoe *Arabidopsis* dit NLP-patroon herkent. Om de NLP-receptor te identificeren maakten we gebruik van twee methoden (**Hoofdstuk 3**): een reverse genetics screening, waarbij bekende *rlp*- en *rlk*-mutanten gescreend werden voor een lagere ethyleenproductie na nlp-behandeling en een screening voor NLP-gevoeligheid in natuurlijk voorkomende ecotypes van *Arabidopsis*. Drie *Arabidopsis*-ecotypes en de *rlp23*-mutant toonden geen verhoogde ethyleenproductie na NLP-behandeling. Alle drie de ecotypes hadden een frameshiftmutatie in het *RLP23*-gen die leidt tot een prematuur stopcodon. Ter bevestiging dat *RLP23* vereist is voor nlp24-herkenning brachten we dit gen tot expressie in de nlp-ongevoelige plant *Nicotiana benthamiana*, waardoor deze plant nlp24-sensitiviteit verkreeg. RLP's hebben geen intracellulair kinasedomein en gaan daarom een interactie aan met de RLK SOBIR1 om een functioneel receptorcomplex te vormen. SOBIR1 blijkt ook van belang voor nlp24-geïnduceerde immuniteit; behandeling van twee *sobir1*-mutanten met dit patroon leidde niet tot een verhoogde ethyleenproductie. Planten waarin *RLP23* of *SOBIR1* uitgeschakeld waren verloren niet alleen de nlp24-geïnduceerde ethyleenaccumulatie maar ontbeerden ook de verhoogde resistentie tegen *H. arabidopsidis* na nlp24-behandeling. Het is daarom waarschijnlijk dat RLP23 de NLP-receptor is en dat SOBIR1 een complex vormt met deze receptor.

Behalve dat nlp24 valse-meeldauwresistentie in het behandelde plantenweefsel induceert, bleken systemische, onbehandelde bladeren ook verhoogd resistent. Dit leek erg op systemisch verworven resistentie (systemic acquired resistance, SAR). SAR-mutanten bleken inderdaad niet in staat om een een systemische resistentie te verwerven na nlp24-behandeling. Dat houdt in dat nlp24-patroonherkenning leidt tot SAR. Ten slotte bestudeerden we in dit hoofdstuk of de expressie van *HaNLP's* in een *rlp23*-mutantachtergrond zou bijdragen aan de vatbaarheid van *Arabidopsis* voor valse meeldauw en het necrotrofe schimmelpathogeen *Botrytis cinerea*. Dit werd echter niet waargenomen en er zijn dus geen aanwijzingen dat NLP's bijdragen aan de vatbaarheid van de plant. De functie van niet-cytotoxische NLP's is dus nog steeds onbekend.

Hoewel we nu weten hoe NLP's herkend worden door *Arabidopsis*, is er nog weinig bekend over wat er gebeurt nadat een RLP een patroon heeft herkend. Om nieuwe genen te identificeren die mogelijk een rol spelen in NLP-geïnduceerde immuniteit (NLP-triggered immunity, NTI) zijn we een forward genetic screen gestart (**Hoofdstuk 4**). Hierbij maakten we gebruik van de hierboven beschreven induceerbare *HaNLP3-Arabidopsis*-lijn (XVE:*NLP3*) opdat we mutanten konden identificeren die een gereduceerd NTI-fenotype (decreased NTI, DNI) hebben. XVE:*NLP3*-zaden werden hiertoe behandeld met ethylmethaansulfonaat (EMS): een organische verbinding die willekeurige genetische mutaties introduceert. De eerste generatie afkomstig van deze zaden (M2), werd gescreend voor een normale groeiontwikkeling na inductie van het *NLP3*-transgen. De geselecteerde vermeende *dni*-mutanten werden in de daaropvolgende generatie getest voor nlp24-gevoeligheid en voor resistentie tegen valse-meeldauwinfectie na *NLP3*-inductie. Planten die tijdens *NLP3*-expressie normaal ontwikkelden, vatbaar waren voor *H. arabidopsidis* én ongevoelig waren voor nlp24 werden vervolgens getest voor mutaties in *RLP23* en *SOBIR1*. Binnen de *dni*-mutantpopulatie ontdekten we verschillende nieuwe *rlp23*- en *sobir1*-mutanten die in de toekomst gebruikt zouden kunnen worden om een beter begrip te vormen van de interacties die plaatsvinden tussen het nlp24-ligand, RLP23 en SOBIR1 en mogelijk andere componenten die van belang zijn bij NTI. Uiteindelijk werden er 4 *dni*-mutanten geselecteerd die geen mutatie hebben in *RLP23* en *SOBIR1* én die aan de hierboven beschreven voorwaarden voldeden. Van deze 4 *dni*-mutanten hebben we het genoom gesequencet en de genmutaties ten opzichte van de XVE:*NLP3*-ouderplant bepaald. Toekomstige studies moeten uitwijzen welke van deze genmutaties de oorzaak zijn van het geobserveerde *dni*-fenotype.

Arabidopsis is niet de enige plantensoort die nlp24 herkent. Zo resulteerde nlp24-behandeling van gecultiveerde sla (*Lactuca sativa*) ook in verhoogde ethyleenproductie en resistentie tegen de valse meeldauw van sla, *Bremia lactucae* (**Hoofdstuk 5**). Bovendien is gecultiveerde sla in staat om een breder scala van nlp-peptiden te herkennen. Een nlp-peptide afgeleid van een type 2 NLP die het geconserveerde AIMY-motief niet heeft, activeerde wel een immuunreactie in sla terwijl deze afwezig was in *Arabidopsis*. Sla en *Arabidopsis* zijn niet nauw aan elkaar verwant, het lijkt er dus op dat deze planten onafhankelijk van elkaar het vermogen om NLP's te herkennen hebben ontwikkeld. Dit wordt ondersteund door het gegeven dat het slagenoom geen *RLP23*-ortholoog lijkt te coderen. Fylogenetische analyse toonde tevens aan dat *RLP23* onderdeel lijkt te zijn van een Brassicaceae-specifieke clade. Verder onderzoek werd ingezet om te pogen de NLP-receptor van sla te identificeren. Ten eerste werden wilde slasoorten op nlp24-gevoeligheid getest. De uitkomst was verrassend: de meeste wilde slasoorten accumuleren geen ethyleen na nlp24-behandeling. Gebruikmakende van terugkruisingslijnen (backcross-inbred lines, BIL's) van gecultiveerde sla x *Lactuca saligna* CGN05271 (een wilde sla-accessie) hebben we getracht de nlp24-gevoeligheid op het slagenoom in kaart te brengen. Echter

bleken alle BIL's, net als de ouderlijn, gecultiveerde sla, gevoelig te zijn voor nlp24. Omdat deze BIL-populatie 96% van het *L. saligna*-genoom omvat, zou het kunnen dat de NLP-receptor in een regio zit die niet in deze populatie aanwezig is. Om hierin een beter inzicht te krijgen maakten we gebruik van een gecultiveerde sla x *L. saligna* F2-populatie. Deze populatie werd ook getest voor nlp24-gevoeligheid. Slechts 4 van de 93 geteste F2-planten waren niet gevoelig voor nlp24. Dit lijkt te duiden op een genetisch model waarin 2 ongekoppelde dominante genen redundante NLP-receptoren coderen. Verdere merkeranalyse van de F2-populatie verschafte geen duidelijkheid over een mogelijke genomische regio die gelinkt kon worden aan NLP-perceptie. Vervolgonderzoek aan de nakomelingen van de ongevoelige F2-planten zou kunnen helpen de locatie van de NLP-receptor te ontcijferen.

In het laatste hoofdstuk, **Hoofdstuk 6**, geef ik een kort historisch overzicht van de veranderende visies op het gebied van pathogeenherkenning over de laatste 2 eeuwen. Verder vat ik het werk, beschreven in deze thesis, samen en geef ik een overzicht over hoe de kennis die in deze thesis vergaard is, toegepast kan worden voor het kweken van duurzame ziekteresistente gewassen.

Summary

Plants can detect pathogens on the inside and outside of their cells. Two types of plasma membrane receptors are employed by plants to detect pathogens extracellularly: receptor-like kinases (RLKs) and receptor-like proteins (RLPs). These receptors recognize specific, pathogen-derived molecules called patterns. Subsequently, pattern recognition leads to the activation of plant immunity and, in many cases, to effective pathogen suppression and resistance.

Many pathogens belong to the class of oomycetes. Worldwide, oomycete pathogens, such as the downy mildews and *Phytophthora* species, cause major crop losses and the cultivation of oomycete-resistant crops therefore, is a major focal point of plant breeders. In **Chapter 1**, previously described extracellular patterns of oomycetes with a (hemi-) biotrophic lifestyle are discussed in detail.

In **Chapter 2**, we describe the discovery of one of these patterns, the family of necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs). NLPs are secreted by several, mainly plant-associated, bacteria, fungi and oomycetes. As the name suggests, NLPs are best known for their cytotoxic activity: cells of dicotyledonous plants that are exposed to these proteins, die (necrosis). For necrotrophic pathogens, that feed on dead plant tissue, these NLPs are thus very useful. In the last decade, however, many NLPs have been identified in (hemi-)biotrophic plant pathogens that do not kill their host. For example, it was demonstrated that the NLPs of the downy mildew *Hyaloperonospora arabidopsidis* (HaNLPs), an obligate biotrophic pathogen of the model plant *Arabidopsis thaliana*, are not cytotoxic. In a search for the function of these proteins, we generated transgenic *Arabidopsis* plants that express these *HaNLPs*. Quite surprisingly, expression of most *HaNLPs* led to a reduced growth phenotype. Growth inhibition has often been associated with the activation of immunity: energy normally invested in plant development is used by the immune system. This proved to be the case here too: *HaNLP*-expressing plants were not only smaller but also highly resistant to *H. arabidopsidis*. To study this surprising effect independently of the growth phenotype, we introduced the HaNLP3 gene under the control of an inducible promoter in *Arabidopsis*. Interestingly, induced HaNLP3 expression also resulted in high resistance to downy mildew as well as the activation of defense-related genes.

The full protein is not always required to be functional as a proteinaceous pattern. For NLPs, the central domain of these proteins proved to be sufficient for the activation of immunity. This 24-amino acid peptide, called 24, contains two highly conserved regions: the heptapeptide motif and the AIMY motif. The latter motif is only highly conserved in type 1 NLPs. Treatment of plants with nlp24 peptides derived from type 1 NLPs of fungal, bacterial, and oomycete (HaNLP3) origin, led to the production of the defense-related

plant hormone ethylene and high resistance to *H. arabidopsidis*. However, we did not observe these responses after treatment with an nlp peptide derived from a type 2 NLP that lacked the AIMY motif. NLPs derived from fungi, bacteria and oomycetes thus act as molecular patterns in *Arabidopsis*.

Next, the question arose how *Arabidopsis* recognizes this NLP-derived pattern. To identify the NLP receptor, we used two methods (**Chapter 3**): a reverse genetic screen, in which known *rlp* and *rlk* mutants were screened for a lower ethylene production after NLP treatment and a screen for NLP-sensitivity in naturally occurring ecotypes of *Arabidopsis*. Three *Arabidopsis* ecotypes and an *rlp23* mutant showed no increased ethylene production after NLP treatment. All three ecotypes had a frameshift mutation in *RLP23* that leads to a premature stop codon. To confirm that RLP23 is required for nlp24 recognition we expressed this gene in the nlp24-insensitive plant *Nicotiana benthamiana*. Strikingly, *RLP23* conferred nlp24-sensitivity. RLPs do not have an intracellular kinase domain and therefore rely on the RLK SOBIR1 to form a functional receptor complex. SOBIR1 also appears to be required for nlp24-triggered immunity; nlp24 treatment did not lead to increased ethylene production in two independent *Arabidopsis sobir1* mutants. Furthermore, *rlp23* and *sobir1* plants not only lacked nlp24-induced ethylene accumulation but were also unable to trigger *H. arabidopsidis* resistance in response to nlp24. Therefore, it is likely that RLP23 recognizes nlp24 and that SOBIR1 interacts with RLP23 to form a bipartite receptor complex.

The pattern nlp24 did not only trigger downy mildew resistance in treated leaves. Systemic, nlp24-untreated leaves were also found to be highly resistant. This was reminiscent of systemic acquired resistance (SAR). Indeed, SAR mutants were unable to trigger systemic immunity after nlp24 treatment, demonstrating that nlp24 recognition leads to SAR. Finally, we studied whether the expression of *HaNLPs* in *rlp23* mutant background would contribute to downy mildew and *Botrytis cinerea* (a necrotrophic fungal pathogen) susceptibility. We did not observe a difference in susceptibility after *HaNLP* expression in *rlp23* plants. Therefore, the function of non-cytotoxic NLPs remains elusive.

Although we now know how NLPs are recognized by *Arabidopsis*, little is known what happens after NLP recognition. To identify new genes that play a role in NLP-triggered immunity (NTI), we started a forward genetic screen (**Chapter 4**). We used the inducible *HaNLP3-Arabidopsis* line (XVE:*NLP3*) described above to identify mutants that have a decreased NTI (DNI) phenotype. For this purpose, XVE:*NLP3* seeds were treated with ethyl methanesulphonate (EMS), an organic compound that introduces random genetic mutations. The first generation of these EMS-treated seeds (M2), were screened for normal growth development after *NLP3* induction. The selected putative *dni* mutants were tested in the following generation for nlp24-sensitivity and for downy mildew resistance after *NLP3* induction. NLP3-expressing plants that developed normally, were not resistant to

H. arabidopsidis, and were nlp24-insensitive, were subsequently tested for mutations in *RLP23* and *SOBIR1*. Within the *dni* mutant population, we discovered several novel *rlp23* and *sobir1* mutant alleles that, in future, could be used to gain a better understanding of the interactions between the nlp24 ligand, RLP23 and SOBIR1 and possibly other components involved in NTI. Ultimately, 4 *dni* mutants were selected with wildtype *RLP23* and *SOBIR1* that meet the criteria described above. We sequenced these 4 *dni* mutants and determined gene mutations relative to the XVE:*NLP3* parental line. Future research will reveal which of these gene mutations are causal to the observed *dni* phenotype.

Arabidopsis is not the only plant species that recognizes nlp24. For example, nlp24 treatment of cultivated lettuce (*Lactuca sativa*) also resulted in increased ethylene production and resistance to the lettuce downy mildew, *Bremia lactucae* (**Chapter 5**). Interestingly, cultivated lettuce is able to recognize a broader range of peptides than *Arabidopsis*: in contrast to *Arabidopsis*, an NLP peptide derived from a type 2 NLP that lacks the conserved AIMY motif does trigger immunity in lettuce. Lettuce and *Arabidopsis* are not closely related, so it is likely that these plants have independently evolved the ability to recognize NLPs. This was corroborated by the fact that the lettuce genome does not seem to encode an *RLP23* ortholog. Furthermore, a phylogenetic analysis showed that *RLP23* appears to be part of a Brassicaceae-specific clade. To try to identify the NLP receptor in lettuce, wild lettuce species were tested for nlp24-sensitivity. Surprisingly, most wild lettuce species did not accumulate ethylene after nlp24 treatment. Next, we tried to map nlp24-responsiveness on the lettuce genome using a backcross inbred line (BIL) population of nlp24-sensitive cultivated lettuce and nlp24-insensitive *Lactuca saligna* CGN05271 (a wild lettuce accession). Strikingly, all BILs were nlp24-responsive. The BIL population covers 96% of the *L. saligna* genome, therefore, it is possible that the NLP receptor is located in a genomic region that is not represented by this population. We took another approach to home in on the NLP receptor by using a cultivated lettuce x *L. saligna* F2 population. Remarkably, only 4 of the 93 tested F2 plants were nlp24-insensitive. This points to a genetic model in which 2 unlinked dominant genes encode redundant NLP receptors. Further marker analysis of the F2 population did not yield a possible genomic region that could be linked to NLP perception. Hopefully, testing the offspring of the nlp24-insensitive F2 plants will help decipher the location of the NLP receptor.

In the final chapter (**Chapter 6**), I give a brief historical perspective on the evolving views of pathogen recognition over the last 2 centuries. Furthermore, I summarize the work described in this thesis and I give an overview of how the newly acquired knowledge in this thesis can be applied for breeding sustainable disease-resistant crops.

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Curriculum Vitae

Tom Raaymakers was born on 30 January, 1987 in Lierop, municipality of Someren. He completed his secondary education at the St.-Willibrord Gymnasium in Deurne in 2005. In the same year, he started with the Bachelor Biology at the University of Utrecht. He obtained his Bachelor of Science degree in 2009, and subsequently enrolled in the Master program Environmental Biology at the University of Utrecht. During his master studies, he performed two internships. His first internship was at the Plant-Microbe Interactions (PMI) group under supervision of Prof. Dr. Guido van den Ackerveken. There, he mainly worked on the conformation of putative interactions between downy mildew RxLR effector proteins and *Arabidopsis thaliana* target proteins. The second internship took place at the Commonwealth Scientific and Industrial Research Organisation (CSIRO), in Canberra, Australia. He investigated the molecular basis of rust disease resistance in flax under supervision of Dr. Maud Bernoux and Dr. Peter Dodds. His research primarily focused on the structural and functional analysis of the L6 flax rust resistance protein. During his master studies, he was admitted to the 'MSc Talent Programme' of the Graduate School of Experimental Plant Sciences (EPS), for which he wrote and subsequently defended a PhD proposal on PAMP-triggered immunity to oomycetes in *Arabidopsis*. In 2012, he graduated and, with Prof. Dr. Guido van den Ackerveken, applied for a PhD grant with a rewritten proposal entitled 'Dissecting oomycete-induced immunity in *Arabidopsis* deploying a non-toxic NEP1-like protein as PAMP'. In early 2013, he started at the PMI group as a research technician, working on mapping a lettuce resistance gene required for recognition of a downy mildew effector protein and the research described in this thesis. At the same time, his application for a PhD project was granted by The Netherlands Organization for Scientific Research (NWO). In June 2013, he started as a PhD candidate under supervision of Prof. Dr. Guido van den Ackerveken, continuing the work described herein. During his PhD studies, he was an active member of the EPS PhD council, in which he helped organize events specifically aimed at EPS PhD candidates.

List of Publications

Oome, S.*, Raaymakers, T.M.*, Cabral, A., Samwel, S., Böhm, H., Albert, I., Nürnberger, T., and Van den Ackerveken, G. (2014). Nep1-like proteins from three kingdoms of life act as a microbe-associated molecular pattern in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 16955–16960.

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