Downy mildew effector recognition and host protein interaction in lettuce

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Cover design: Martien Kuijer
Lay-out: Alexandra Pelgrom
Printed by: Ridderprint BV | www.ridderprint.nl
ISBN: 978-90-393-7010-0

The work presented in this thesis was performed in the Plant-Microbe Interactions group at Utrecht University.

This work is part of the research programme “Open technology” with project number STW12683, which is (partly) financed by the Netherlands Organisation for Scientific Research (NWO).

Downy mildew effector recognition and host protein interaction in lettuce

Valse meeldauw effector herkenning en gastheer eiwit interactie in sla

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties in
het openbaar te verdedigen op
maandag 2 juli 2018 des middags te 4.15 uur

door

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te Eindhoven

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Table of contents

Abbreviations and glossary

C/G/R/YFP: cyan/ green/ red/ yellow fluorescent protein

CNL: NLR with an N-terminal coiled-coil domain

CRN: Crinkler or crinkling- and necrosis-inducing protein. Type of effector that is predominantly found in oomycetes with a necrotrophic life stage.

cv: cultivar

***Dm* gene**: downy mildew resistance gene

Effector: protein secreted by pathogens to promote establishment and maintenance of an infection in the host

ETI: effector-triggered immunity

ETS: effector-triggered susceptibility

GAL4 AD: GAL4 activation domain

GAL4 DBD: GAL4 DNA-binding domain

hpRNA: hairpinRNA

HR: hypersensitive response, which results in localized cell death

LOD score: logarithm of odds score

M/PAMP: microbe/ pathogen-associated molecular pattern

MRC: major resistance cluster

MTF: membrane-associated transcription factor

NLR: nucleotide-binding and leucine-rich repeat receptor. These proteins recognize effectors or effector-mediated alterations to host proteins.

PRR: pattern recognition receptor

PTI: pattern-triggered immunity, which occurs upon activation of PRRs

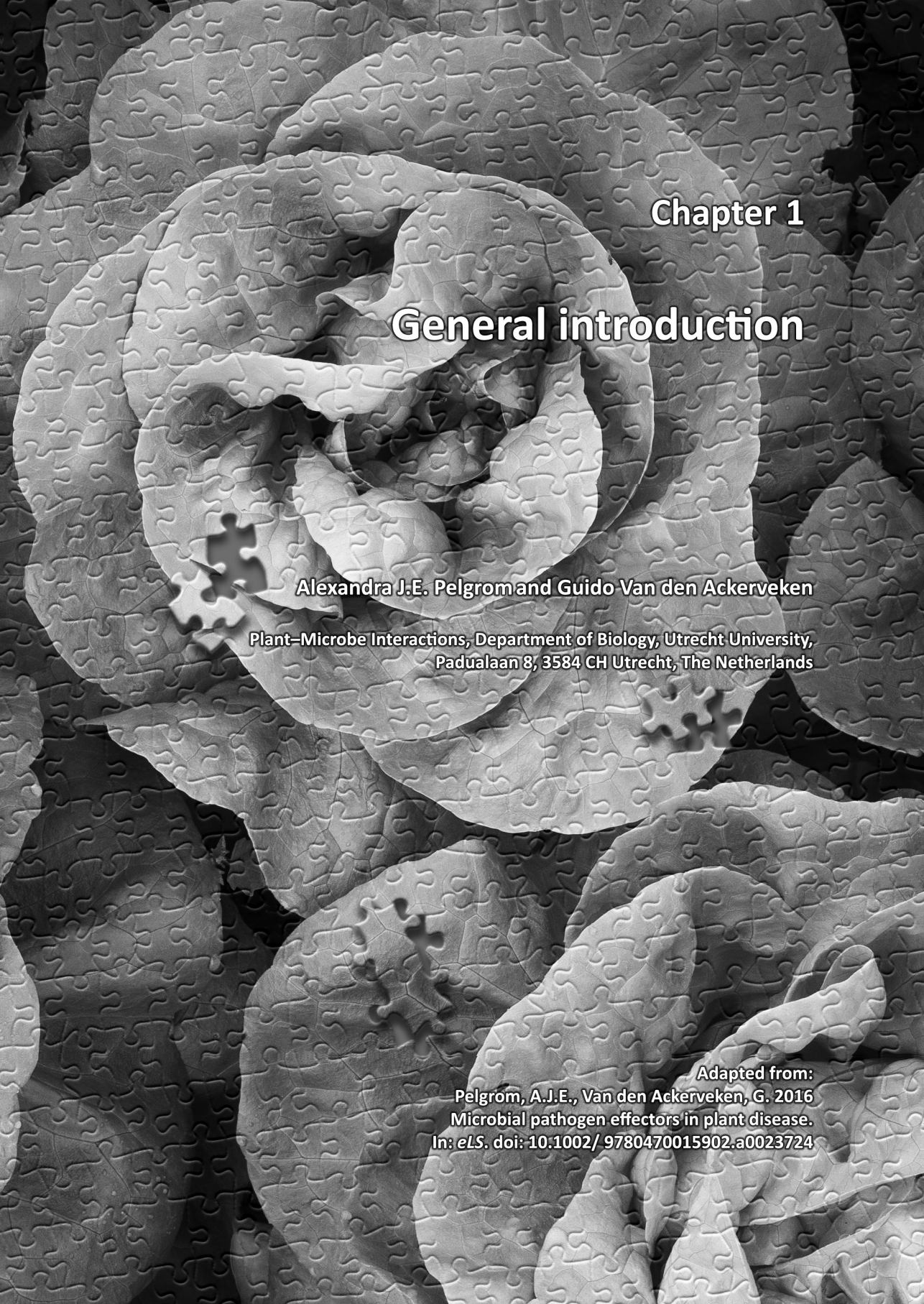
QTL: quantitative trait locus

R protein: resistance protein. These often confer monogenic dominant resistance.

***S* gene**: susceptibility gene. Plant genes that facilitate and support pathogen infection.

TNL: NLR with an N-terminal Toll/ Interleukin-1 receptor domain

Y2H: yeast-two-hybrid



Chapter 1

General introduction

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Adapted from:
Pelgrom, A.J.E., Van den Ackerveken, G. 2016
Microbial pathogen effectors in plant disease.
In: *eLS*. doi: 10.1002/ 9780470015902.a0023724

Abstract

Microbial plant pathogens use secreted effector proteins for successful infection of their host. This evolved state is rather exceptional as most microbes do not cause disease on the vast majority of plant species. An important primary activity of effectors is to interfere with a range of plant immune processes to evade and suppress pathogen detection, or to block immune signaling and downstream responses. Furthermore, effectors can enhance disease susceptibility by altering cellular processes and modulating host transcription. For most of these activities, effectors specifically target plant proteins that are central in these processes. An advanced virulence strategy is the post-translational modification by effectors of plant targets to change their activity or stability. The knowledge gathered on the molecular mechanisms underlying effector-triggered susceptibility of plants provides great potential for novel approaches of resistance breeding.

Key words

biotic stress; immune signaling; pathogen; disease resistance; effectors; effector-triggered immunity; plant pathology; resistance protein; protein modification; phosphorylation; ubiquitination

Key concepts

- Pathogens secrete and/or translocate effector proteins to promote plant disease.
- Besides their primary role in promoting disease, effectors or effector-modified plant proteins can be recognized by resistance proteins to activate an effector-triggered immune response.
- Many effectors block pathogen-associated molecular pattern (PAMP)-triggered immunity and/or effector-triggered immunity.
- Other effectors rewire signaling pathways and reprogram the plant cell to promote pathogen growth.
- Certain effectors can affect the activity or function of host proteins by post-translational modifications e.g. (de)phosphorylation or targeting for proteasomal degradation.

Introduction

Plant pathogenic bacteria, fungi, and oomycetes have diverse lifestyles and infection strategies, but have in common that they attempt to colonize and live at the expense of their host. Essential in infection by these microbial pathogens is the evasion or suppression of the host immune system and the modulation of host processes. To achieve this, pathogens secrete effector proteins that are collectively required to promote disease. On the other hand, effectors can also be potent triggers of the plant immune system and work against the pathogen producing them. In the molecular arms race between plants and their pathogens, effector genes are thus under constant evolutionary pressure.

In this review we discuss how bacteria, fungi and oomycetes use protein effectors to manipulate plant processes to their benefit. The immense and expanding number of identified effectors, different host targets, and mechanisms of action make it impossible to be comprehensive in this review. We have, therefore, chosen to provide general concepts with prime examples, highlighting the diversity and power of effector activities.

The plant immune system

During infection, conserved microbe- or pathogen-associated molecular patterns (M/PAMPs), e.g. flagellin, lipopolysaccharides, chitin, or other pathogen-derived molecules, can be recognized extracellularly by pattern recognition receptors (PRRs) at the plant plasma membrane. Most plants resist infection through detection of PAMPs by PRRs resulting in pattern-triggered immunity (PTI), which is associated with calcium influx, an oxidative burst, callose deposition and activation of a MAPK cascade to induce defense gene expression (Nicaise *et al.*, 2009). Adapted pathogens evade detection or suppress PTI by translocating effector proteins into host cells to cause disease, known as effector-triggered susceptibility (ETS). In turn, within the host species, specialized polymorphic intracellular receptors (NLRs) containing a Nucleotide binding and Leucine-rich Repeat domain have evolved that can recognize pathogen effectors and induce effector-triggered immunity (ETI). ETI is an amplified PTI response, which is often observed as a hypersensitive response (HR) that is associated with localized programmed cell death at the site of the attempted infection (Jones & Dangl, 2006).

NLR proteins, also referred to as plant Resistance (R) proteins, mediate recognition of single pathogen effectors, thereby fitting the gene-for-gene model. However, this high level of specificity in combination with a limited repertoire of NLR proteins (~150 in Arabidopsis) cannot explain how plants fend off a wide range of pathogens with unrelated effectors. This led to the hypothesis that R proteins do not always directly interact with pathogen effectors, but instead 'guard' other plant proteins, the guardees that are modified by one or more effectors. This model has been extended by introducing the concept of plant decoy proteins that have evolved to mimic aspects of guardees but are optimized for R protein activation. Interestingly, a subset of NLRs contains a variable integrated domain that is required for effector detection. These domains are therefore referred to as integrated decoys (Cesari *et al.*, 2014).

Generally, guardees are conserved plant proteins that are important for pathogen infection. This makes them valid targets for effector proteins and may drive convergent evolution of multiple, unrelated effectors to target the same plant protein. Indeed, extensive effector-target protein-protein interaction assays have shown that a number of

Arabidopsis proteins is targeted by diverse, sequence-unrelated effectors from pathogens of three kingdoms of life (Mukhtar *et al.*, 2011; Weßling *et al.*, 2014). Targeting these highly connected hubs with central regulatory roles allows pathogens to effectively suppress immune responses and rewire signaling.

Pathogens have evolved many different methods to effectively deal with the plant immune system (detailed later in Figure 2) allowing them to cause disease. In the following sections we present generalized concepts of effector activities that have allowed microbes to become pathogenic on plants.

Effector secretion and translocation

Many microbial effectors exert their function inside the plant cell, while others function in the apoplast, the free diffusional space outside of the plant cell membrane (Figure 1). Gram-negative bacterial pathogens often translocate effectors directly into the host cytoplasm via their Type III Secretion System (Galán & Wolf-Watz, 2006). A syringe-like structure traverses the bacterial inner and outer membranes and the plant cell wall resulting in a channel between the pathogen and host cytoplasm. Machine learning algorithms, developed on N-termini of reported type III effectors, have been used to predict Type III Secretion System signal sequence in candidate effectors (Arnold *et al.*, 2009; Samudrala *et al.*, 2009).

Fungi and oomycetes do not possess injection systems but translocate effectors in two steps that each use distinct amino acid motifs for targeting. First, secretion from the pathogen is ensured by a signal peptide or alternative motif on effector proteins. Secondly, the secreted effector requires a motif to facilitate translocation across the plant cell membrane. Once inside the host cell, dedicated cell sorting motifs, e.g. nuclear localization signals, can mediate further transport of effectors to their final subcellular destination.

Although a signal peptide is sufficient for secretion of apoplastic effectors, an additional host-translocation motif located directly downstream of the signal sequence is generally considered to be required to cross the plant cell membrane. In oomycete effectors two major classes of host translocation motifs have been identified: the bipartite RxLR-dEER motif in RXLR-like effectors and LxLFLAK motif in Crinklers (CRNs). It was shown that secretion of Avr3a by the oomycete *Phytophthora infestans* does not require the bipartite RXLR-dEER motif, but its mutation abolishes uptake into host plant cells (Whisson *et al.*, 2007). Also, the LxLFLAK containing N-termini of CRN 2, 8 and 16 mediate translocation (Schornack *et al.*, 2010).

Fungal effector prediction is complicated by the absence of conserved sequence motifs or structural folds within and between species. An exception is the identification of an N-terminal [YFW]xC motif in effector candidates of the barley powdery mildew fungus. [YFW]xC effector candidate gene expression was upregulated in haustoria-rich epidermal tissue compared to spores and hyphae. Nevertheless, it is unclear whether these proteins are translocated into host cells or remain apoplastic (Godfrey *et al.*, 2010).

A machine learning method, EffectorP, was recently developed to improve effector prediction. Training sets of known effector and non-effector sequences are used for pattern learning based on protein features, e.g. many apoplastic fungal effectors are relatively small and cysteine-rich proteins. The authors confirmed the relevance of low molecular weight and high cysteine content as prediction criteria, but found that protein net charge, and serine and tryptophan abundance are additional important discriminative features. However, EffectorP cannot distinguish apoplastic from translocated effectors (Sperschneider *et al.*, 2016).

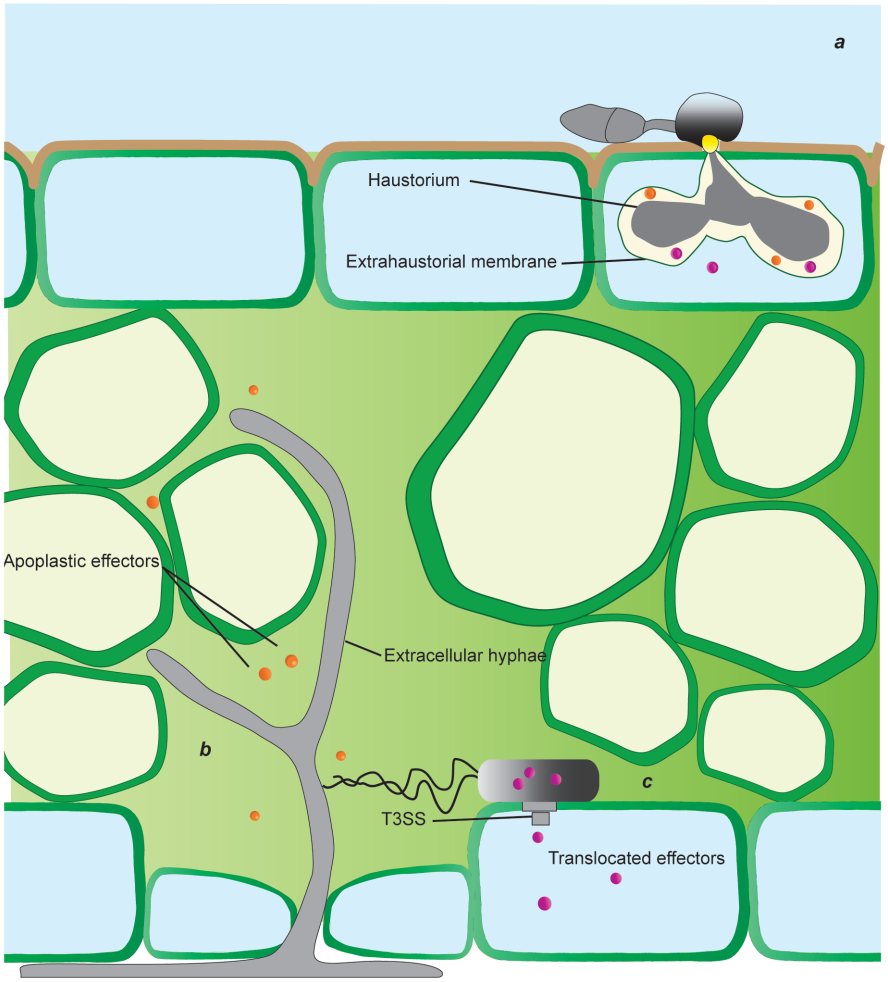

Figure 1, Plant invasion and effector delivery strategies of bacteria and filamentous pathogens. The delivery mechanisms and sites of action of effector proteins is largely determined by the pathogen lifestyle as shown in this schematic drawing of a cross section through an infected plant leaf. a) Filamentous fungal and oomycete pathogens may directly penetrate the cuticle and cell wall. In order to gain access to the host cytoplasm, extracellular pathogens may form haustoria: specialized feeding structures that remain separated from the host by the host-derived extrahaustorial membrane. Effectors that are secreted from the haustorium, remain extracellularly (orange), or may be translocated into the host (purple). b) Other filamentous pathogens enter via stomata. Hyphae can grow between the cells in the apoplast or invade the plant cells and spread intracellularly whilst remaining separated from the plant cytoplasm by the plant-derived extra-invasive hyphal matrix. c) Bacteria frequently gain access through stomata or wounds. Many Gram-negative species use a Type III Secretion System (T3SS) to translocate effectors directly into the host cytoplasm.

Evading and suppressing detection in the apoplast

Oomycetes and fungi secrete a range of molecules into the apoplast, e.g. toxins, cell wall-degrading enzymes and effectors. In this section, we focus on apoplastic effectors that evade or suppress pathogen detection and defense by the plant (see example in Figure 2a).

Plants produce papain-like cysteine proteases that degrade non-self-proteins as part of their defense. To circumvent breakdown of their secreted proteins, pathogens secrete an array of protease inhibitors into the apoplast that target papain-like cysteine proteases, e.g. the Avr2 effector of the fungus *Cladosporium fulvum* that inhibits the tomato papain-like cysteine protease Rcr3. Interestingly, in resistant *Cf-2* tomato lines the Rcr3 protein is guarded, enabling Avr2 detection and subsequent activation of ETI. Similarly, *P. infestans* produces a family of cystatin-like protease inhibitors. These EpiC effectors inhibit a variety of papain-like cysteine proteases including tomato Rcr3 and Pip1 but also the Arabidopsis C14 protease that focally accumulates around haustoria during infection. Another *P. infestans* effector, AvrBlb2, acts in the plant cytoplasm to prevent C14 secretion (Krüger *et al.*, 2002; Bozkurt *et al.*, 2011).

Molecular patterns of pathogens that are exposed during infection form a source of PAMPs. Chitin and peptidoglycan are indispensable components of the fungal and bacterial cell wall, respectively. However, the release of chitin oligomers and peptidoglycan fragments, aided by plant hydrolases, effectively activates PTI. To evade detection of these fragments *C. fulvum* secretes the Avr4 and Ecp6 effectors that have complementary activities. Avr4 binds to chitin on the fungal cell wall, thereby preventing secreted plant chitinases access to their substrate (Van den Burg *et al.*, 2006). Chitin oligosaccharides that, nevertheless, are released, are sequestered by LysM domains in Ecp6 proteins that occur in many fungal pathogens. This lowers the amount of free chitin oligomers that act as ligands for plant PAMP receptors (De Jonge *et al.*, 2010).

Interference with immune signaling

Many translocated effectors suppress immune responses (see Figure 2d). Interference of effectors with signaling, e.g. that initiated by perception of bacterial flagellin by the Arabidopsis receptor Flagellin-Sensing 2 (FLS2), is observed at different levels. Firstly, pathogens can deploy effectors to degrade the PAMP. Monomeric flagellin, but not filamentous flagellin, can be degraded by the *Pseudomonas syringae* alkaline protease AprA. AprA cleaves within the 22 amino acid epitope of flagellin (flg22) that is recognized extracellularly by FLS2 (Pel *et al.*, 2014).

Secondly, effectors can influence the accumulation of receptor complexes at the membrane. *P. syringae* effector HopUI transfers an ADP-ribose moiety to the RNA-binding protein GRP7. This blocks the interaction of GRP7 with *FLS2* mRNA and reduces accumulation of FLS2 protein levels during pathogen infection (Nicaise *et al.*, 2013).

The membrane localized receptor complex is the third level at which effectors can block signal transduction, e.g. of the FLS2-BAK1 complex, which activates PTI in response to flagellin. BAK1 functions as a co-receptor in complexes with different receptors that have diverse roles in immunity or plant growth, by activating downstream components through its cytoplasmic kinase domain. Additionally, the cytoplasmic kinase BIK1, associates with FLS2 and BAK1 in the cytoplasm. BIK1 undergoes sequential BAK1-mediated transphosphorylation and autophosphorylation within the activated complex and acts as a positive regulator of

immunity (Lin *et al.*, 2014). The bacterial effector AvrPtoB also associates with the FLS2-BAK1 complex and exerts its E3 ubiquitin ligase activity on FLS2 to target it for degradation. BAK1 was also found to interact with AvrPtoB but is not a major target for ubiquitination (Göhre *et al.*, 2008). Another *P. syringae* effector AvrPphb, a cysteine protease, proteolytically cleaves BIK1 to further block PTI signaling (Zhang *et al.*, 2010).

Activated PRR complexes undergo endocytosis that is associated with attenuation of signaling due to degradation or post-translational modifications. However, research in mammals demonstrated that signal transduction can continue in endosomal vesicles. Also, in plants, effectors could affect immune signaling from endocytic compartments. In plant cells expressing the *P. infestans* effector Avr3a, the number of endosomal vesicles with activated FLS2 was reduced by almost half. At the same time flg22-triggered defense gene activation and reactive oxygen species (ROS) production were strongly reduced. ROS act as signaling molecules to promote defense gene activation and HR. Avr3a interacts *in vitro* with the dynamin-related protein 2 that is required for FLS2 endosomal vesicle formation. The authors suggest that Avr3a interferes with vesicular trafficking to block FLS2 signaling (Chaparro-Garcia *et al.*, 2015).

Activation of PAMP receptors, such as FLS2, triggers mitogen-activated protein kinase (MAPK) cascades. MAPK-mediated phosphorylation of plant proteins, including transcription factors and enzymes, initiates a wide range of defense responses. In the MEKK1, MKK4/MKK5 and MPK3/MPK6 cascade at least two *P. syringae* effectors are active. HopF2 prevents MKK5-mediated activation of MPK6. The ADP-ribosyltransferase activity of HopF2 targets the region of MKK5 that is required for defense gene activation suggesting that ADP-ribosylation blocks MKK5 activity (Wang *et al.*, 2010). Just downstream, effector HopAI1 inactivates MPK3 and MPK6 through dephosphorylation and thereby quenches flg22-induced immune responses (Zhang *et al.*, 2007).

As a countermeasure, to detect effectors that interfere with PTI, the plant NLR SUMM2 guards MPK4. Inactivation of MPK4 by HopAI1 in Arabidopsis containing SUMM2 thus elicits an ETI response (Zhang *et al.*, 2012).

In contrast to the stacking of effector-mediated interference observed in the FLS2 signaling cascade, RPM1-interacting protein 4 (RIN4) is single-handedly targeted by at least five *P. syringae* effectors (AvrB, AvrRpm1, AvrRpt2, AvrPto and HopF2). RIN4 is a central player in the PTI response. It regulates stomatal aperture in a complex with plasma membrane H^+-ATPases. Plants close their stomata in response to PAMP detection to limit bacterial entry. Interestingly, *rin4* mutants do no longer re-open stomata in response to coronatine, a toxin produced by virulent strains of *P. syringae.* Moreover, *rin4* mutants display enhanced resistance to a type III secretion system deficient *P. syringae* mutant and increased callose deposition upon PAMP perception. Thus, RIN4 acts as a negative regulator of PTI.

AvrB promotes RPM1-Induced protein kinase-mediated RIN4 phosphorylation at threonine 21 and 166, and serine 160. Triple phosphorylated RIN4 promotes H^+-ATPase activity leading to increased stomatal aperture and thus bacterial growth (Lee *et al.*, 2015). Flg22 recognition induces phosphorylation at serine 141 to relieve RIN4-mediated suppression of PTI, e.g. visible as increased callose deposition, which also occurs in a RIN4 S141 phosphomimic. However, AvrB-induced phosphorylation overrules flg22-induced pS141 returning the plant to a state of PTI repression, e.g. leading to reduced callose deposition (Chung *et al.*, 2014).

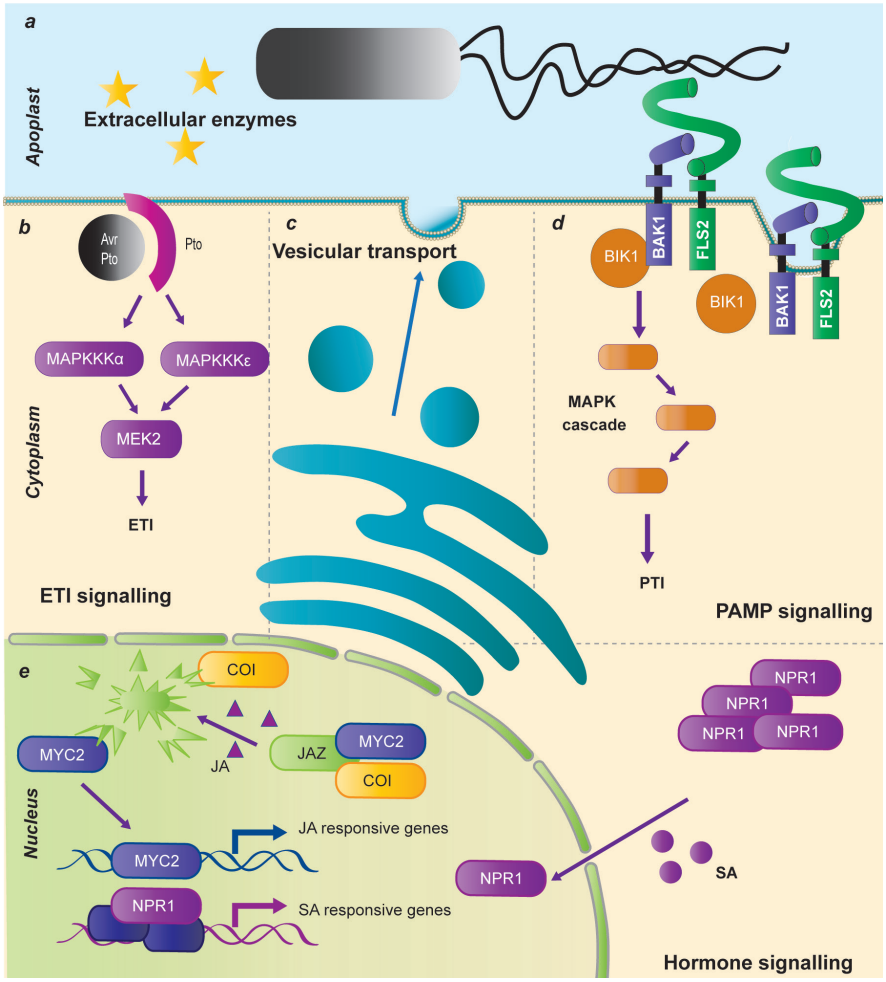

Figure 2, Examples of subcellular processes in a plant cell that are targets of pathogen effectors. a) Plants secrete a range of papain-like cysteine proteases into the apoplast to degrade non-self proteins. b) Recognition of effectors by resistance proteins leads to effector-triggered immunity. c) The secretory pathway delivers proteins and cell wall components to the apoplast constituting a first line of defence. d) Detection of PAMPs, e.g. bacterial flagellin, by PRRs in the plant cell membrane activates a MAPK cascade that results in a PTI response. e) The hormone salicylic acid (SA) accumulates in response to biotrophic pathogens, and acts together with NPR1 monomers that travel into the nucleus. There, activated NPR1 interacts with TGA transcription factors to promote transcription of SA-responsive genes. Another important defense hormone, jasmonic acid (JA), mediates the COI-dependent degradation of JAZ proteins. MYC2 is released in the process and drives the transcription of JA-responsive genes.

RIN4 is also an important player in ETI where it acts as a guardee. In resistant Arabidopsis accessions, both induction of phosphorylation by *P. syringae* effector AvrRpm1 or AvrB, and proteolytic cleavage by AvrRpt2, trigger a RPM1- or RPS2-dependent ETI response, respectively. This response can be suppressed by another effector, AvrPphb, that cleaves the kinase RPM1-Induced protein kinase, thereby preventing AvrB-induced phosphorylation of RIN4 and subsequent recognition by RPM1 (Russell *et al.*, 2015).

Suppression of cell death

The hypersensitive response that follows effector recognition restricts the growth of biotrophic pathogens and is often associated with programmed cell death (see Figure 2b). Suppression of cell death and other ETI responses can render resistance genes ineffective. Initiation of effector-triggered cell death by several resistance proteins is partially mediated by MAPKs. Recognition of *P. syringae* AvrPto by tomato Pto activates the MAPKKKα and MAPKKKε proteins that are proposed to converge on MEK2. The *P. infestans* effector PexRD2 is able to suppress MAPKKKε-mediated cell death, but not cell death mediated by overexpression of MAPKKKα or constitutively active MEK2 (King *et al.*, 2014). On the other hand, *Xanthomonas euvesicatoria* effector XopQ effectively suppresses MAPKKKα or constitutively active MEK2-mediated cell death, but not MAPKKKε mediated cell death (Teper *et al.*, 2013). These data suggest that activation of both MAPKKK pathways is required for AvrPto/Pto-triggered HR since expression of either effector is sufficient for inhibition of for AvrPto/Pto-triggered HR (Teper *et al.*, 2013; King *et al.*, 2014).

The *P. infestans* effector Avr3a suppresses cell death induced by the elicitin infestin1 and several R protein/effector pairs such as Cf-9/Avr9, Cf-4/Avr4 and Pto/AvrPto. The host ubiquitin E3 ligase CMPG1 is critical in this process. Activation of CMPG1 leads to proteasomal degradation of itself and its substrates. Furthermore, the E3 ligase activity of CMPG1 is required for infestin1-triggered cell death. Interaction with effector Avr3a stabilizes the host E3 ligase and thereby suppresses cell death (Gilroy *et al.*, 2011). Although effector-mediated interference in both the CMPG1 and MAPKKKα/ε pathways is sufficient to abolish AvrPto-induced HR, these pathways are not known to converge. Moreover, infestin1-, Cf-4/Avr4-, and Cf-9/Avr9-triggered HR responses could not be suppressed by PexRD2 or XopQ, respectively, precluding direct overlap.

Altering hormonal signaling

Salicylic acid (SA) confers local and systemic resistance against (hemi-)biotrophic pathogens, whereas jasmonic acid (JA) and ethylene (ET) induce resistance to necrotrophs. Cross-talk between the SA and JA branch of the immune system allows for optimization and prioritization of immune responses. Several plant hormones such as auxin, abscisic acid, gibberellins and cytokinins further fine-tune responses. For example, auxin and abscisic acid antagonize SA signaling and vice versa (Pieterse *et al.*, 2012). The flexibility in this extensive signaling network provides an opportunity for pathogens to shift the balance in their favor.

Biotrophic microbial attackers deploy effectors to attenuate or subvert SA metabolism and responses (see Figure 2e). The corn smut fungus *Ustilago maydis* secretes an effector, Cmu1, that acts as a chorismate mutase. Chorismate is a precursor of SA biosynthesis and its conversion to prephenate by Cmu1 reduces its availability for SA production (Djamei *et al.*, 2011). Also, *in planta* expression of the bacterial effector *XopJ*

leads to a reduction in SA levels and SA marker gene expression. *X. campestris* effector XopJ proteolytically degrades the proteasomal subunit RPT6. In the absence of RPT6, proteasome activity is impaired leading to accumulation of the ubiquitinated form of NPR1, a positive regulator of SA signaling. Ubiquitinated NPR1 could interfere with activation of NPR1 target genes and reduce immune responses (Üstün & Börnke, 2015).

Instead of decreasing SA levels, effector HaRxL44 of the downy mildew *Hyaloperonospora arabidopsidis* (*Hpa*) shifts the balance from an SA response to a JA and ET based response, that is tailored to defend against necrotrophic pathogens and has limited effectiveness against biotrophic pathogens. HaRxL44 interacts with Mediator subunit 19a and causes its proteasomal degradation. Mediator subunit 19a is part of a larger mediator complex that bridges the gap between transcriptional regulators and the transcription machinery. Expression of *HaRxL44* or loss of *Mediator subunit 19a* leads to an increase in JA/ET marker gene expression and reduced resistance to *Hpa* and other biotrophs (Caillaud *et al.*, 2013).

JA signaling is also induced by the *P. syringae* effectors HopX1 and HopZ1a. Both effectors promote degradation of JAZ proteins, which are negative regulators of the JA pathway. The subsequent activation of JA/ET defense responses antagonizes the SA pathway through molecular cross-talk (Jiang *et al.*, 2013; Gimenez-Ibanez *et al.*, 2014). Several *P. syringae* strains take it a step further by producing coronatine, a phytotoxin that structurally mimics the bioactive form of JA. Coronatine binding to the F-box protein coronatine-insensitive 1 promotes association and subsequent degradation of JAZ proteins, thereby promoting JA signaling (Tanaka *et al.*, 2015).

The necrotrophic fungus *Botrytis cinerea* benefits from induction of SA to antagonize JA. *B. cinerea* produces an exopolysaccharide that promotes SA accumulation and NPR1 mediated suppression of JA–induced defense genes (El Oirdi *et al.*, 2011).

Modifying host vesicular transport

Transport by vesicle trafficking between different intracellular compartments and the extracellular environment is important for plant immunity. The plant secretory or exocytic pathway is required for delivery of plant proteins mediating the first line of defense at the plasma membrane or in the apoplast (see Figure 2c). The *P. syringae* effector HopE1 targets the microtubule network to contribute to virulence. HopE1 interacts with microtubule-associated protein 65 in a calmodulin-dependent manner, which becomes redistributed from the microtubule network to the cytoplasm. Both *microtubule-associated protein 65-1* mutants and *HopE1* overexpressing plants display reduced secretion of pathogenesis-related protein PR-1 and diminished callose deposition (Guo *et al.*, 2016).

Secretion is also impaired by the *P. infestans* effector Avr1 that interacts with Sec5, a subunit of the exocyst complex. Sec5 is required for tethering of vesicles to their target membrane. Silencing of *Sec5* enhances susceptibility to *P. infestans* and abolishes callose deposition upon *P. syringae* infiltration. *In planta* expression of *Avr1* mimics the loss of *Sec5* as callose deposition is impaired suggesting that Avr1 manipulates plant immunity through interaction with Sec5 (Du *et al.*, 2015).

Other cellular traffic is affected by the *P. infestans* effector, PexRD54, that interacts with the autophagy-related protein ATG8CL, and colocalizes to autophagosomes. During autophagy cellular components are degraded to recycle building blocks and energy. PexRD54 stimulates ATG8CL-mediated autophagy as expression of *PexRD54* increases the number

of ATG8CL-labelled autophagosomes. However, PexRD54 outcompetes the endogenous selective autophagy cargo receptor Joka2 in this process, suggesting it may steer autophagy towards defense related components (Dagdas *et al.*, 2016).

Transcriptional reprogramming of the cell

Activation of immune pathways induces transcriptional reprogramming to activate defense responses. Pathogens therefore deploy nuclear-localized effectors that can modulate nuclear processes. The term 'nucleomodulin' has emerged to describe effectors that modify chromatin structure, affect epigenetic regulation or alter transcription (Bierne & Cossart, 2012).

Many *Xanthomonas* species encode transcription activator-like effectors (TALEs) that are injected into plant cells via the Type III Secretion System. The overall structure of TALEs is conserved; at the N-terminus, a type III translocation signal required for transport into the plant cell, and at the C-terminus nuclear localization signals and an acidic transcriptional activation domain for import into the nucleus and activation of plant gene transcription, respectively. The middle region consists of a variable number of near-perfect repeats of 33-35 amino acids long that mediate DNA binding. The identity and specificity of TALEs is largely determined by residues 12-13 within each repeat. These are referred to as the repeat-variable diresidues and almost 20 distinct combinations of amino acids occur at this position. The repeat-variable diresidues are the only part of the repeat that directly interact with the DNA helix, with the remainder of the repeat fanning out behind the repeat-variable diresidues. Each repeat-variable diresidue associates with a different affinity with adenine, cytosine, guanine and thymine. Some repeat-variable diresidues interact almost exclusively with a single nucleotide, such as NI with adenine or HD with cytosine. Others have similar binding affinities for several nucleotides, as NN can bind both guanine and adenine, and NS binds all four nucleotides. The combination of highly specific and more promiscuous repeat-variable diresidues within a repeat region results in both binding specificity and flexibility (Bogdanove *et al.*, 2010).

Although the TALE DNA binding cipher has been broken, relatively little is known about the target genes of TALEs. AvrBs3 was the first TALE for which targets were identified based on induced expression. These *UPA* (*upregulated by AvrBs3*) genes included putative auxin–induced genes and α-expansins. Of these, *UPA20*, a basic helix-loop-helix transcription factor, is postulated to be a direct target as its induction does not require *de novo* protein synthesis and interaction of AvrBs3 with an *UPA20* promoter fragment was found using electrophoretic mobility shift assays. Overexpression of *UPA20* is sufficient to induce the hypertrophy (cell enlargement) phenotype characteristic for plant tissue transiently overexpressing *AvrBs3* (Kay *et al.*, 2007).

Besides activating plant genes that increase plant susceptibility to disease, TALEs can also be responsible for their own demise. Transcription of the pepper *Bs3* and rice *Xa27 R* genes is specifically induced by the TAL effectors AvrBs3 and AvrXa27, respectively, due to compatible binding elements in the *R* gene promoter regions. This type of *R* gene is also referred to as executor gene, because the product does not recognize an effector or guardee, but only functions to execute an immune response or cell death. In line with this function, these executor genes do not share sequence resemblance to NLRs (Bogdanove *et al.*, 2010).

Also, the oomycete-specific Crinkler effectors predominantly reside in the nucleus upon translocation into host cells (Schornack *et al.*, 2010). The nuclear-localized *P. sojae* effector PsCRN108 contains a helix-hairpin-helix motif that is associated with DNA binding. *Arabidopsis* lines overexpressing PsCRN108 displayed reduced accumulation of almost half of the *heat shock proteins* transcripts. Heat shock proteins aid in proper folding of proteins and prevent the formation of protein aggregates. In line with their function, *heat shock protein* expression is upregulated in response to various abiotic and biotic stresses. The authors propose that PsCRN108 suppresses basal defenses by binding to heat shock elements in *heat shock protein* promoters, thereby interfering with the binding of endogenous transcription factors. Markedly, *PsCRN108* overexpression lines of *N. benthamiana* and Arabidopsis display enhanced disease susceptibility (Song *et al.*, 2015).

Whereas the effectors described above reprogram host cells themselves, another effective strategy is to interfere with host transcription factors. The potato membrane-bound NAC transcription factors NPT1 and NPT2 are associated with the endoplasmic reticulum in their dormant state but are activated upon perception of *Phytophthora.* Subsequently, relocalization of the transcription factors to the nucleus occurs, presumably to induce defense-associated transcriptional reprogramming. To negate these effects, the *P. infestans* RXLR effector Pi03192 associates with the NPTs at the endoplasmic reticulum membrane, where they maintain dormant so that translocation to the nucleus does not occur (McLellan *et al.*, 2013).

Sweet rewards

To acquire nutrients from the host, pathogens modify expression of the evolutionary conserved family of plant *SWEET* genes that encode sugar transporters. The *X. oryzae* TAL effector PthXo1 was shown to directly interact with the rice *SWEET11* (*OsSWEET11*) promoter and activate transcription. Loss of *PthXo1* expression or mutations in the *OsSWEET11* binding element leads to reduced pathogen growth. Pathogenicity can be regained by expression of effector *AvrXa7* that activates *OsSWEET14* (Chen *et al.*, 2010).

Exploitation of host sugar transporters is not limited to *Xanthomonas* as *P. syringae* infection induced expression of *AtSWEET4, AtSWEET5, AtSWEET7, AtSWEET8, AtSWEET10, AtSWEET12* and *AtSWEET15.* Also, the fungal pathogen *Golovinomyces cichoracearum* induced a distinct, but partially overlapping *AtSWEET* subset (Chen *et al.*, 2010). As these latter pathogens do not have TALEs, the mechanism of *SWEET* gene induction is unknown.

Future perspectives and applications

As more pathogen effectors are discovered and their modes of action in supporting disease susceptibility in plants are unraveled, the obtained knowledge can be used to develop new methods for durable disease resistance. Here three strategies are presented to illustrate the potential of effector-assisted breeding for resistance.

Knowledge of the effector repertoire of a pathogen can assist breeders in the identification of new *R* genes and advance the characterization of previously found ones. Firstly, *R* gene identification can be accelerated by screening germplasm with effectors using transient expression systems such as *Agrobacterium tumefaciens*. A similar approach can identify *R* genes with extended effector specificity when different allelic variants of an effector are tested. Secondly, new cultivars or wild progenitors can be classified based on effector responses. Natural *R* gene stacks can be broken down to a collection of effector recognition specificities (to Mendelize *R* genes). Additionally, when *R* gene resistance is quantitative and only a partial resistance effect is observed in the field, breeding is simplified when it can be linked to an easier quantifiable trait such as effector recognition. For example, field resistance mediated by *Rpi-Smira2* in potato cultivar 'Sarpo Mira' is associated with recognition of RXLR effector AvrSmira2 (Rietman *et al.*, 2012). Lastly, the identification of effectors that are essential for pathogen virulence by spatio-temporal monitoring of effector repertoires allows for rationalized *R* gene deployment and may provide more durable resistance (Vleeshouwers & Oliver, 2014).

A second recently emerged strategy focuses on the plant targets of effectors, in particular plant genes required for pathogen infection. Loss of these so-called *Susceptibility* (*S*) genes confers resistance to pathogen infection. In a screen for interactors of the HopZ family of *P. syringae* effectors the Arabidopsis MLO2 protein was identified as target. Interestingly, *mlo* resistance has been successfully used in barley against powdery mildew infection for over 70 years. Similar resistance is observed in Arabidopsis *mlo2* mutants. *S* genes will be deployed only if the benefit gained by increased pathogen resistance outweighs the negative effect of gene loss. Alternatively, a germplasm set can be screened for alleles of the *S* gene in which the protein function is maintained but effector interaction is lost (Dangl *et al.*, 2013).

Similar to the concept of *R* gene stacking, TAL effector binding elements in executor gene promoter regions can be stacked. The promoter of *Xa27* in rice conferring resistance to *AvrXa27*-containing *X. oryzae* strains was expanded with six additional TALE binding elements. The rice plants gained resistance to a variety of strains that are virulent on wildtype *Xa27* plants. The difficulty in expanding promoter regions is the risk of unintentionally introducing endogenous regulatory elements that may be activated in response to environmental or developmental cues (Hummel *et al.*, 2012).

The technological progress made in the last decade, especially the availability of low-cost large-scale sequencing techniques, has enabled researchers to determine the effector repertoire of many pathogens. The identification of plant targets for pathogen effectors has subsequently taken flight. With interest we await how these fascinating scientific discoveries will be translated to disease resistant crops in the future.

Thesis outline

The aim of this thesis is to explore the interactions between effectors of the plant pathogenic oomycete *Bremia lactucae* and proteins of lettuce, the pathogen host, to gain a better understanding of the molecular mechanisms underlying host susceptibility. As discussed in this introductory **Chapter 1**, effectors manipulate host proteins involved in a broad range of cellular processes to establish a successful infection. But, effectors can be caught in the act by intracellular receptors that activate host immune responses. Since these two aspects of effectors – manipulation of the host by the effector and recognition of effectors by the host – define the outcome of the battle between lettuce and *B. lactucae*, they were both investigated in my research project.

In **Chapter 2**, the response of >150 lettuce lines to transient expression of 14 *B. lactucae* effectors is described. This resulted in the identification of three *B. lactucae* effectors that trigger immune responses in specific lettuce lines. Subsequently, the response to effector BLR38 was mapped in segregating F2 and F3 populations and two unlinked loci for BLR38 recognition were identified that act cooperatively. Recognition of BLR38 was associated with resistance to five *B. lactucae* races but not to the race it was cloned from, Bl:24. This raises the possibility that other effectors in Bl:24 suppress plant responses following effector recognition.

In **Chapter 3** the execution of a yeast-two-hybrid (Y2H) screen is described that aimed to elucidate the targets of *B. lactucae* effectors in lettuce. To investigate the relevance of the identified protein-protein interactions, selected effectors and their targets were expressed as fluorophore fusion proteins in *N. benthamiana* and their subcellular localization was determined. Interestingly, co-expression of effectors with their targets induced relocalization to and colocalization in the plant cell nucleus for four effector-target pairs.

A NAC transcription factor with a C-terminal transmembrane domain was targeted by four *B. lactucae* effectors in the Y2H screens (**Chapter 3**) and was further explored in **Chapter 4**. Y2H assays between *B. lactucae* effectors and orthologous NAC proteins from potato and Arabidopsis indicated that NAC proteins may be conserved targets of oomycete effectors. Furthermore, relocalization of the lettuce NAC protein from the endoplasmic reticulum membrane to the nucleus was induced using *P. capsici* culture filtrate treatment but was reduced upon co-expression of *B. lactucae* effectors. Finally, the role of the lettuce NAC protein in susceptibility to *B. lactucae* infection was studied in transgenic lines with silencing constructs. Silencing of the NAC in lettuce did not alter susceptibility to *B. lactucae* infection, but this finding does not preclude a role for NAC proteins in disease development.

In **Chapter 5** the most important findings are summarized and placed in a broader context. Furthermore, suggestions for future research and possible applications are presented.

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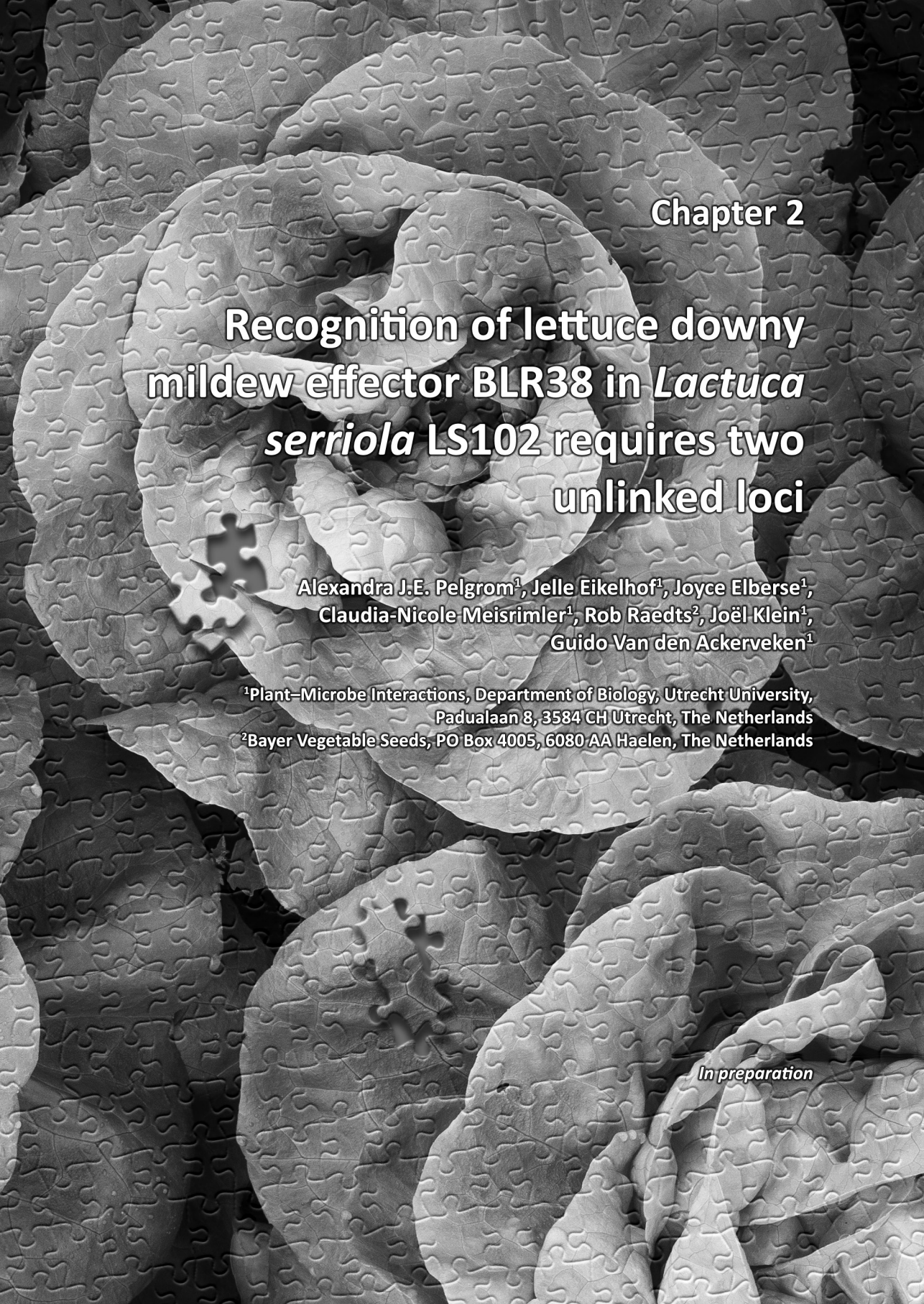

Chapter 2

Recognition of lettuce downy mildew effector BLR38 in *Lactuca serriola* LS102 requires two unlinked loci

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In preparation

Summary

- Plant pathogenic oomycetes secrete effector proteins to suppress host immune responses. Resistance genes may recognize effectors and activate immunity, which is often associated with a hypersensitive response (HR). Transient expression of effectors in plant germplasm and screening for HR has proven a powerful tool in the identification of new resistance genes.
- In this study, a set of fourteen effectors from the lettuce downy mildew *Bremia lactucae* race Bl:24 was screened for HR induction in over 150 lettuce accessions. Three effectors - BLN06, BLR38 and BLR40 – were recognized in specific lettuce lines.
- Recognition of effector BLR38 in *Lactuca serriola* LS102 did not co-segregate with resistance against race Bl:24 but was linked to resistance against multiple other *B. lactucae* races.
- Two unlinked loci are both required for effector recognition and are located near known major resistance gene clusters. Gene dosage affects the intensity of the BLR38-triggered HR but is of minor importance for disease resistance.

Introduction

Plants rely on their innate immune system to fend off a wide range of pathogens. Initial detection of pathogens occurs through recognition of conserved motifs, collectively referred to as pathogen-associated molecular patterns (PAMPs), by specialized receptors on the plant cell surface and the subsequent activation of pattern-triggered immunity (PTI). Plant pathogens can evade or suppress PTI by secreting effector molecules that act in the plant apoplast or intracellularly, thereby inducing a state of effector-triggered susceptibility. However, resistant host genotypes can recognize host-translocated effectors by intracellular nucleotide-binding and leucine-rich repeat receptors (NLRs) and launch an effector-triggered immune response that is frequently associated with a hypersensitive response (HR), a form of localized cell death (Jones & Dangl, 2006).

Two classes of host-translocated effectors have been discovered in plant pathogenic *Phytophthora* species and downy mildews: the Crinkler (CRN) effectors and RXLR(-like) effectors (Anderson *et al.*, 2015). Crinklers are modular proteins, most of which have an N-terminal signal peptide, followed by LXLFLAK and DWL domains. Analysis of 315 *Phytophthora* CRNs resulted in the identification of 36 conserved C-terminal domains (Haas *et al.*, 2009). The N-terminus of RXLR-like effectors is characterized by a signal peptide followed by an RXLR or related motif, such as GKLR or QXLR, in the first 40 amino acids after the signal peptide cleavage site, and in some cases an EER motif (Stassen *et al.*, 2013). The C-terminal effector domains of many *Phytophthora* RXLR effectors contain conserved W, Y and L sequence motifs (Jiang *et al.*, 2008). Crystal structure analysis of two seemingly sequence-unrelated *Phytophthora* effectors revealed a conserved α-helical fold, the WY domain, to which the previously identified W and Y sequence motifs map. The WY domain is present as a single module or repeat in ~44% of *Phytophthora* RXLR effectors (Boutemy *et al.*, 2011).

Host-translocated effectors may activate the immune system by interacting directly with NLRs or through modification of host proteins that are 'guarded' by NLRs (Van Der Biezen & Jones, 1998; Dangl & Jones, 2001). NLRs can be classified into two main categories based on their N-terminal domain: Toll/ Interleukin-1 receptor (TIR) -type NLRs (TNLs) and coiled-coil (CC)- type NLRs (CNLs). The TIR and CC domains are followed by a nucleotide-binding domain that forms the core of the resistance protein, and C-terminal leucine-rich repeats. Resistance was thought to be mediated predominantly by single NLRs. However, in recent years the idea has emerged that NLRs can operate in pairs or networks (Wu *et al.*, 2017), in which the 'sensor' NLR is responsible for effector detection and the 'helper' NLR initiates immune signaling (Cesari *et al.*, 2014; Sukarta *et al.*, 2016; Zhang *et al.*, 2017). These pairs have evolved in both mono- and dicotyledonous plants. The genetically linked rice pair *RGA4/RGA5* confers resistance against the fungal pathogen *Magnaporthe oryzae.* The 'sensor' CNL RGA5 negatively regulates the 'helper' CNL RGA4 in the absence of pathogen effectors to prevent RGA4-mediated autoimmune responses (César *et al.*, 2014). Negative regulation limits expansion of NLR pairs as loss of the 'sensor' NLR would be detrimental for the plant. A network composed of multiple 'sensor' and 'helper' CNLs that operate through positive regulation is present in members of the asterids clade (e.g. coffee, pepper and tomato) but not in the rosids clade (e.g. Arabidopsis and soybean) (Wu et al., 2017). The asterids network is proposed to have evolved from a single NLR pair over 100 million years ago (Wu *et al.*, 2017). Independently, the Arabidopsis ADR1 family evolved, consisting of three CNLs, that act redundantly as 'helper' NLRs to initiate immune signaling

in cooperation with several CNL or TNL 'sensors' (Bonardi *et al.*, 2011).

Genome analysis of lettuce (*Lactuca sativa*) cultivar Salinas identified 47 CNLs and 189 TNLs dispersed over all nine chromosomes of lettuce, although the majority of NLRs reside in five major resistance clusters (MRCs) on chromosomes 1, 2, 3, 4, and 8 (Christopoulou *et al.*, 2015a). Lettuce MRCs contain, for example, downy mildew (*Dm*) resistance genes that confer protection against specific races of the oomycete *Bremia lactucae* (Christopoulou *et al.*, 2015a; Parra *et al.*, 2016; Giesbers *et al.*, 2017). MRC2 alone contains eight *Dm* genes, including *Dm3. Dm3*-mediated resistance is conferred by the CNL *RGC2B* (Shen et al., 2002). Substantial yield loss due to infection of lettuce with the oomycete *B. lactucae* is an important agricultural problem and introgression of *Dm* genes is a major focus of commercial breeding programs. However, resistance mediated by newly introgressed *Dm* genes is rapidly broken by constantly evolving *B. lactucae* races. Wild lettuce species such as *L. saligna*, *L. serriola* and *L. virosa* are exploited as sources of new resistance genes (Parra *et al.*, 2016).

Large scale *Agrobacterium tumefaciens*-mediated transient expression of effectors has proven a useful tool in the identification and dissection of effector recognition specificities and disease resistance genes (Vleeshouwers *et al.*, 2008; Wroblewski *et al.*, 2009; Stassen *et al.*, 2013; Vleeshouwers & Oliver, 2014; Giesbers *et al.*, 2017). Previously, recognition of four *B. lactucae* effectors that were transiently expressed via *A. tumefaciens* in a *Lactuca* germplasm set, was reported (Stassen *et al.*, 2013; Giesbers *et al.*, 2017). Effectors BLG01 (GKLR motif), BLN08 (no RXLR motif) and BLR31 (RXLR motif) were specifically recognized in multiple *L. saligna* accessions. BLR31 recognition also co-segregated with resistance to *B. lactucae* race 24 (Bl:24) and was mapped to MRC2 (Giesbers *et al.*, 2017). Interestingly, recognition of BLG01 and BLN08 was not linked to resistance to the effector-producing race (Stassen *et al.*, 2013; Giesbers *et al.*, 2017). Finally, BLG03 (GKLR motif) is recognized in *L. sativa* cv. Amplus and UCDM2 that harbor the resistance gene *Dm2*. Although recognition of BLG03 did not result in resistance against Bl:24, recognition did co-segregate with resistance against *B. lactucae* race Bl:5 (Stassen *et al.*, 2013).

In this study, fourteen *B. lactucae* effectors were expressed in a lettuce germplasm set and the response of specific lettuce lines to three effectors is described. Interestingly, the response to RXLR effector BLR38 in *L. serriola* LS102 was mapped to two unlinked loci. Detailed analysis of BLR38-induced responses revealed that the identified QTLs are both required and confer gene dosage-dependent HR. BLR38 recognition did not co-segregate with resistance against race Bl:24 but was linked to resistance against multiple other *B. lactucae* races.

Materials & Methods

Plant growth conditions and *B. lactucae* maintenance

Lettuce seed germination as well as *B. lactucae* maintenance and disease assays were performed under short day growth conditions (9 h of light (100 µE/m2/s)) at 16°C. To maintain high humidity during infection, a tray with infected plant material was closed with a transparent lid, a dish with water was placed inside and the edges were sealed with tape. *Nicotiana benthamiana* plants and germinated lettuce seedlings were grown under long day conditions (16 h of light, 70% humidity) at 21°C. *L. serriola* LS102 is also known as LS-102 and corresponds to accession number CGN24780 at the Centre for Genetic Resources (Wageningen, the Netherlands).

Candidate effector identification and cloning

To expand the *B. lactucae* effectorome, Illumina-based RNAseq was performed on mRNA derived from *B. lactucae* spores and *B. lactucae*-infected lettuce seedlings as described (Giesbers *et al.*, 2017). To identify novel effector candidates, proteins in the secretome (Giesbers *et al.*, 2017) were analyzed with a Perl script using regular expressions for RXLR-like, dEER and LXLFLAK motifs. Secondly, a homology search of the secretome against a database composed of known effector sequences from *B. lactucae, P. cinnamomi, P. infestans, P. parasitica, P. ramorum, P. sojae, P. andina, Pseudoperonospora cubensis* and *Hyaloperonospora arabidopsidis* was conducted. WY motifs were identified using an HMM model (Boutemy *et al.*, 2011) and, after manual inspection, a cut-off E-value of 0.001 was set for the best motif within an effector sequence. Effector coding sequences were PCR amplified from *B. lactucae* race 24 cDNA from the signal peptide cleavage site and cloned in pENTR/D-TOPO (Invitrogen) with a new start codon. The resulting entry clones were recombined with binary vector pK2GW7 using LR clonase to generate untagged effector constructs suitable for transformation in *Agrobacterium tumefaciens* strain C58C1 (pGV2260) according to (Stassen *et al.*, 2013). The primers used for cloning are listed in Supplemental table 1.

Accession numbers

Effector coding sequences were deposited under the following Genbank numbers: BLR32 (MG686566), BLR33 (MG686567), BLR35 (MG686568), BLR36 (MG686569), BLR37 (MG686570), BLR38 (MG686571), BLR40 (MG686572), BLC01 (MG686573), BLN01 (MG686574), BLN03 (MG686575), BLN04 (MG686576), BLN05 (MG686577), BLN06 (MG686578), and BLQ04 (MG686579).

Effector recognition assays in lettuce

A. tumefaciens strains harboring *B. lactucae* effector constructs were grown in LB medium o/n at 28°C and 200 rpm. Cells were spun down for 10 min at 4000 rpm and resuspended in induction medium (8.5 gr/L Na_2HPO_4 x $2H_2O$, 3 gr/L KH_2PO_4, 0.5 gr/L NaCl, 1 gr/L NH_4Cl, 1% (w/v) glucose, 50 µM acetosyringone, rifampicin (50 µg/ml), carbenicillin (50 µg/ml) and spectinomycin (100 µg/ml)) to an OD_{600}<1. Cultures were incubated for 3-4 hours at 28°C and 200 rpm. Cells were spun down for 10 min at 4000 rpm and resuspended in infiltration medium (0.5x MS salts, 10 mM MES, 0.5 % (w/v) sucrose, 0.5 % (w/v) fructose, 150 µM acetosyringone, pH 5.6) to an OD_{600} of 0.4. Leaves of three-weeks-old lettuce plants were infiltrated with bacterial suspensions and scored 3-5 days after infiltration. Infiltration sites were scored from 0 (no chlorosis) to 4 (severe necrosis). Plants that clearly responded to

the negative control YFP or failed to respond to the positive control, *Phytophthora sojae* necrosis-inducing protein (PsojNIP) (Qutob *et al.*, 2002), were left out of the analysis.

RNA isolation
Seedlings were flash-frozen in liquid nitrogen and ground using mortar and pestle or the TissueLyser (Qiagen) to a fine powder. Total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma Aldrich) and treated with DNase I (Thermo Fisher Scientific) to remove genomic DNA. cDNA was synthesized using RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific).

Time course quantitative RT-PCR
Three to four-day-old *L. sativa* cv. Olof seedlings were sprayed with tap water or a spore suspension of *B. lactucae* race Bl:24 (~100 spores/µl). Cotyledons were collected at 3 hours, 1 day, 3 days or 6 days after spraying. Relative transcript levels were determined in three biological replicates per experiment (each replicate being measured in two technical replicates) using SYBR Green PCR Master mix (ThermoFisher Scientific) and the ViiA7 Real-Time PCR system (ThermoFisher Scientific). *B. lactucae ACTIN* transcript levels were normalized to *L. sativa ACTIN* transcript. Effector gene expression levels were normalized to *B. lactucae ACTIN*. The primers used for qRT-PCR are listed in Supplemental table 1.

Leaf disc assays
Leaf discs were punched from four-week-old lettuce plants, placed upside down in a transparent tray on four layers of soaked filter paper and sprayed with a spore suspension of *B. lactucae*. The spore density differed between experiments and races and was 50-150 spores/µl. At 8-11 days after inoculation the percentage of leaf disc area covered in sporangiophores was scored, unless indicated otherwise.

Genotyping
Ten leaf discs (6 mm Ø) were punched per plant and placed in a 96-wells tray. Leaf discs were dried by placing a silica bag on top of the plate and applying a vacuum to the sealed package. DNA was isolated using the sbeadex maxi plant kit (LGC) and verified on the NanoDrop-8000 spectrophotometer. Genotyping was carried out using the KASP genotyping chemistry (LGC) on Fluidigm chips. Data was analysed using the Fluidigm SNP genotyping software. A genetic linkage map was constructed using JoinMap 4.0 software (Van Ooijen, 2006) and quantitative trait loci were detected using MapQTL 5.0 software (Van Ooijen, 2004).

To determine the smallest mapping interval on chr 4, BLR38 unresponsive plants homozygous for *L. serriola* LS102 alleles at the QTL on chr 8 were selected. In these plants, BLR38 unresponsiveness was caused by homozygous *L. sativa* GreenTowers alleles on chr 4 at marker positions closely linked to BLR38 recognition. After delineating the interval on chr 4, the smallest mapping interval on chr 8 was determined with BLR38 unresponsive plants that were homozygous *L. serriola* LS102 on chr 4. For the markers defining the smallest mapping intervals on chr 4 and chr 8, the single nucleotide polymorphism (SNP) with its flanking sequence is listed in Supplemental table 1.

Sequencing of effector alleles

Primers (Supplemental table 1) were designed on the 5' and 3' untranslated regions of *BLR38* mRNA originating from Bl:24. *BLR38* was amplified using Phusion High-Fidelity DNA polymerase from cDNA prepared from seedlings heavily infected with *B. lactucae* races Bl:22, Bl:23, Bl:24 Bl:26, Bl:28 or Bl:31. PCR products containing attB sites were recombined in a modified pGemTEasy vector containing the pDONR201 Gateway recombination site (pGemTEasy^{mod}), in a BP clonase reaction. Per race ten transformed *E. coli* DH5α colonies were selected for plasmid DNA isolation and subsequent Sanger sequencing. Single nucleotide polymorphisms, identified by comparison with the Bl:24 *BLR38* reference sequence, were considered when detected in at least three independent sequences.

Effector localization in *N. benthamiana*

Open-ended constructs of BLN06, BLR38 and BLR40 were cloned in (pGemTEasy^{mod}). Entry clones were recombined with pB7YWG2 using LR clonase to generate constructs with the effector fused at the C-terminus to YFP. *A. tumefaciens* strains were resuspended in infiltration buffer (10 mM MES, 10 mM MgCl2, and 150 µM acetosyringone, pH 5.6) to an OD of 0.4 per strain. An *A. tumefaciens* strain carrying pB7WGC2 (free CFP) was co-infiltrated with strains harboring effector fusion constructs in the leaves of four to five-week-old *N. benthamiana* plants. Leaf sections were examined at 2-3 dpi using a Zeiss LSM 700 laser scanning microscope. Plant cell walls were stained by 7-10 min incubation in 5 mg/ml propidium iodide (PI) solution. CFP, YFP and PI were excited at 405 nm, 488 nm and 555 nm respectively. Emitted light of both CFP and YFP was captured using a 490-555 nm band-pass filter, and emitted light of PI was captured using a 560 nm long pass filter.

Results

BLN06 and BLR38 are recognized in *L. serriola* LS102

Previously, sixteen novel *B. lactucae* effectors were identified from transcriptome sequences, though from this set only two effectors, BLN08 and BLR31, were described so far (Giesbers *et al.*, 2017). The remaining fourteen effectors include the first identified *B. lactucae* Crinkler (BLC), eight effectors with the canonical RXLR motif (BLRs), one effector containing a QXLR motif (BLQ), and six effectors that only contain an EER-like domain (BLN) (Table 1 and Supplemental table 2). Putative WY domains were detected in two BLR effectors and two BLN effectors (Table 1).

To determine if these fourteen effectors are specifically recognized in lettuce, coding sequences were amplified starting from the position corresponding to the predicted signal peptide cleavage site, cloned, and transiently expressed in a lettuce germplasm set consisting of 158 accessions and lines using *A. tumefaciens*. After the initial large-scale screen (Supplemental table 3), effectors that triggered necrosis or chlorosis were validated in a second screen and responses to three effectors were confirmed (Supplemental table 4). Effector BLR40 triggered a robust HR in *L. sativa* cv. Design (Supplemental figure 1a&b), and effectors BLN06 and BLR38 were both recognized in *L. serriola* LS102 (Figure 1a&b). Several lines of evidence suggest that recognition of BLN06 and BLR38 in *L. serriola* LS102 is based on different mechanisms. Firstly, the response to BLN06 was visible as chlorosis and was considerably weaker than the response to BLR38 that induced strong necrosis. Secondly, BLN06 was also recognized in *L. sativa* NunDm17 and RYZ2164; two lettuce cultivars that did

not respond to BLR38 (Figure 1b). Furthermore, *BLN06* and *BLR38* encode proteins of 502 and 260 amino acids, respectively, that share sequence homology with other *B. lactucae* effectors (Supplemental figure 2) but not with each other. Finally, C-terminal YFP fusion proteins of BLN06 and BLR38 showed a different subcellular localization pattern *in planta*. BLN06-YFP and BLR40-YFP accumulated in the plasma membrane, whereas BLR38-YFP was exclusively present in the nucleus (Supplemental figure 3a). Recognition of BLN06-YFP and BLR38-YFP in *L. serriola* LS102 was not significantly different from untagged versions (Supplemental figure 3b).

Expression of *BLN06, BLR38* and *BLR40* during infection

BLN06, *BLR38* and *BLR40* transcripts were originally identified in lettuce seedlings infected with *B. lactucae* race Bl:24 indicating that these effectors are expressed during infection. To determine if expression of *BLN06*, *BLR38* and *BLR40* varies at different infection stages, *L. sativa* cv. Olof seedlings were infected with race Bl:24 and samples were taken at 3 hours, 1 day, 3 days and 6 days after inoculation. *B. lactucae* biomass increased rapidly during this period and a ~6-fold higher transcript abundance of *B. lactucae ACTIN* compared to lettuce *ACTIN* was observed at 6 dpi (Supplemental figure 4). From 6 dpi onward sporulation occurred on susceptible cotyledons. The relative abundance of *BLN06*, *BLR38* and *BLR40* transcript compared to *B. lactucae ACTIN* decreased slightly during the time course (Supplemental figure 4) but did not show signs of strong down-regulation. Thus, transcription of these effectors could not be associated with specific infection stages.

Table 1, Overview of newly identified *B. lactucae* effectors.

Crinkler effectors

Effector	*Protein length*	*Signal peptide*	*LXLFLAK motif*		*HVLVXXP motif*	
			Motif	Start position	Motif	Start position
BLC01	198	1-15	LRLFLAK	49	HVLVVP	114

RXLR-like effectors

Effector	*Protein length*	*Signal peptide*	*RXLR-like motif*		*EER-like motif*		*WY domains*
			Motif	Start position	Motif	Start position	
BLN01	652	1-18	-	-	EER	48	yes
BLN03	169	1-21	-	-	EER	52	no
BLN04	147	1-23	-	-	EER	59	no
BLN05	491	1-18	-	-	EER	50	no
BLN06	502	1-20	-	-	EER	51	yes
BLQ04	531	1-15	QILR	27	EER	54	no
BLR32	146	1-22	RLLR	44	DER	56	no
BLR33	270	1-22	RRLR	50	DER	64	no
BLR35	514	1-16	RSLR	47	EER	58	no
BLR36	476	1-23	RALR	55	EER	68	yes
BLR37	814	1-33	RRLR	42	-	-	yes
BLR38	260	1-18	RLLR	46	-	-	no
BLR40	145	1-22	RRLR	44	EER	56	no

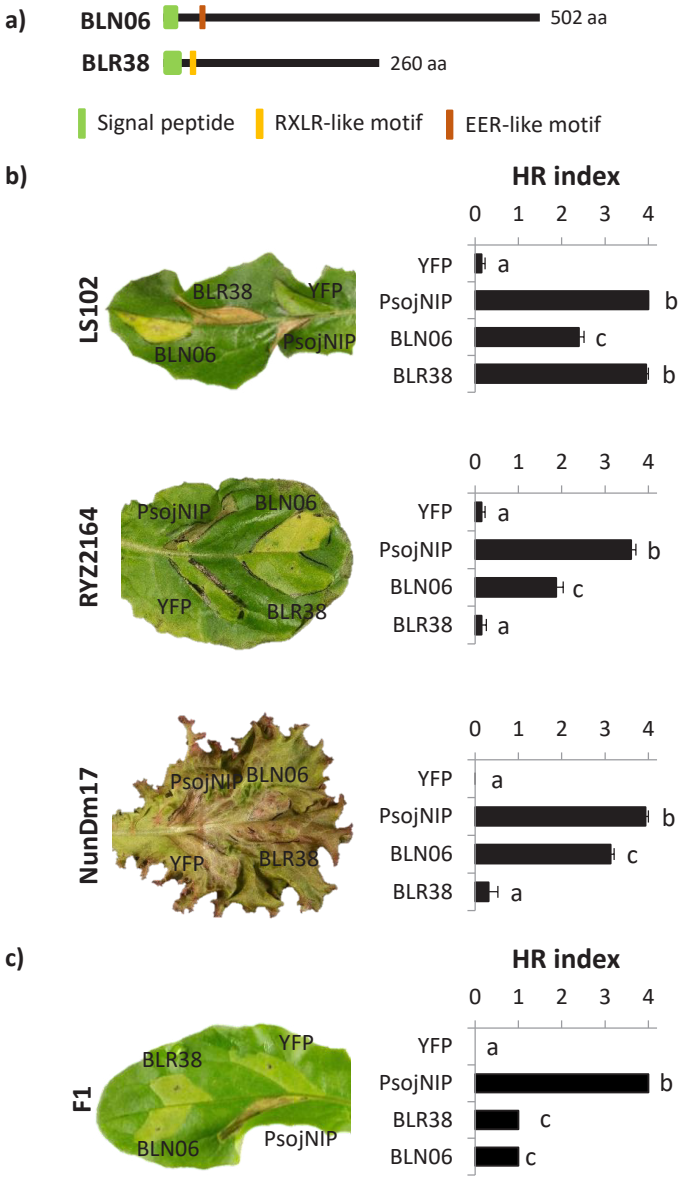

Figure 1, BLN06 and BLR38 are recognized in specific lettuce lines. a) Schematic representation of *B. lactucae* effectors BLN06 and BLR38. b) BLR38 and BLN06 are both recognized in *L. serriola* LS102, and BLN06 is also recognized in *L. sativa* cv. RYZ2164 and NunDm17. Hypersensitive responses were scored 5 days after infiltration of *Agrobacterium*. Bars represent the mean + SE from 16-20 infiltration sites. c) Hypersensitive responses were scored 5 days after infiltration of *Agrobacterium* in F1 plants of *L. serriola* LS102 x *L. sativa* GreenTowers. Bars represent the mean + SE from 8 infiltration sites. Statistical differences were assessed using ANOVA with *post-hoc* Tukey testing.

Recognition of BLR38 requires two unlinked loci

To determine if recognition of BLN06 and BLR38 is inherited as a dominant trait, *L. serriola* LS102 was crossed with *L. sativa* cv. GreenTowers that does not recognize BLN06 or BLR38 and is susceptible to *B. lactucae* infection. Transient *Agrobacterium*-mediated expression of *BLN06* and *BLR38* in the resulting F1 plants and F2 population led to two striking observations. First, BLN06 responsiveness segregated in an unresponsive (28.7%), chlorotic (64.8%) and necrotic (6.5%) fraction in the F2 population, whereas only chlorosis was observed in F1 plants (Figure 1c) and *L. serriola* LS102 (Figure 1b). Secondly, expression of *BLR38* in F1 plants resulted consistently in intermediate response levels (i.e. chlorosis) (Figure 1c), which is indicative of a semi-dominant trait. In the F2 population responsiveness segregated in an unresponsive (47.2%), chlorotic (21.3%) and necrotic (31.5%) fraction. Subsequent Chi-square analysis did not support the hypothesis of a single semi-dominant gene responsible for BLR38 recognition (tested ratio 1:2:1, $\chi^2 = 37.8$, *df*=2, 95% confidence cut-off $\chi^2 \leq 5.991$) suggesting that recognition of this effector is genetically more complex.

To map loci mediating recognition of BLN06 and BLR38, quantitative trait locus (QTL) analysis was performed on the F2 population. No significant loci were identified for BLN06 recognition. In contrast, mapping of BLR38 recognition revealed a QTL at the bottom of chromosome (chr) 4 with a logarithm of odds (LOD) score of 21 (Figure 2a) and a weaker QTL at the top of chr 8 with a LOD score of 5 (Figure 2b) that explain 56.3% and 17.5% of the variance in this F2 population, respectively.

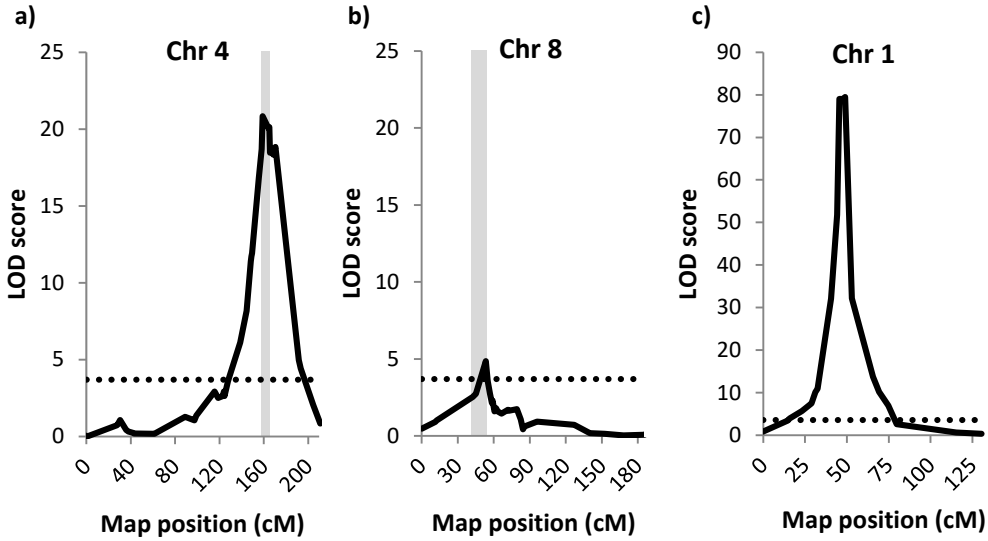

Figure 2, Recognition of BLR38 maps to loci on chromosome 4 and 8, whereas resistance to *B. lactucae* Bl:24 maps to chr 1. Recognition of BLR38 maps to a locus a) on the bottom of chr 4 and b) on the top of chr 8. The smallest mapping intervals required for the recognition of BLR38 are depicted with vertical grey bars. c) Resistance to Bl:24 in an F2 population of *L. serriola* LS102 x *L. sativa* GreenTowers maps to the *Dm17* locus on chromosome 1. The significance threshold for the logarithm of the odds (LOD) scores is depicted as a dashed horizontal line.

The substantial difference in LOD score between the QTL on chr 4 and chr 8 may reflect unequal contribution of the QTLs to BLR38 recognition. To explore this, the smallest mapping intervals (Figure 2a&b) were determined (see Materials & methods for details). Markers mLT19011 and mLT6534 on chr 4 and mLT9544 and mLT4241 on chr 8 delineated the smallest mapping intervals that span 18.7 Mbp and 8.1 Mbp, respectively in *L. sativa* cv. Salinas reference genome v8. A simplified genotype based only on these markers could be assigned to 85 of the 108 F2 plants using 'A' and 'B' for the *L. serriola* LS102 alleles at chr 4 and chr 8, respectively, and 'a' and 'b' for the *L. sativa* cv. GreenTowers alleles. Clear differences in genotype distribution per BLR38 response class were observed. In the absence of at least one of the *L. serriola* LS102 loci either at chr 4 and/or chr 8 (genotypes: aabb, AAbb, aaBB, aaBb, Aabb) plants were, without exception, unresponsive to BLR38 (Figure 3b). *Agrobacterium* infiltration sites of heterozygous (AaBb) plants turned predominantly chlorotic (15/23), but also no response (4/23) and necrosis (4/23) were observed (Figure

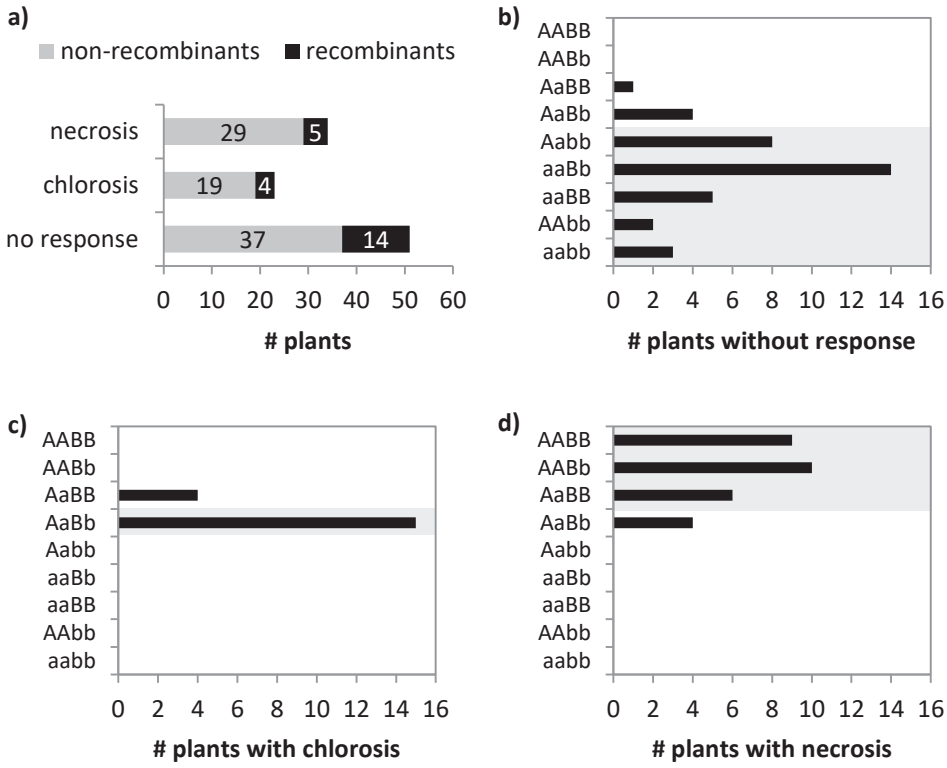

Figure 3, Gene dosage is an important factor in BLR38 recognition. BLR38 was transiently expressed using *Agrobacterium* in an F2 population of *L. serriola* LS102 x *L. sativa* GreenTowers. a) BLR38 recognition in the whole F2 population and the subpopulation without recombinants. Recombinants are defined here as plants with recombinations within the smallest mapping interval on chr 4 and/ or chr 8 delineated by mLT19011 and mLT6534 on chr 4 and mLT9544 and mLT4241 on chr 8. b), c) and d) The distribution of non-recombinants by genotype and response to transient expression of BLR38. Grey blocks indicate the genotypes most frequently observed to result in b) no response, c) chlorosis and d) necrosis upon transient expression of BLR38. A and B = *L. serriola* LS102 genotype locus chr 4 and chr 8 respectively, a and b = *L. sativa* GreenTowers genotype locus chr 4 and chr 8 respectively.

3c). As expected, a necrotic response was observed in plants homozygous for the *L. serriola* LS102 alleles at both loci (Figure 3d). Surprisingly, plants heterozygous for the *L. serriola* LS102 allele at chr 8 showed a strong response to BLR38 (AABb, 10/10 necrosis), whereas plants heterozygous at chr 4 showed a lower number of necrotic infiltration sites (AaBB, 6/11 necrosis), suggesting that the gene product of chr 4 is more rate limiting than that of chr 8. The observed requirement for both loci is confirmed by the segregation ratio in the F2, where the response segregates in a 7:4:5 (unresponsive: chlorosis: necrosis) ratio (tested ratio 7:4:5, $\chi^2 = 0.89$, *df*=2, 95% confidence cut-off $\chi^2 \leq 5.991$). In conclusion, the *L. serriola* LS102 loci on chr 4 and chr 8 are semi-dominant and are both required to mediate recognition of BLR38.

F3 analysis confirms a digenic model for BLR38 recognition

To further substantiate the digenic model, responsiveness to BLR38 was assessed in F3 progeny of selected F2 plants. As expected, F3 families #6 and #28 that lack the *L. serriola* LS102 allele at chr 4 or chr 8 (F2 genotypes aaBB and AAbb) remained unresponsive to BLR38, whereas a severe necrotic response was observed in all F3 plants from family #27 that were homozygous for the *L. serriola* LS102 allele at both chromosomes (F2 genotype AABB) (Table 2). F3 progeny of heterozygous (AaBb) F2 parents are expected to segregate similarly to the F2 population in a 7:4:5 (unresponsive: chlorosis: necrosis) ratio. Four families indeed segregated in three response classes, but only F3 families #108 and #113 segregated in the expected 7:4:5 ratio. In F3 families #69 and #117 a higher fraction of unresponsive (observed: 54% and 52%; expected: 43.75%) infiltration sites were observed and a lower fraction of chlorotic sites (observed: 8% and 14%; expected: 25%) (Table 2). This tilting towards the extremes may be related to fixation of modifier loci due to a reduction

Table 2, Response of selected *L. serriola* LS102 x *L. sativa* GreenTowers F3 families to transient expression of BLR38.

Non-segregating families for BLR38 recognition

F3 family	F2 genotype[1]	Expected (unresponsive: chlorotic: necrotic)	Observed (unresponsive: chlorotic: necrotic)	χ^2 (cut-off=5.991)
#6	AAbb	16:0:0	28:1:0	0.75
#28	aaBB	16:0:0	30:0:0	0
#27	AABB	0:0:16	0:0:29	0

Segregating families for BLR38 recognition

F3 family	F2 genotype[1]	Expected (unresponsive: chlorotic: necrotic)	Observed (unresponsive: chlorotic: necrotic)	χ^2 (cut-off=5.991)
#69	AaBb	7:4:5	14:2:10	15.98
#108	AaBb	7:4:5	13:5:6	4.42
#113	AaBb	7:4:5	14:5:10	3.21
#117	AaBb	7:4:5	15:4:10	6.81

[1] A and B = *L. serriola* LS102 genotype locus chr 4 and chr 8 respectively, a and b = *L. sativa* GreenTowers genotype locus chr 4 and chr 8 respectively.

in heterozygosity from 50% in F2 plants to 25% in F3 plants. Altogether, the F3 analysis supports a digenic model to describe BLR38 recognition.

Resistance to Bl:24 in *L. serriola* LS102 is independent of BLN06 or BLR38 recognition and mediated by *Dm17*

The BLR38 and BLN06 recognizing line *L. serriola* LS102 is resistant to infection by *B. lactucae* race Bl:24 from which *BLR38* and *BLN06* were cloned. To test if recognition of BLN06 or BLR38 is genetically linked to resistance, disease assays with *B. lactucae* race Bl:24 were performed on the *L. serriola* LS102 x *L. sativa* GreenTowers F2 population. Eighty out of 108 F2 plants were resistant to Bl:24 indicating the presence of a single dominant resistance gene (tested ratio 3:1, χ^2 =0.05, *df*=1, 95% confidence cut-off $\chi^2 \leq 3.84$). Resistance to Bl:24 segregated in the BLN06 and BLR38 responsive subpopulations in the same 3:1 ratio as observed for the F2 population as a whole (Figure 4). This demonstrates that BLN06 and BLR38 recognition is not linked to resistance to Bl:24.

The BLN06 recognizing lines *L. serriola* LS102 and *L. sativa* NunDm17 possess *Dm17,* which confers resistance against races Bl:1-Bl:7, Bl:10-Bl:26 and Bl:28 (Parra *et al.*, 2016). To confirm that the observed single dominant resistance against Bl:24 in the *L. serriola* LS102 x *L. sativa* GreenTowers F2 population was mediated by *Dm17*, QTL analysis was performed. Disease resistance to Bl:24 was mapped to a single, highly significant peak at the top of chr 1 with a LOD score of 79 (Figure 2c) corresponding to the reported location of *Dm17* (Maisonneuve *et al.*, 1994).

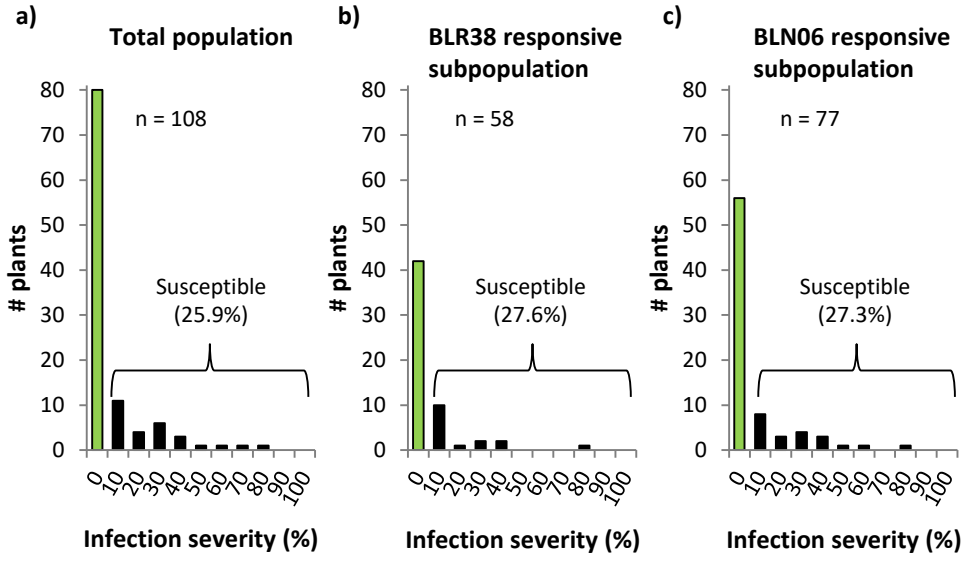

Figure 4, BLN06 and BLR38 recognition is independent of resistance to Bl:24. a) Resistance to Bl:24 in an F2 population of *L. serriola* LS102 x *L. sativa* GreenTowers. Leaf discs were infected with Bl:24 (60 spores/µl) and the percentage of leaf disc surface covered in sporangiophores was scored at 8 days after inoculation. Infection classes start at 0 %, and then increase in 10% increments. b) Resistance to Bl:24 in F2 plants that scored ≥ 1 (on a scale from 0 = no response to 4 = severe necrosis) upon transient expression of BLR38 or c) BLN06 using *Agrobacterium*. Two infiltration sites per plant were scored 4-6 days after infiltration with the highest score considered leading. Green bars represent fully resistant plants (no sporulation).

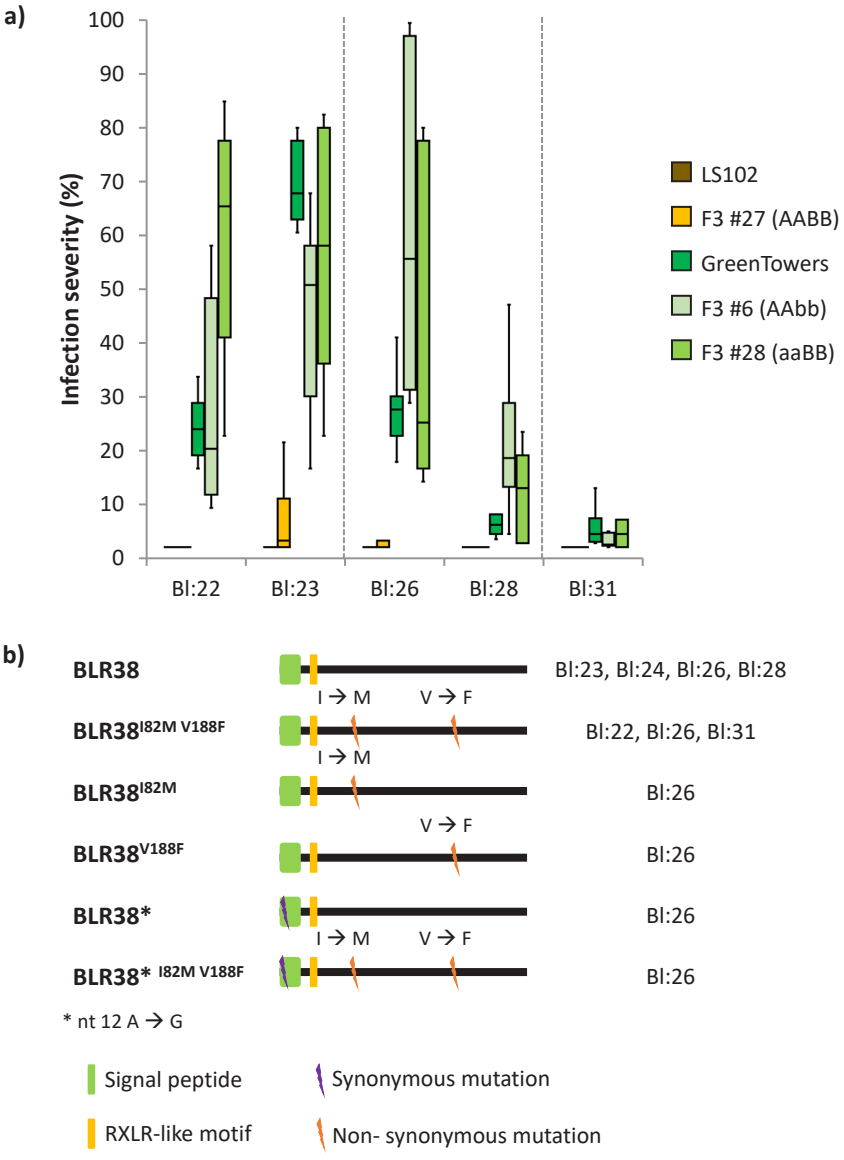

Figure 5, Recognition of BLR38 induces resistance against multiple *B. lactucae* races. a) Susceptibility of BLR38 recognizing *L. serriola* LS102 x *L. sativa* GreenTowers F3 family #27 and BLR38 non-recognizing F3 families #6 and #28 to *B. lactucae* races Bl:22, Bl:23, Bl:26, Bl:28 and Bl:31. Data from three independent experiments with distinct batches of plants and *B. lactucae* races is depicted separated by grey lines. Leaf discs were punched from seven plants per line or family and were sprayed with 80-100 spores/µl of Bl:22 or Bl23, ~150 spores/µl of Bl:26 or Bl:28, or 100 spores/µl of Bl:31 and scored at 10-11 days after inoculation. b) Allelic variation of *BLR38* in *B. lactucae* races. RNA was isolated from infected seedlings and *BLR38* was cloned using 5'UTR and 3'UTR binding primers. The top allele represents the reference sequence originating from Bl:24.

BLR38 recognition is linked to resistance to other *B. lactucae* races

Previously, recognition of effector BLG03 was found to co-segregate with resistance against *B. lactucae* race Bl:5 but not Bl:24 (Stassen *et al.*, 2013). Thus, the absence of linkage between BLR38 recognition and resistance against Bl:24 did not preclude linkage between BLR38 recognition and resistance to other *B. lactucae* races. To explore this possibility, leaf disc assays were performed on F3 families with races Bl:22, Bl:23, Bl:26, Bl:28 and Bl:31. To exclude confounding effects of *Dm17* in the disease assays, F3 families lacking *Dm17* were selected from crosses between *L. serriola* LS102 and *L. sativa* cv GreenTowers (family #27, 6 and 28) and CobhamGreen. BLR38 recognizing family #27 (F2 genotype AABB) displayed moderate to strong resistance against races Bl:22, Bl:23 and Bl:26 (average infection severity = 0.0-4.7%), whereas the BLR38 non-recognizing F3 families #6 (F2 genotype aaBB) and #28 (F2 genotype AAbb) were susceptible to these three races (average infection severity = 26.6-57.1%) (Figure 5a). A similar trend was observed with races Bl:28 and Bl:31 even though the difference between the BLR38 recognizing (average infection severity = 0.0%) and non-recognizing (average infection severity = 1.2-20.1%) families was much smaller due to lower virulence of these *B. lactucae* races. The BLR38-recognizing *L. serriola* LS102 x *L. sativa* cv CobhamGreen F3 families were also moderately to highly resistant to all five tested races. BLR38-non-recognizing *L. serriola* LS102 x *L. sativa* cv CobhamGreen F3 families were more susceptible, although infection levels varied strongly (Supplemental figure 5). In summary, these results demonstrate that recognition of BLR38 is linked to resistance against races Bl:22, Bl:23, Bl:26, Bl:28 and Bl:31.

Allelic variation in effector genes between races of a pathogen species can affect recognition in the pathogen host. To determine if allelic variation in *BLR38* is present in *B. lactucae* races, the coding sequence was amplified from races Bl:22, Bl:23, Bl:24, Bl:26, Bl:28 and Bl:31 using primers designed on the 5' and 3' untranslated regions of the Bl:24-derived *BLR38* transcript. Races Bl:23, Bl:24 and Bl:28 were all homozygous for the Bl:24 allele. Races Bl:22 and Bl:31 were homozygous for a second allele with two single nucleotide polymorphisms (SNPs) in the effector domain resulting in amino acid substitutions at position 82, isoleucine (I) to methionine (M), and position 188, valine (V) to phenylalanine (F). Surprisingly, six variants including the Bl:24 sequence and Bl:22/Bl:31 variant (encoding *BLR38* $^{I82M\ V188F}$) were identified in cDNA of Bl:26 (Figure 5b). Yet, the non-synonymous SNPs did not affect effector recognition in *L. serriola* LS102 (Supplemental figure 6) suggesting that the identified alleles have not evolved to escape detection by the plant immune system.

Gene dosage plays a limited role in BLR38-induced resistance

To further substantiate linkage between BLR38 recognition and resistance against *B. lactucae*, a leaf disc assay with Bl:22 was performed on *L. serriola* LS102 x *L. sativa* GreenTowers F3 family #108 that segregates for the two loci required for BLR38 recognition. Individual F3 plants were tested for BLR38 recognition and genotyped using additional markers in the previously identified QTL intervals on chr 4 and chr 8. Mapping of either disease resistance or BLR38 recognition resulted in maximum LOD scores at the same marker positions on chr 4 and chr 8 confirming previous results. Maximum LOD scores associated with resistance were 3.3 on chr 4 and 7.6 on chr 8, whereas for BLR38 recognition they were 6.4 on chr 4 and 12.2 on chr 8 (Supplemental figure 7).

To determine if resistance against Bl:22 is dependent on gene dosage, simplified genotypes were assigned to 88 out of 115 plants. These plants were not recombinant between markers mLT35950571 and mLT105577576 on chr 4 and markers mLT48854237

and mLT4241 on chr 8 that flank the reduced smallest mapping intervals (Supplemental figure 7). In all five susceptible (average infection severity = 39.8 – 48.0%) F3 genotypes the *L. serriola* LS102 allele was absent on chr 4 and/or chr 8 (Figure 6c). These plants were also unresponsive to BLR38 (Figure 6a). Heterozygous plants (AaBb) were moderately resistant (average infection severity = 6.5%). AABB, AaBB and AABb plants were highly resistant (average infection severity = 0.03-1.34%) (Figure 6c). When plants were grouped by BLR38 response class, BLR38 unresponsive plants were significantly more susceptible to Bl:22 (average infection severity = 42.1%) than necrotic (average infection severity =

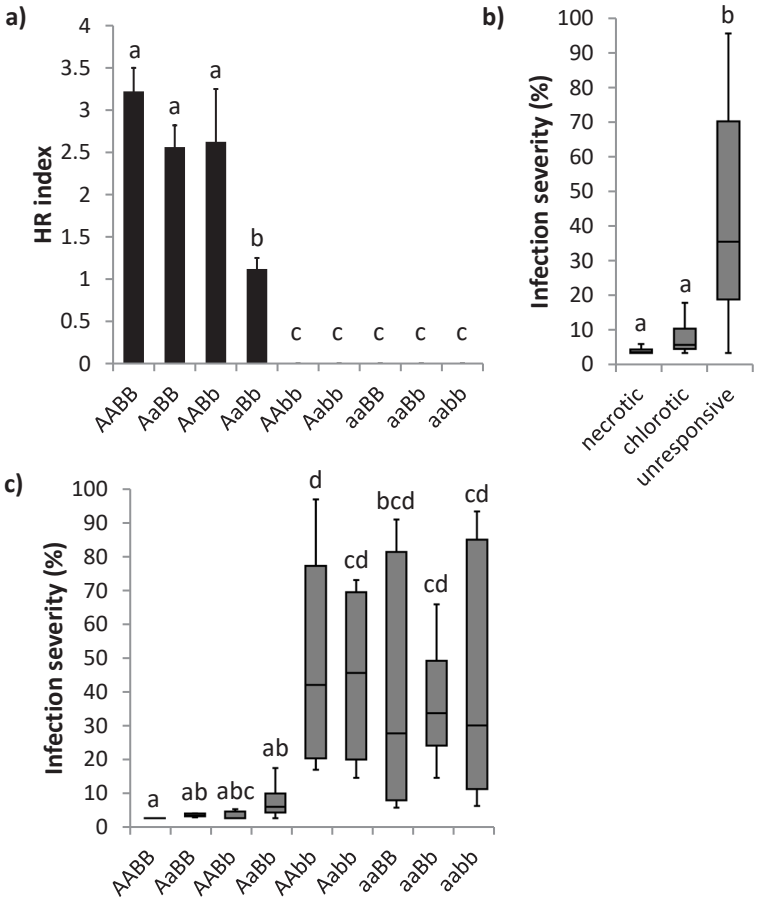

Figure 6, Gene dosage plays a minor role in resistance to race Bl:22 in BLR38 recognizing plants. a) Recognition of BLR38 in *L. serriola* LS102 x *L. sativa* GreenTowers F3 family #108. Genotypes were assigned based on markers mLT35950571 and mLT105577576 on chr 4 and mLT48854237 and mLT4241 on chr 8. Hypersensitive responses were scored 5-6 days after *Agrobacterium*-mediated transient transformation. On the same plants a leaf disc assay was performed with Bl:22 (100 spores/µl) and the percentage of leaf disc surface covered in sporangiophores was scored 10 days after inocualtion. Plants were grouped b) based on the highest score of two infiltration sites and were divided over three response classes: unresponsive (score =0), chlorotic (score =0.5-2) or necrotic (score ≥2.5). Or c) plants were grouped based on genotype. Statistical differences were assessed using ANOVA with *post-hoc* Tukey testing.

0.9%) and chlorotic (average infection severity = 5.7%) responders (Figure 6b). However, susceptibility of the chlorotic responders was not significantly different from that of the necrotic responders. Thus, gene dosage plays a more limited role in resistance to *B. lactucae* than in BLR38 effector recognition.

The QTLs are located near major resistance clusters

The majority of resistance loci in cultivated lettuce is located on five major resistance clusters (MRCs) on chr 1, 2, 3, 4 and 8 (McHale *et al.*, 2009; Christopoulou *et al.*, 2015a,b). The position of the loci mediating BLR38 recognition was compared with the position of MRCs in the *L. sativa* cv. Salinas reference genome v6 (Christopoulou *et al.*, 2015a,b) to determine if there is overlap with resistance gene clusters. The smallest mapping interval on chr 4 partially overlapped with the upstream border of MRC4 (Figure 7a), whereas the smallest mapping interval on chr 8 was located just downstream of MRC8A (Figure 7b). Based on genotyping of F3 family #108, the smallest mapping intervals on chr 4 and chr 8 spanned 14.2 Mbp and 5.5 Mbp (genome v8) respectively (Reyes-Chin-Wo *et al.*, 2017). Genes within these intervals were screened for the presence of TIR, NB-ARC and LRR domains that are characteristic for NLRs. Both regions contained a TNL (Supplemental table 5), thus it is probable that the corresponding regions in the *L. serriola* LS102 genome also contain NLRs that could cooperatively mediate BLR38 recognition.

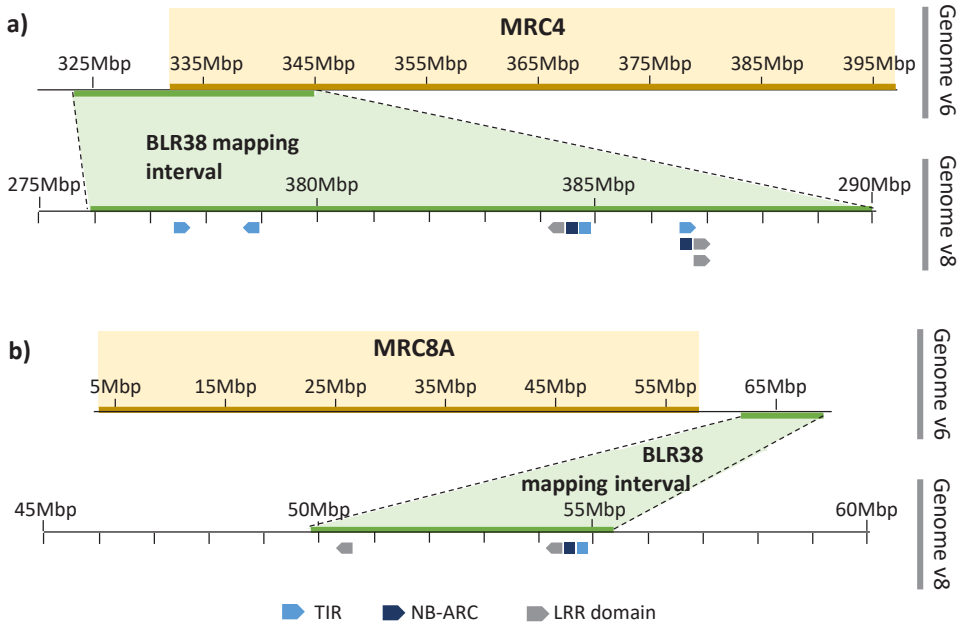

Figure 7, Graphical representation of the mapping intervals conferring BLR38 recognition relative to major resistance clusters. The top panels show the position of a) MRC4 and b) MRC8A as reported by (Christopoulou *et al.*, 2015a,b). The position of the smallest mapping intervals for BLR38 recognition are depicted according to *L. sativa* cv. Salinas genome v6 and v8. The bottom panels indicate the position of genes containing TIR (light blue), NB-ARC (dark blue) and LRR (grey) domains (not to scale).

Discussion

In this study, recognition of *B. lactucae* effectors BLN06 and BLR38 in the wild lettuce accession *L. serriola* LS102 is described. Though effector recognition is frequently associated with single dominant resistance (*R*) gene loci (Flor, 1971; Vleeshouwers *et al.*, 2008; Michelmore & Wong, 2008), recognition of BLR38 required two unlinked loci that displayed incomplete dominance resulting in a 7:4:5 (unresponsive: chlorotic: necrotic) ratio in the F2. Similarly, a study on nonhost resistance against *P. infestans* in pepper found that, although the loci involved in recognition of five *P. infestans* effectors were dominant, the response to PexRD24, PexRD46 and PexRD50 segregated in a 7:9 (unresponsive: necrotic) ratio consistent with two unlinked loci whose gene products interacted complementary (Lee *et al.*, 2014). Yet our data contrast with previously characterized *B. lactucae* effector responses that mapped to single loci (Stassen *et al.*, 2013; Giesbers *et al.*, 2017). Also, lettuce responses to multiple bacterial type III effectors from *Pseudomonas* and *Ralstonia* pathovars segregated as single dominant loci (Wroblewski *et al.*, 2009).

Recognition of BLR38 was not genetically linked to resistance against *B. lactucae* race Bl:24, from which the effector was cloned, but was linked to resistance against races Bl:22, Bl:23, Bl:26, Bl:28 and Bl:31. Six *BLR38* alleles were identified in these *B. lactucae* races including the Bl:24-derived allele. The three BLR38 variants with amino acid substitutions were still recognized in *L. serriola* LS102 indicating that *BLR38* allelic variation in these races did not evolve to overcome resistance. Also, recognition of effectors BLG01, BLG03, BLN06 and BLN08 was not genetically linked to resistance against Bl:24, whereas recognition of BLR31 did provide resistance to Bl:24 (Stassen *et al.*, 2013; Giesbers *et al.*, 2017). It has previously been postulated that *B. lactucae* expresses additional effectors that suppress effector recognition or downstream immune signaling (Stassen *et al.*, 2013; Giesbers *et al.*, 2017). Considering that recognition of at least five Bl:24-derived effectors may be suppressed, it is possible that key pathways involved in effector-triggered immunity are targeted by other Bl:24 effectors. Similarly, *P. infestans* RXLR effector PexRD2 and *Xanthomonas euvesicatoria* type III effector XopQ target distinct branches of the MAPK signaling pathway involved in activation of ETI and are thereby able to suppress HR induced by various effectors (Teper *et al.*, 2013; King *et al.*, 2014).

Despite a clear effect of gene dosage on BLR38 recognition in F2 and F3 plants, the observed difference in infection severity between plants homozygous or heterozygous for the *L. serriola* LS102 loci was not significant. Gene dosage-dependent resistance against *B. lactucae* was previously reported for *Dm6* (Crute & Norwood, 1986) and *Dm17* under extreme disease pressure (Maisonneuve *et al.*, 1994). Indeed, gene dosage effects may be more pronounced under specific circumstances (e.g. at the seedling stage) or at high disease pressures, when transcription from a single allele is more likely to become the rate-limiting determinant of the immune response than under low disease pressure circumstances. Incomplete dominance of resistance to *BLR38*-expressing *B. lactucae* races could be apparent under specific environmental conditions and/or disease pressures. The gene dosage effects observed in BLR38 transient expression assays strongly suggest that transcript levels of the loci conferring HR to this effector are indeed rate-limiting under these circumstances resulting in chlorosis in heterozygous plants.

The loci mediating BLR38 recognition were mapped in a segregating F2 and F3 population to chr 4 and chr 8. The equivalent intervals in the *L. sativa* cv. Salinas reference genome contain several genes that encode proteins associated with resistance due to the

presence of TIR, NB-ARC and LRR domains. Thus, *NLRs* are candidate genes for mediating BLR38 recognition and inducing disease resistance against several *B. lactucae* races. Multiple NLRs have been reported to operate in pairs (Ashikawa *et al.*, 2008; Yuan *et al.*, 2011; Saucet *et al.*, 2015; Wu *et al.*, 2016) and the two components are postulated to have distinct functions: one acts as a 'sensor' and is responsible for recognition of the pathogen effector or effector-modified plant protein, whereas the other NLR acts as 'helper' or 'executor' and mediates activation of downstream signaling pathways (Sukarta *et al.*, 2016; Wu *et al.*, 2017). The 'sensor' NLR repertoire of a plant is highly divergent to allow for identification of a broad range of pathogens. On the contrary, 'helper' NLRs benefit from a certain amount of redundancy and conservation to ensure robustness in the immune system (Wu *et al.*, 2017). To determine if the loci mediating BLR38 recognition constitute a 'sensor' and 'helper' NLR pair, first, sequencing of the corresponding regions in the *L. serriola* LS102 genome is required. Then, candidate genes could be amplified, cloned and transiently co-expressed with BLR38 to complement F3 families that lack the *L. serriola* LS102 allele at chr 4 or chr 8 (aaBB or AAbb). Alternatively, the loci mediating BLR38 recognition could constitute an R protein and an effector target. In this model, the R protein mediates indirect recognition of the effector by monitoring an effector target with a function in host susceptibility ('guardee') or an effector target that has no function in the host other than activating the guarding R protein through interaction with the effector ('decoy') (van der Hoorn & Kamoun, 2008).

Our large-scale transient expression assay in lettuce germplasm showed that BLN06 is not only recognized in *L. serriola* LS102 but also in *L. sativa* NunDm17 and RYZ2164. Although the link with resistance gene *Dm17*, that is present in all three lines, seemed obvious, BLN06 responsiveness and *Dm17* were not genetically linked. BLN06 responsiveness segregated in a 3:1 (responsive: unresponsive) ratio in the *L. serriola* LS102 x *L. sativa* GreenTowers F2 population suggesting a simple single gene model, yet, interval mapping failed to identify significant QTLs. Unexpectedly, a small number (6.5%) of *Agrobacterium* infiltration sites in F2 plants displayed necrosis, a much stronger response than observed in *L. serriola* LS102 or in F1 plants. Transgressive segregation, i.e. the offspring of a cross displays a more extreme phenotype than the parents, is generally attributed to complementary gene action among multiple loci. The extreme phenotypes can arise when alleles of opposing effects, present in the parents, are recombined in offspring and result in allele combinations with an additive effect (Rieseberg *et al.*, 1999; Bell & Travis, 2005; Nelson *et al.*, 2018). Since allele combinations can be selected for, these can get fixed in populations, thereby making transgressive phenotypes heritable (Rieseberg *et al.*, 1999). It is possible that this occurred in *L. sativa* NunDm17, which could explain our finding that *Agrobacterium* infiltration sites in *L. sativa* NunDm17 (derived from *L. serriola* LS102 (Maisonneuve *et al.*, 1994; Parra *et al.*, 2016)) turned predominantly necrotic. Thus, recognition of BLN06 may be a more complex trait than anticipated and more research is required to determine the number of loci involved and their individual contribution.

Acknowledgements

This project (#12683) was part of the Open Technology Programme and supported by the Netherlands Organization for Scientific Research (NWO) in collaboration with Wageningen University (Wageningen, The Netherlands), Bayer (Nunhem, The Netherlands), Enza zaden (Enkhuizen, The Netherlands), Syngenta (Enkhuizen, The Netherlands), RijkZwaan (De Lier, The Netherlands) and Vilmorin & Cie (La Ménitré, France). We would like to thank Marcel van Verk for bioinformatics support.

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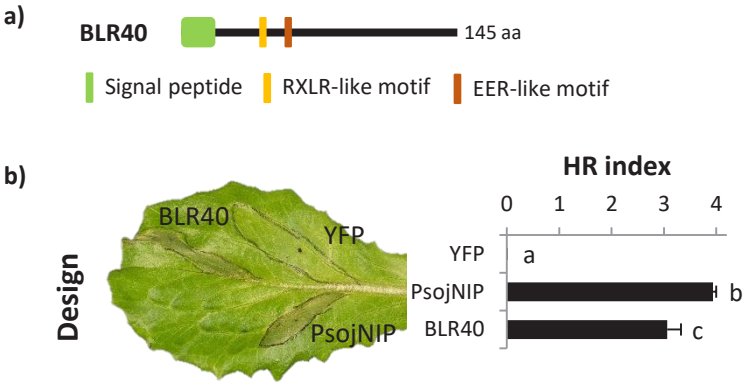
Supplemental data



Supplemental figure 1, BLR40 is specifically recognized in *L. sativa* cv. Design. a) Schematic representation of *B. lactucae* effector BLR40. b) BLR40 is recognized in *L. sativa* cv. Design. Hypersensitive responses were scored 5 days after infiltration of *Agrobacterium*. Bars represent the mean + SE from 16 infiltration sites. Statistical differences were assessed using ANOVA with *post-hoc* Tukey testing.

Alignment of BLN06 with BLN01

```
BLN06   MTLLHCWLLLVGHLASTAYADFITKDFKS--LPPPAYDTNATQALVPYNAALEERNGPSS
BLN01   MNL--RVLVLAVFLAATTRGDLATDDVNSTSLRLPSIEKRAD----GYGNAFNEEKV---
        *.*    *:*. .**:*: .*: *.*.:*  *  *: :..*      *. *::*.:

BLN06   STALLQYI--DHKPG-LMKKLLAGLSIRFAP---PTMKVISTPTDMLRIDKIKNNIIKSS
BLN01   -LNLRQITDPSNFPNAHKKHLVAAVSKFWVKPRIPQEDLAQQTFKVFHVERVKSNLFTSK
           * *    .: *.   *:*:*.:*  :.    *  .: .   .:::::::*.*::.*.

BLN06   QWKRWARSLLEQNSM-QNSHVIITKKMMDDLKPTNFFLVLLEASKDKNTKAVAKILEETQ
BLN01   RWIKWVERVKVAIPDRTKRDEELTRIMVEEFGLPLFFQKLHEASKNPETSETAKYFERLE
        :* :*.. :        : .  :*: *::::    **  * ****: :*. .** :*. :

BLN06   FARWCTIEHESISPMEFYDVLQLNLNEPIAYMERLPVLLRYWKY----YKSVHSPMSSVA
BLN01   YDRLL---KNKVFPEEFREQLGLQHLES------DIELTRYYLKFRSRFLMLATPVEELK
        : *     ::.: * ** : * *:  *          * **:      :  : :*:..:

BLN06   TPKD------IIDKQTVNRFGPYWKHT-GEIAELLRLDFDSDSFFNHPARNVWLDLMKTY
BLN01   VPKLLKEVMDEPTKRTIELFGEFGKEEPDMVAKMFELKFNNEREVEHPLFNIWIDFMMAY
        .**          *:*:: ** : *.  . :*:::.*.*:.:  .:**  *:*:*:* :*

BLN06   LDDTKTAEPLMIKTFQLLGNAAAKNLQNNVYSPIHFAERWIQANLQPIDVVTILGLD--I
BLN01   LDEKFVASATFLQTFRLLESSAAAGNKRSLQIKETFSRRWISSDRSLKDVAKMLQLGKNQ
        **:. .*.  :::**:** .:** . :..:     *:.***.:: .  **..:* *.

BLN06   HDSNLATNSAFSFLKVFIEKFLVNHPEADTTVVKIFSRLGSDESEKALALRKSFVSFFLR
BLN01   ADSSWINRLELQLYMSYMRKYILTHRKADPMLSWVLSKLVGLKHERSRKYQTRFVNFFR-
         **.  ..  :.:   ::.*:::.* :**  :  ::*:* . : *::   :. **.**

BLN06   TPKFTPKTVMSIFDLTISADYVEKNPVWAIWMEYVNVYLVKNKVCPEGPLADTLEFLGST
BLN01   -DNFQPSEVLTILRLDNEGGQIKNRPLVEFGLQYVASFLLKNPKAEPSI-LKTLKLLCHH
          :* *. *::*: *  ... :::.*:  : ::**  :*:**  .  .   .**::*

BLN06   AAADGVV-------------------------------------------------------
BLN01   GNSDDMFAFAKLWVSLETTPETFLKMLGIKKIDASILNHDLRDLWLAFLAHYGARLPSQA
        . :*.:.

BLN06   ----------------------RKKSIELLYSSWSGKTSQDTRIKQFLTAAARLNQLEL*
BLN01   LPHEMLQTINHLTTSAMMDNRSSREALAKIFHFWSRKNLTQDDMFKMLRLHTFRPHYFIN
                               ::::  ::  ** *.  :  : ::*   :   :  :

BLN06   -------------------------------------------------------------
BLN01   PLLSTWDMYQLTFVTMHPTEPQQQLSDMIFRCFNVNDVEKLTAGAKHIPDMLHSKVKTVV


BLN06   ---------------
BLN01   KELQDKVDHFFGRT*
```

Supplemental figure 2, Alignments of BLN06, BLR38 and BLR40 with related *B. lactucae* effectors. tBLASTn was performed with the amino acid sequences of BLN06, BLR38 and BLR40 against the transcriptome set derived from *L. sativa* cv. Olof infected with Bl:24 material, with a cut-off of $1e^{-05}$. Hits were compared with the *B. lactucae* effecterome to identify related effectors. Alignments were created using Clustal Omega.

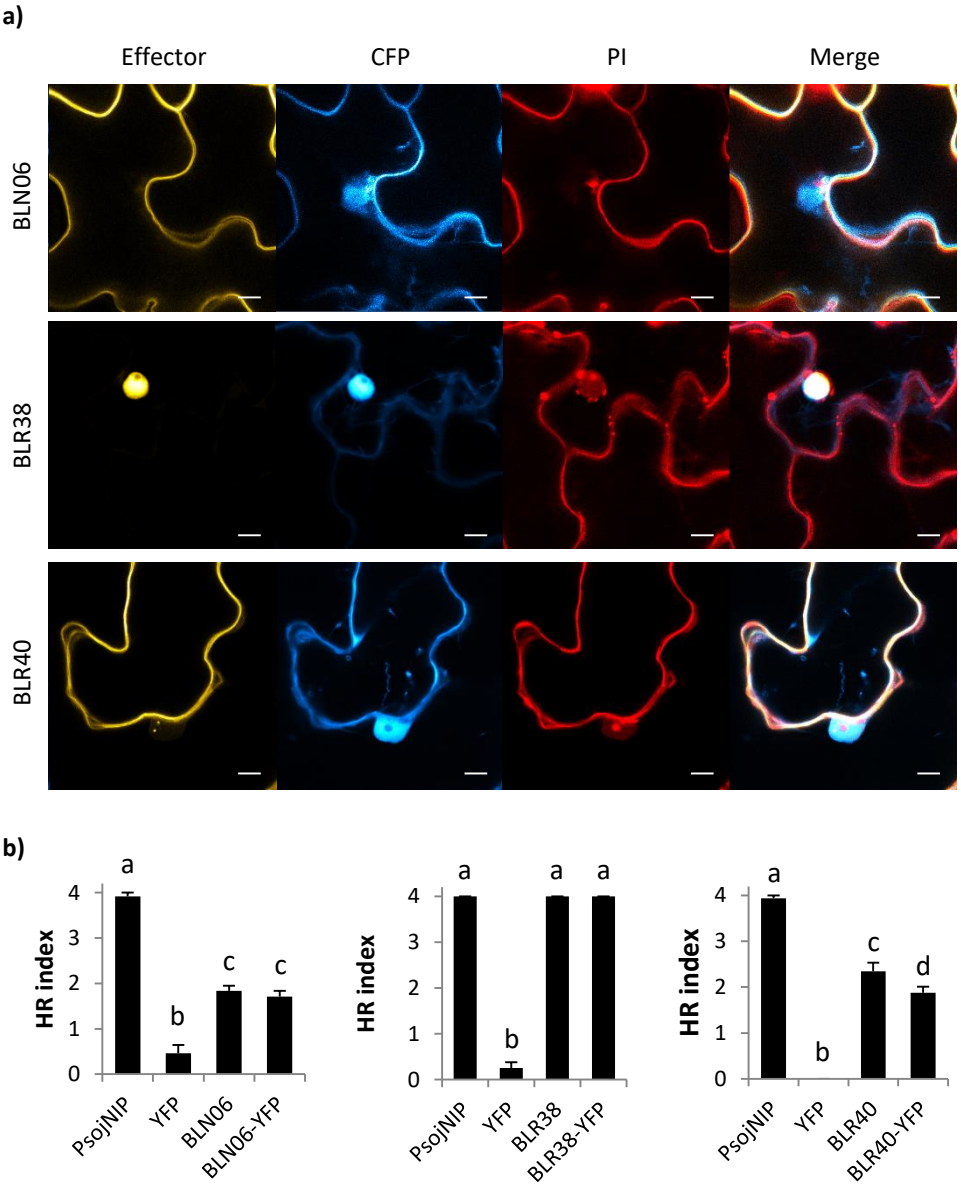
Alignment of BLR38 with BLR17, BLR28 and BLR33

```
BLR38   -MHCTVFFLLIACAKSSYGQTRSVSTAK----SESKSDEYSYNSDAIDQSRLLRGAVNPV
BLR33   MLFSVFFFLVATCVKSSYGHSVAVSTRD-PENIALQLHEYASIPETIETIRRLRGALAHD
BLR28   -MLRVVLFLVAACAKTSYSHTVALSTRN-SQYIASKANEHATIPEDINLNRRLRKAAVIT
BLR17   -MFSTVLFLVAACAKSSYGHSVSIFTRDSTKYIASNFDEYSTIPEDIDAKRRLREAVGVD
         :  ..:**: :*.*:**.:: :: * .       : .*::   : *:  * ** *

BLR38   SENMALRKFIFDMPEMLRPEH-----------F------EPIFINPAAVKEVIKDYLAYG
BLR33   ATAYDERMFFENAANKMYAIAQKTR--LSAAAVKKLIPESQEKKLLSYYLTKMKGFIKDR
BLR28   EVAETLESIIEAF-NPLRTLRSDV---RSEMSSKTKLEQDAMLKEPSFYFRMLKPFSEFR
BLR17   GIARDAEKTFADIRHSLDRLNKDFVRSNSFKNINPLITAEALVKQPSFYQEAFLPLITAR
              .  :    . :                         .      :     :

BLR38   EALCGSGYDPRLLALFGVRPTVLKQELMKAKGVTLSVMPSSRKRPRALDEVESEVENFRN
BLR33   EALYSITSSYDELALLGVTPDLFRTRLLSVESP---------------EVAAARAIEYEE
BLR28   IRACFEVYEIDTLILFGTSPHLLKQYIQNGIPRGIL-----PESVTVLATTGEKLKRFQR
BLR17   GIKCSKDFEFATMALLGVSPGTLRQKIQVAAQQPSN-----IWSYQTSEYNEHFVASYKK
                .   : *:*. *  ::  :                                 :..

BLR38   VLKDFFIPPTTTNPSKLIPDDIETLVPEHVSAHFN-----SLVYLMYFAVLHF-DSQELA
BLR33   YIKNICFVSSEKNPCKTVTELV---ESNKFDKIKELMEKHSIAELLIGALTNLNKVNHLD
BLR28   QFDIFFNPPTGSKPSKPSRAWPYARGPQVQANFKKI-YSSDHIKFLAYAFHHLDDVNILA
BLR17   YLDVVFMAPTISESSAFVKKHISMSIPEPSEMTYKL-LNAAVIQLLQLAIRNVDDVNELA
         :. .    : .: .           :      :         ::  *. :. . : *

BLR38   TMSSSVLLKYALQKNLLLREKIESGTLGEWERDFRLMRVLNVYKSEQT*-----
BLR33   KLTTRIQLKYVLENTPELGPLLSR-DVNELLKDPSVSKIFSRFL-TFVYGIPS*
BLR28   KLSSSIIYRFVLDNFKECRATIRYGTVEDWYKHPMLNKLLRVHEVCRKFGI*--
BLR17   KLATSVTFKYALEHDEEFSTIMMFGNLEAWISNPVLNKLLMVHQVLLKS*----
        .::: :  ::.*::       :    :     .  : :::  .
```

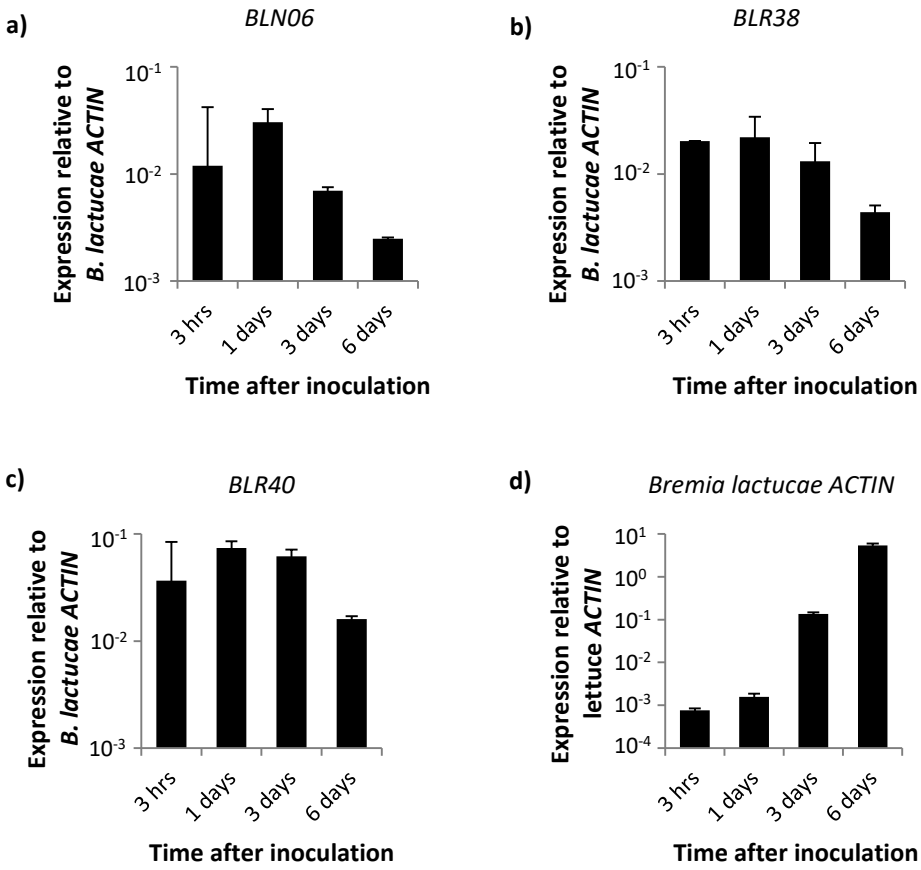
Alignment of BLR40 with BLR19, BLR31 and BLR32

```
BLR40   MLLSRAISVVALLACICCGVHTQDSKADLGTLRT-TDSAIITSQRRLRTSVDLVDNEERF
BLR31   MLLSRAISVLALLACIRCGVHAQNTEQNLKTQLT-TDSAMITSQRLLRTSVDFKDSEER-
BLR32   MNLIRAMFVAALVACTRNGVHAKASEADLVTLLT-TNSEIVTSQRLLRTSAEPDDNDERA
BLR19   MLLSRAISVVALLACIXCGAHAQDSEVDLGTLLTTLDSSMVTSQRLLRTSVDLDNNEERV
        * * **: * **:**   *.*:: :: :* *  *  :* ::**** ****.:  :.:**

BLR40   RWPFQQFFKDRWHRKQIKTYFRDQK-----------DNVSEGLV-EQLIARHGLKNVEKV
BLR31   -WPTES----SRIRSAIKDYFREFP-----------EKVSIAMA-IRQIDAHGVRHVEKV
BLR32   I--NIPFITNYNQRTRIKAPLKELKKDSNILSNVKQRLALERVVRKEIYNRGGYQNAKKV
BLR19   KWPFQNLVTDYLNQKAIRKSLVNQAKKTVN---AHDENVLEEAV-KKEINAGRVKNVKQA
                    :. *:  : :               .    .  .       ::.::.

BLR40   LSEVKFPLAVQISIRKILVNYKGKQAFTRPHLTPADTL*-------
BLR31   LSQYKFPAADQGNIRLAIIHHKAPK*--------------------
BLR32   LEETDVNDPGRAILNSHVNLYKWFHNVDK*----------------
BLR19   LSKLKNGDPAKAKLQRLY-NAEILRNLPKTHNSGQVRISRDKVSR*
           *.: .     :  :.      :  :
```

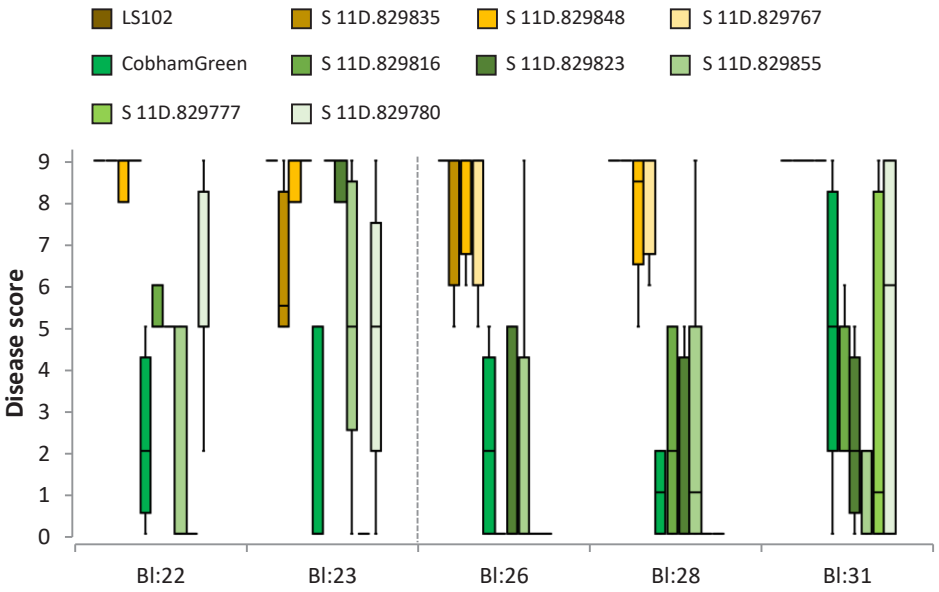
Supplemental figure 2 continued.



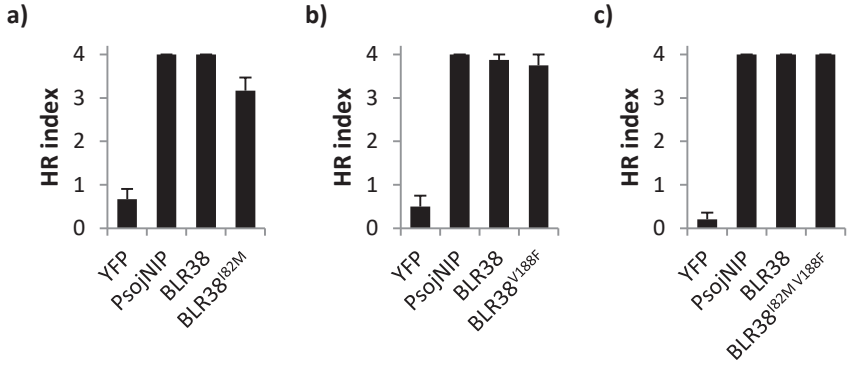
Supplemental figure 3, Subcellular localization of effectors *in planta.* a) *B. lactucae* effectors BLN06-YFP, BLR38-YFP and BLR40-YFP were co-expressed with free CFP in *N. benthamiana* using *Agrobacterium*-mediated transient transformation. Infiltration sites were cut out 2 days after infiltration and incubated in a 5mg/ml propidium iodide (PI) solution to stain the cell wall. Effectors BLN06 and BLR40 are localized at the plasma membrane, whereas BLR38 resides in the nucleus. Free CFP was used as marker for the cytoplasm and the nucleus. Bars are 10 µm. b) BLN06-YFP and BLR38-YFP were expressed in *L. serriola* LS102 alongside untagged versions, and BLR40-YFP was expressed in *L. sativa* cv. Design alongside untagged BLR40. Bars represent the mean + SE from 12-16 infiltration sites. Statistical differences were assessed using ANOVA with *post-hoc* Tukey testing.



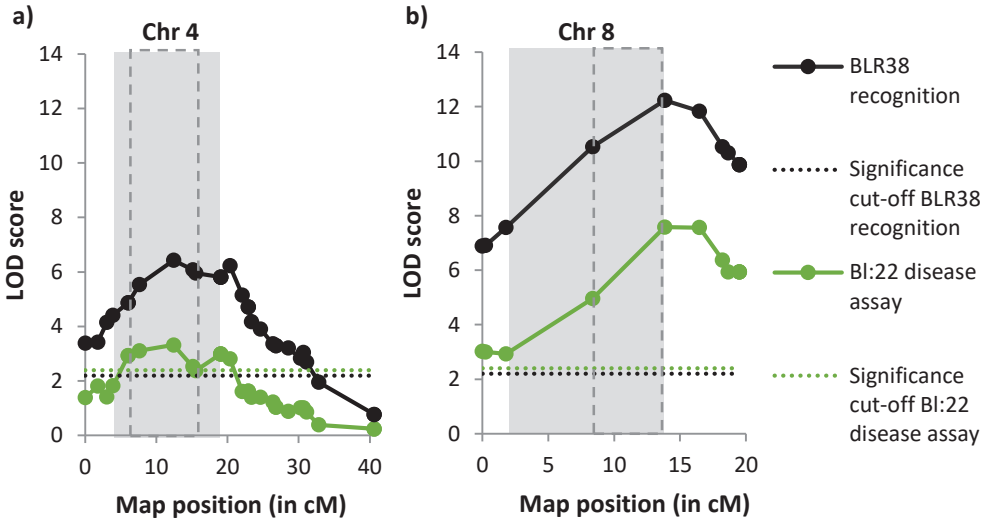
Supplemental figure 4, Expression of effectors *BLN06*, *BLR38* and *BLR40* during *B. lactucae* infection. *B. lactucae* effectors a) *BLN06*, b) *BLR38* and c) *BLR40* are expressed during infection of *L. sativa* cv. Olof with BI:24. Seedlings were harvested 3 hours, 1 day, 3 days and 6 days after inoculation. Data of three biological replicates and two technical replicates are depicted as mean + SD. Effector transcript levels were calculated relative to *B. lactucae ACTIN*. d) *B. lactucae ACTIN* transcript levels were calculated relative to *L. sativa ACTIN*.



Supplemental figure 5, Susceptibility of BLR38 recognizing and non-recognizing families to multiple *B. lactucae* races. Leaf disc assays were performed on BLR38 recognizing (in brown/yellow) and non-recognizing (green) *L. serriola* LS102 x *L. sativa* CobhamGreen F3 families with various *B. lactucae* races (50 spores/µl). Data from two independent experiments with distinct batches of plants and *B. lactucae* races is depicted separated by a grey line. Leaf discs were scored at 11 days after inoculation. Scores: 0 = fully susceptible, 2 = 75% sporulation, 5 = some spots with sporulation, 6 = sporulation on the leaf disc edge only, 8= resistant with necrosis, 9= fully resistant.



Supplemental figure 6, BLR38 alleles are recognized in *L. serriola* LS102. BLR38 alleles a) BLR38^{I82M}, b) BLR38^{V188F} and c) BLR38$^{I82M\ V188F}$ with non-synonymous SNPs in the effector domain are recognized in *L. serriola* LS102. Hypersensitive responses were scored 3 days after infiltration of *Agrobacterium*. Bars represent the mean + SE from 6-8 infiltration sites.



Supplemental figure 7, Fine-mapping of BLR38 responsiveness on chromosome 4 and 8. Resistance to Bl:22 and recognition of BLR38 were fine-mapped in *L. serriola* LS102 x *L. sativa* GreenTowers F3 family #108 on a) chr 4 and b) chr 8. The previously defined smallest mapping intervals required for the recognition of BLR38 are depicted with vertical grey bars. The new smallest mapping intervals are indicated with dashed grey lines within the previous intervals. The significance thresholds for the logarithm of the odds (LOD) scores are depicted as dashed horizontal lines.

Supplemental table 1, Primers used in this study.

pENTR/D-TOPO cloning primers

Gene	*Orientation*	*Sequence*[1]
BLC01	Fwd	*CACCATG*GCTTTTCCTATCGATATTGATACGAATGAGTTGG
BLC01	Rev	TCAAAAAGGCGAGACATAAAAGAAGCACGTA
BLN01	Fwd	*CACCATG*GACCTCGCAACAGACGACGTG
BLN01	Rev	CTATGTACGTCCAAAAAAATGATCTAC
BLN03	Fwd	*CACCATG*GTGGGCTATCCATCAGACG
BLN03	Rev	TCAAACTGCCGTCATAGC
BLN04	Fwd	*CACCATG*ATTAGCGACGAGATTTCACCAG
BLN04	Rev	CTAATGAATGTAGCTGCTCTCGAC
BLN05	Fwd	*CACCATG*GATTACATTTTGAAGGAAAGACG
BLN05	Rev	TTACAAGTGTAGCATCGCGTTCAA
BLN06	Fwd	*CACCATG*GACTTCATAACGAAGGACTTCAAG
BLN06	Rev	TCATAGCTCGAGTTGATTGAGAC
BLQ04	Fwd	*CACCATG*ATTTATTGTGAGCACGTGTTAAAAG
BLQ04	Rev	TCAGAAATTGTTCTTATGGTAAAGTT
BLR32	Fwd	*CACCATG*AAAGCCTCGGAAGCAGAT
BLR32	Rev	TTACTTGTCGACATTATGAAACCA
BLR33	Fwd	*CACCATG*GCGGTGTCAACGCGTGAT
BLR33	Rev	TTATGAAGGGATACCATAGACGAA
BLR35	Fwd	*CACCATG*AATAGCGTACCGACTTCTGTTGCCTTCG
BLR35	Rev	TTACCGATTGTAACAATGGCGTATTCCACTT
BLR36	Fwd	*CACCATG*GGAAAGCAGCGTCAATGCTTGG
BLR36	Rev	TCAAATTTCGAGAATTCCGGATG
BLR37	Fwd	*CACCATG*ACCGCGCCAAGCACGGAG
BLR37	Rev	CTACAAGAGATCACTTGGCACTTCTAA
BLR38	Fwd	*CACCATG*CAAACGCGGTCGGTATCCA
BLR38	Rev	TTATGTCTGTTCCGACTTGTACACA
BLR40	Fwd	*CACCATG*CAAGACTCGAAAGCAGATCTCG
BLR40	Rev	TTAAAGAGTGTCTGCAGGAGTTAGAT

[1]The TOPO site (CACC) and new START codon (ATG) are in italics.

Additional internal primers for sequencing

Gene	*Orientation*	*Sequence*
BLR37	Fwd	TGCATTTCAAAACAGCCAAA
BLR37	Rev	GAAGCACTGTCTTTGCGTCA

Supplemental table 1 continued.

BP cloning primers for C-terminal YFP fusion constructs

Gene	*Orientation*	*Sequence*[1]
BLN06	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGGACTTCATAACGAAGGACTT-CAAG
BLN06	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTT*AGCTCGAGTTGATTGAGACG
BLR38	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGCAAACGCGGTCGGTATCCA
BLR38	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTT*TGTCTGTTCCGACTTGTACAC
BLR40	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGCAAGACTCGAAAGCAGATCTCG
BLR40	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTT*AAGAGTGTCTGCAGGAGTTAG

[1]The attB1 and attB2 sites are in italics.

BP cloning primers for BLR38 allele identification

Gene	*Orientation*	*Sequence*[1]
BLR38	5' UTR Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*GGCTTCATCCCCTCTCACTT
BLR38	3' UTR Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTT*AGTACTCCCCTTCAGCACATT

[1]The attB1 and attB2 sites are in italics.

qRT-PCR primers

Gene	*Orientation*	*Sequence*
Lettuce *ACTIN*	Fwd	CTATCCAGGCTGTGCTTTCC
Lettuce *ACTIN*	Rev	ACCCTTCGTAGATCGGGACT
Bremia lactucae ACTIN	Fwd	GCGAGAAATTGTGCGTGATA
Bremia lactucae ACTIN	Rev	ACTCGGCTGCAGTCTTCATT
BLN06	Fwd	GTGGGCAATTTGGATGGAG
BLN06	Rev	AGTGTATCGGCTAACGGC
BLR40	Fwd	GGAACAGCTCATAGCACG
BLR40	Rev	CTGCAGGAGTTAGATGTGG
BLR38	Fwd	CACCACAAACCCGTCCAA
BLR38	Rev	AGCAGAACAGATGACGACA

Supplemental table 1 continued.

Genotyping markers

Marker	*Linkage group*	*Variant position genome v8*	*Flanking sequence [reference /alternative nucleotide]*
mLT19011	4	274,187,931	ATTATATTTGTATAGTATTTCTTCTTTGAAAATCTACCCCAGTATAACAATCTATTGCATTTAACCATGAATA-TAAAAATGCTGTTCCTTCCTACATTGG[T/C]TGTTGAACGATAGATTCTGACATAAACCATGTTTTTCCAG-GATTGCCCTTGAAAGGGTCTAGTGGAGGTCAACCAAACTAATTTCAGTCAACCTTCTTGG
mLT6534	4	292,881,001	ACTTAACTTTAATGCAAGTTTACATGAACCCTAGAAAAACAACGAAAAACATCGAAAAAAGGATCAC-GAATTTGGTGACTTCATCGAGCACTGGTAATTT[G/A]AAGAGTGCTTGGTCATCAGTTAGGGGTAAAG-GAAAAGGGTTAGGTAAGAAGATTGTAGAGATAGAAAGCGCGGAAGATTCTAGAATGAAAGCAAG-TAGTG
mLT4241	8	55,420,000	ATTGAAATAAAGAACATTAACAAGAAGAGCTAATTATCGTCGTAAAACTAGCTTATAATCAAAACCGG-ACACCAATGGGGCGAAATTTAAAGACATTATG[A/G]AGAAAAGATCACAGTTCATGATAAACAATGAT-CAAGTTGATTACCCCCATCTCACATTTTGAAAACAAACCTCCATTGTGTTCTTTGTGTGCGAGAATGT
mLT9544	8	47,352,309	AGAATCGTCAAATGCTAGATGCTGTTGGGAACTAAAAGATCGTCAACAAGAAGTTCTATTGAAGACTT-GTTGTATCATTATTCGAGATGCCATGCAATAT[G/T]TTACGCATTTTTTGTAGGCAAATGTAAGACACTTC-GAGAAAATATTATGTTGATCAGTTGCACTCCATTTTTTTTGGAATACAAAAAGTTTCTTAATTAA
mLT35950571	4	275,559,567	GGGGAACAAACAGCAAGGATTGCTTGTAGTATACTCTGTTTTGCCCCATTACTAACCACAACCTGGT-CAG[C/G]TGTGTAGGAAATATCATTCTCATCTGCAATAATAAATGAAGGAAAATACTTMAGAATTAT-GAAGTATATG
mLT105577576	4	289,732,580	AAGACTTCAACAGGAGCTKTGTTGGTGAGGATTCCAAAAACAAAGAGTTTGGGCGTGAMACAAAT-ARACTGGATTACTTTAGTGTCGCTGCTTTTGATGT[C/T]TATGGAGACGATCATCTCACTGGGGCTGC-TAGTTATGGTGGCGGTGGTGGTAGAAGGGATGGTGGCATAGTGTTAGCCCCGGAACAGGAGAGAGGT-GGTG
mLT48854237	8	49,925,846	TAAGAGTAAACCCACCTGGAAAAAAGTCATTCATGGTGTACAGGGACAAACACAAAGTAAATTCTG-GAATCATCTCACTAATGAAATCCATAAAATGTTT[C/G]TCTAGAGGATGCACATCATATTTGGCATTCG-TAATTGATGCAAAGAAGGAGAAAAAGGAGGTGCAGAGTATACCAGTGGTGTGTGATTATCCGGAAGTCT

Supplemental table 2, *B. lactucae* effectors and amino acid sequences.

Effector	*Sequence*[1]
BLN01	MNLRVLVLAVFLAATTRGDLATDDVNSTSLRLPSIEKRADGYGNAFN**EEK**VLNLRQITDPSNFPNAHKKHLVAAVSKFWVKPRIPQEDLAQQTFKVFHVERVKSNLFTSKRWIKWVERVKVAIPDRTKRDEELTRIMVEEFGLPLFFQKLHEASKNPETSETAKYFERLEYDRLLKNKVFPEEFREQLGLQHLESDIELTRYYLKFRSRFLMLATPVEELKVPKLLKEVMDEPTKRTIELFGEFGKEEPDMVAKMFELKFNNEREVEHPLFNIWIDFMMAYLDEKFVASATFLQTFRLLESSAAAGNKRSLQIKETFSRRWISSDRSLKDVAKMLQLGKNQADSSWINRLELQLYMSYMRKYILTHRKADPMLSWVLSKLVGLKHERSRKYQTRFVNFFRDNFQPSEVLTILRLDNEGGQIKNRPLVEFGLQYVASFLLKNPKAEPSILKTLKLLCHHGNSDDMFAFAKLWVSLETTPETFLKMLGIKKIDASILNHDLRDLWLAFLAHYGARLPSQALPHEMLQTINHLTTSAMMDNRSSREALAKIFHFWSRKNLTQDDMFKMLRLHTFRPHYFINPLLSTWDMYQLTFVTMHPTEPQQQLSDMIFRCFNVNDVEKLTAGAKHIPDMLHSKVKTVVKELQDKVDHFFGRT* MRPRKYIVVVLLSIAYTMCLAVGYPSDGTDDSTKSLKSGNRIRSHTPDIGT**EER**TLTWLKSMLRPSSSSAKVTKEIPEVVKSASDFKVRQTLSAITRKLGLHSQRLKRFASWLRKTGERLTRKKVYYAGYIAIVDSSSSQLKRLSVTYGSAFLFVIGFIVLLAFAMTAV* MATMRRICFLFVFNLAVATSTQGISDEISPDIPETSQAVIDGHRLKGSSKVSKATTGS**EER**FIRSQLQFLINKIFGARGVAKVLVEEGPKSGVFNKLKEILVKISKFEMKGDMLYMVYIYTILFLSIPIILGVAMYINHHVESSYIH* MQLLLFLLLIIVLTAVYCDYILKERRHLNQNDGHASLRANFANGIEKDK**EER**GFNIFRKKNPVDKAARWLLEGYKPLLKQQNPIQRGIEIQTWLSVIETEPIMPKDVITNMESYFNYEESLELCLWLVHAKNLKLKNWGIHARDKLNAAVIDEESFPTIFKKNPTVSLMFLEFKDKIHSEDPILLKWFQHFSTHETVIADKICEKDLYTLLSKSIAEMDLLQLLNSLQKKSESQLLAEQMLMFMSNDADISIKVLAAWLKDDNDLTEFWKSFPLDLTQVPLSPAFSVWLISYQQGMQKMGTTESKIFGHLVEQIHRFPVPEQTYVARLHELVAVLVSRVDTNKKTAVAELMLLSDGVNNKDLREFVIRSLAIANFDPLDAINVFGLDKVIAAGTSATVVGELVINWCKYIEGYNKLAAVQFTVDKAYKALVVLLKLTDNTIINLYTALFNSTNRNSVATGLLNLIIENKIYKTKSSDRFAALLNLNAMLHL* MTLLHCWLLLVGHLASTAYADFITKDFKSLPPPAYDTNATQALVPYNAAL**EER**NGPSSSTALLQYIDHKPGLMKKLLAGLSIRFAPPTMKVISTPTDMLRIDKIKNNIIKSSQWKRWARSLLEQNSMQNSHVIITKKMMDDLKPTNFFLVLLEASKDKNTKAVAKILEETQFARWCTIEHESISPMEFYDVLQLNLNEPIAYMERLPVLLRYWKYYKSVHSPMSSVATPKDIIDKQTVNRFGPYWKHTGEIAELLRLDFDSDSFFNHPARNVWLDLMKTYLDDTKTAEPLMIKTFQLLGNAAAKNLQNNVYSPIHFAERWIQANLQPIDVVTILGLDIHDSNLATNSAFSFLKVFIEKFLVNHPEADTTVVKIFSRLGSDESEKALALRKSFVSFFLRTPKFTPKTVMSIFDLTISADYVEKNPVWAIWMEYVNVYLVKNKVCPEGPLADTLEFLGSTAAADGVVRKKSIELLYSSWSGKTSQDTRIKQFLTAAARLNQLEL* MRLLFFLQFVIFATAIYCEHVLKGRQ**QILR**PGGHASSVKALRVNVVKGLEAKQ**EER**GFLPCCSVDPIEKAARQFLRQFDEIVRKRQDAIVRSKEIQPLLSILEDTYTIEETINALEVKMTQTKDALKKDPNQSQWVQLLVHLIQADETNGKQWQFHAKTKLNAFCKDPAHLSSILHLDPTFSLTVLELEDKVKADDAHFLQWFQNFLQNDVNTKAHHNELYYLLFKKLKNDTEMLKLFRSLQRHTETETNAKEMILFMSHDEVISKKVFEEWLNENGENANLVEFWKVLPLALNRPFLSPAFPLWLNAYGRSLKRTQGQEITFHHLLETICANSNEIFDENENPATLLAAFLKIPFHENYAKHSASHVETSSKDVKLCTYANLAKIYLWVSGCSDTRYEKQFYSTLSWSLLPPEYIYFLLDLGKVAGKISERVAIRREKVWLEYIVYYRKTNHKTNSMGMTDYMMKDYVAKNPKRSLESIKALLAKLAKGKNVVSDVLSDDMKLQEVKHAMDNNAHVGTSRDAELYHKNNF* MNLIRAMFVAALVACTRNGVHAKASEADLVTLLTTNSEIVTSQ**RLLR**TSAEPDDN**DER**AINIPFITNYNQRTRIKAPLKELKKDSNILSNVKQRLALERVVRKEIYNRGGYQNAKKVLEETDVNDPGRAILNSHVNLYKWFHNVDK*

Supplemental table 2 continued.

BLR33	MLFSVFFFLVATCVKSSYGHSVAVSTRDPENIALQLHEYASIPETIETI**RRLR**GALAHDATAY**DER**MFFENAANKMYAIAQKTRLSAAAVKKLIPESQEKKLLSYYLTKMKGFIKDREALY-SITSSYDELALLGVTPDLFRTRLLSVESPEVAAARAIEYEEYIKNICFVSSEKNPCKTVTELVESNKFDKIKELMEKHSIAELLIGALTNLNKVNHLDKLTTRIQLKYVLENTPELGPLLSRDV-NELLKDPSVSKIFSRFLTFVYGIPS*
BLR35	MARVLIPALAILLAQANSVPTSVAFELILANGQPEAAAKQNNVSPP**RSLR**SKSQAPV**EER**GIEAFDASKTIVMSDVSPIVLLMDECFGKTLDRDAFLLHENALKRIQLHISDIKQLHQI-ASTYSHLVERYGISDVVHALYSAVLKCDEKVPQTITDLVMQQREAWHLLPGGDEKILELLHMGLDGAKAFANGKVWAFMDYVAWKNLEKVFYTEKQLFRVLLTEFGGEKKLTTILLE-ATSATFDKDVGRTEFIRRSATIRKVKKPLMIKWLEDELTLDQVWTQKEIVQSEDGNLSFGTLAMFRAYLIAYLNGRTFPKMSAATFGSVFVEKALEDSFIRGYESSKSSHTPAFEMLDMI-WRAREASSSEFYEMLQKMDILSVGERFINLKERLDFIKDGSKRSIYQVLRDHHSNNELALLLDQALRAEDETISLIKVGDKEMKTDHQAGKLVLQEIEQMQFETWIEIKKLKDVDVYHN-VFKTTPKEASDLVKGIVKRFSTYKKRKSLKSGIRHCYNR*
BLR36	MRGHNLRFLFLAVIAAAVVLLMAGKQRQCLELISIEYFSKQYIAAVSNVSDPDS**RALR**YASKPGIGT**EER**GIGSSLKNKLYDSAFKWALKFHMNPKLLLYLLNVPFSHPHAEMDRNFGR-WIHYVIKFESFQDEAHRFSESTFYKLLETKLTTEDMAMLFQSLETWPDGERLAGKMQAYMILNAPSRDVMHIAWNRVREPPEHVFRILNLENETIPDRDRLITEWLRYCRSYRETES-AVAFQGLRIPDELFYSELLRLLRKTKADEDIIAFLQSIRNIDGMETFTDHIEAVADRTLLTHEILLKRVEHPETFFNRLHLQNAFLAYNSNFLKWLSYIAVYNKQPGIAPFTMEKAFEFIRSS-SEVRRLDLYFGEFLEAKMKDVPELKEELRKMQTEVFKYWRSLNIKPLTLWNTVNVHKRGRLNTPEMKNSFAYRSIQSYTKYFAQHLDNTDVSNKVLIEIADGELYKASKLASGILEI*
BLR37	MTLKPTLKACQCTFFLLWGVASLLCIETATVNATAPSTEGH**RRLR**QLVNPTLNETNYRSEDRVFPSGVGLKRTAGIPFETRPFKMSKSEADAVIKRIYHHANEHIDIALKNSNILSSNILAK-KHIERIIAEKVVALKSQPQSLPFGLAFQENVFKDWERRGITIEAAYEKLSLEHEINLGVGDSVMEVWFSFACHLKKEKYSTQFLLSKLLEHFLFEPFALFNFLVRLHSNATMSATKNLVLE-LIKELLERHSRLRIDIFTSLFTDQDLDTILKSPSFEVYANYVARYANEAEIKEKFLYLKEHTNPIQLAKHVAAFQNSQMDAAMAMKFKSCLIDVWHSDGYLESQVYQMLQLDVQDGKGL-NEPLLDLWAEYYQQYADGTVQWSSQLLSRLKLLYSNAELANLVGTPAKTNPFYPTFKMEVLLDELLKDWMARYDLTSASVLFTELYNGVDFKDVLSTPTTCVWFEFDLKLRKRENPGL-KAISLVNLEAAEVDLANKDAVEANLGNKIDAKTFLEDASYEAFLQHLLDFYHVTELNVAQLAAENIAHESLRPVATALQLALIGRWGPKEIIKVFQVLNLNFDKSAELLTNPLLEFSLDVLC-FIGIDDAKTVLRTTLKEEQIINLLSLPRRDSYLQEALFNLLTDMELWKSPEKLFQLKLQDARDTLFENPFLPFWDHFMENIHKESDTPDVENHYFAMLMIILNNGYDEPYLERLLAQ-VRNSDMTINSIILSQNASIAKALQEQLWEWNFKTSEEVFQLLGLDQTYYDPRLSKMYPYWLSYHANLNSKLPENRQILANDVIAKCALEVPSDLL*
BLR38	MHCTVFFLLIACAKSSYGQTRSVSTAKSESKSDEYSYNSDAIDQS**RLLR**GAVNPVSENMALRKFIFDMPEMLRPEHFEPIFINPAAVKEVIKDYLAYGEALCGSGYDPRLLALFGVRPT-VLKQELMKAKGVTLSVMPSSRKRPRALDEVESEVENFRNVLKDFFIPPTTTNPSKLIPDDIETLVPEHVSAHFNSLVYLMYFAVLHFDSQELATMSSSVLLKYALQKNLLLREKIESGTLGE-WERDFRLMRVLNVYKSEQT*
BLR40	MLLSRAISVVALLACICCGVHTQDSKADLGTLRTTDSAIITSQ**RRLR**TSVDLVDN**EER**FRWPFQQFFKDRWHRKQIKTYFRDQKDNVSEGLVEQLIARHGLKNVEKVLSEVKFPLAVQI-SIRKILVNYKGKQAFTRPHLTPADTL*
BLC01	MMVKLICAIVDIAGAAFPIDIDTNELVGDFKKVIKAENSRTIACDAND**LR**L**FLAK**TDGRWLTEFEVQNGVADISVFEELDVVGAPLNMIGLSEETVSSVAITKELVKAKKTPLHVLVVP-SEPVQLQRKLWLVTGTVVNALGSKGIRRHLHLMASLHIGFYDPTRRVDNKNVAFWYEAKNLCFHVLFKSSTCFFYVSPF*

[1] Signal peptides are underlined; RXLR-like sequences, EER-like sequences and LXLFLAK sequences are in bold.

Supplemental table 3, Overview of effector responses.

Germplasm set		Effector response [1]																														
		BLC01			*BLR40*			*BLN01*							*BLN03*			*BLN04*			*BLN05*			*BLN06*			*BLQ04*		
Accession/ line	*Lactuca* species	1	2	3	1	2	3	1A	1B	2	3A	3B			1	2	3	1	2	3	1	2	3	1	2	3	1	2	3			
CGN09357	*aculeata*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.3			-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0			
CGN15692	*aculeata*	-	0.0	0.8	-	0.0	0.0	-	-	0.0	0.1	1.6			-	0.0	0.1	-	0.0	0.0	-	0.0	0.8	-	0.0	0.8	-	0.0	0.1			
CGN04664	*altaica*	-	-	0.0	-	1.0	2.0	-	-	-	0.0	0.0			-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	-	1.0	-	-	0.0			
CGN15711	*altaica*	-	0.0	0.0	-	0.0	0.3	-	-	0.0	0.0	0.0			-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.3			
R201		-	0.0	-	-	0.0	-	-	-	0.0	-	-			-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-			
ANGIE (8002RZ)	*sativa*	-	-	0.0	-	-	1.3	-	-	-	0.8	1.0			-	-	0.5	-	-	0.3	-	-	0.0	-	-	2.0	-	-	0.0			
CAROLUS 4166RZ		-	-	0.0	-	-	0.9	-	-	-	0.0	0.0			-	-	0.0	-	-	0.0	-	-	0.9	-	-	1.2	-	-	1.2			
LUCIUS 4155RZ		-	-	0.3	-	-	0.3	-	-	-	0.3	0.3			-	-	0.0	-	-	0.3	-	-	0.0	-	-	0.3	-	-	0.3			
MARKIES 4287RZ		-	-	0.0	-	-	0.0	-	-	-	0.0	0.3			-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.5	-	-	0.0			
PENELOPE 42175R		-	-	1.0	-	-	0.0	-	-	-	0.5	1.0			-	-	0.0	-	-	0.3	-	-	0.0	-	-	1.0	-	-	0.0			
PI 491204		-	-	0.5	-	-	0.0	-	-	-	0.0	0.0			-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0			
PI 491226		-	-	0.0	-	-	0.0	-	-	-	0.6	0.6			-	-	0.0	-	-	0.6	-	-	0.1	-	-	0.6	-	-	0.0			
WILBUR 8542RZ		-	-	0.8	-	-	0.3	-	-	-	0.0	0.8			-	-	-	-	-	0.0	-	-	0.0	-	-	0.8	-	-	0.0			
CGN17444	*saligna*	-	0.0	-	-	0.0	-	-	-	0.0	-	-			-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0			
CGN04662	*saligna*	0.0	-	0.8	0.0	-	0.3	0.5	0.0	-	0.3	0.3			0.0	-	0.8	0.0	-	0.3	0.0	-	0.3	0.0	-	0.3	0.0	-	0.3			
CGN05147	*saligna*	0.0	0.0	0.4	0.0	0.0	0.4	0.0	0.0	0.0	0.2	0.2			0.0	0.0	0.7	0.0	0.0	0.7	0.0	0.0	0.2	0.0	0.0	0.7	0.0	0.0	0.2			
CGN05157	*saligna*	0.0	0.0	0.2	0.0	0.3	0.2	0.0	0.0	0.0	0.0	0.2			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0			
CGN05265	*saligna*	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0			
CGN05267	*saligna*	0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0	0.0	0.3	0.8			0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.3	0.0	0.0	0.3			
CGN05271	*saligna*	0.0	0.0	0.2	0.5	0.0	0.2	0.0	0.0	0.0	0.0	0.0			0.3	0.0	0.0	0.3	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.2			
CGN05282	*saligna*	0.0	-	0.2	0.0	-	0.0	0.0	0.0	-	0.0	0.0			0.0	-	0.0	0.0	-	0.2	0.0	-	0.0	0.0	-	0.7	0.0	-	0.0			
CGN05301	*saligna*	0.0	0.0	0.4	0.0	0.0	0.2	0.0	0.0	0.0	0.4	0.2			0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.2	0.0	0.0	0.4	0.0	0.0	0.2			
CGN05304	*saligna*	0.0	0.5	0.2	0.0	1.0	-	0.0	0.0	0.0	0.4	0.0			0.0	0.0	2.4	0.0	0.5	0.0	0.0	1.0	0.0	0.0	0.5	0.4	0.0	0.0	-			
CGN05306	*saligna*	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.5			0.0	0.0	-	0.0	0.0	2.5	0.8	0.0	0.0	0.3	0.0	0.3	0.0	0.0	0.5			
CGN05308	*saligna*	0.0	-	0.0	0.3	-	0.8	0.0	0.0	-	0.3	0.3			0.0	-	0.3	0.0	-	0.6	0.0	-	0.1	0.0	-	1.1	0.0	-	0.6			
CGN05309	*saligna*	0.0	0.0	1.6	0.0	0.0	1.6	0.0	0.0	0.0	1.1	1.1			0.0	0.0	0.3	0.0	0.0	1.8	0.0	0.3	0.3	0.0	0.0	0.8	0.0	0.0	0.6			
CGN05310	*saligna*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			1.5	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0			
CGN05311	*saligna*	0.3	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
CGN05313	*saligna*	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0			0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0			
CGN05314	*saligna*	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
CGN05315	*saligna*	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0			
CGN05317	*saligna*	0.0	0.0	0.3	0.0	0.5	0.3	0.0	0.0	0.5	0.5	0.0			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.5	0.3	0.0	0.0	0.3			
CGN05318	*saligna*	0.0	0.0	1.7	0.0	0.0	1.4	0.0	0.0	0.0	0.7	0.0			0.5	0.0	0.2	0.8	0.0	0.7	0.0	0.5	0.0	0.0	0.0	1.4	0.0	0.5	0.7			
CGN05320	*saligna*	0.0	0.0	0.2	0.0	0.0	0.7	0.0	0.0	0.5	0.2	0.2			0.0	0.5	0.2	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.2			
CGN05321	*saligna*	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1			0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.6	0.0	0.0	0.0			
CGN05322	*saligna*	0.0	0.0	0.2	0.8	-	0.4	0.0	0.0	0.0	0.4	0.4			0.8	-	0.2	0.3	-	0.2	0.0	-	0.2	0.0	0.0	0.2	0.8	0.0	0.4			
CGN05323	*saligna*	0.0	0.0	0.3	0.0	0.3	0.3	0.0	0.0	0.0	0.3	0.3			0.0	0.0	0.1	0.5	0.0	0.6	0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0	0.3			
CGN05324	*saligna*	0.3	0.0	0.5	0.0	0.0	0.0	0.5	0.3	0.0	0.0	0.0			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
CGN05325	*saligna*	0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0	0.0	0.3	0.3			0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.0	0.3	0.0	0.0	0.5	0.0	0.0	0.8			
CGN05327	*saligna*	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0			0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
CGN05329	*saligna*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.3	0.0	0.0	0.0			
CGN05330	*saligna*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			0.0	0.0	0.0	0.0	0.0	0.5	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
CGN05796	*saligna*	0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0			0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0	0.0	0.3	0.0	0.3	0.0	0.0	0.3			
CGN05882	*saligna*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
CGN05895	*saligna*	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.3	0.0			0.0	0.5	0.0	0.0	0.5	0.3	0.0	0.5	0.3	0.0	0.0	0.3	0.0	0.0	0.0			
CGN05947	*saligna*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
CGN09311	*saligna*	0.8	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0			0.0	0.0	0.5	0.0	0.0	0.0	1.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
CGN09313	*saligna*	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2			0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.5	0.2	0.0	0.0	0.2	0.0	0.0	0.7			
CGN09314	*saligna*	0.3	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0			0.5	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.5	0.0	0.0			
CGN10888	*saligna*	0.0	-	0.0	0.0	-	0.3	-	0.0	-	0.3	0.3			0.0	-	0.3	0.0	-	0.3	0.0	-	0.3	0.3	-	0.3	0.0	-	0.3			
CGN11341	*saligna*	0.0	0.0	0.3	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.3	0.3	0.0	0.0	0.0	0.0	0.0			
CGN13326	*saligna*	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0			0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
CGN13327	*saligna*	0.0	-	-	0.0	-	-	0.0	0.0	-	-	-			0.0	-	-	0.0	-	-	0.0	-	-	-	-	-	0.0	-	-			
CGN13330	*saligna*	0.0	-	0.3	0.0	-	0.0	0.0	-	-	1.8	0.0			0.0	-	0.0	0.0	-	-	0.0	-	1.8	0.0	-	0.3	0.0	-	0.3			
CGN15705	*saligna*	0.0	0.0	-	0.0	0.0	-	0.0	0.0	0.0	-	-			0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-			
CGN15726	*saligna*	0.0	-	0.0	0.0	-	0.5	0.0	0.0	-	0.5	0.0			0.0	-	0.0	0.0	-	0.5	0.0	-	0.5	0.0	-	0.5	0.0	-	0.5			
CGN20697	*saligna*	0.0	-	0.5	0.0	-	0.0	0.0	0.0	-	0.5	0.5			0.0	-	0.0	0.0	-	0.5	0.0	-	0.0	0.0	-	0.5	0.0	-	0.5			
HRI11140	*saligna*	-	-	0.0	-	-	0.0	-	-	-	0.0	0.0			-	-	0.5	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0			
LJ85314	*saligna*	-	-	0.0	-	-	0.0	-	-	-	0.0	0.0			-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.3	-	-	0.0			
LJ85314	*saligna*	-	-	1.0	-	-	0.0	-	-	-	0.0	0.0			-	-	0.1	-	-	0.4	-	-	0.3	-	-	0.8	-	-	0.0			
PI491000	*saligna*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.5			-	0.0	0.0	-	0.0	0.5	-	3.0	0.0	-	0.0	0.5	-	0.0	0.0			
PI491207	*saligna*	0.0	0.0	0.7	0.0	0.0	0.2	0.0	0.0	0.0	0.7	0.2			0.0	0.5	0.0	0.0	0.0	0.2	0.0	1.0	0.2	0.0	0.0	0.2	0.0	0.0	0.2			
PI491208	*saligna*	-	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
PI503623	*saligna*	0.0	0.0	0.2	0.0	0.5	0.2	0.0	0.0	0.0	0.2	0.0			0.0	0.0	0.2	0.0	0.0	0.2	0.0	0.5	0.2	0.0	0.0	0.2	0.0	0.0	0.2			
PI509523	*saligna*	0.0	-	0.0	0.0	-	0.0	0.0	0.0	-	0.0	0.0			0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.5	0.0	-	0.0			
PI509524	*saligna*	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5			0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.5	0.5	0.0	0.0	0.5	0.0	0.0	0.0			
UC94ISR1	*saligna*	1.0	-	-	0.0	-	-	0.8	0.0	-	-	-			0.0	-	-	0.8	-	-	0.0	-	-	0.0	-	-	0.0	-	-			
UC94US104	*saligna*	-	-	0.0	-	-	0.0	-	-	-	0.5	0.5			-	-	0.0	-	-	0.5	-	-	0.0	-	-	0.5	-	-	0.0			
AMPLUS	*sativa*	-	0.0	0.1	-	0.0	0.0	-	-	0.0	0.8	0.1			-	0.0	0.8	-	0.0	0.8	-	0.0	0.8	-	0.0	0.8	-	0.0	0.0			
ARGELES	*sativa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.0			-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.8	-	0.0	0.5			
BALESTA	*sativa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.3			-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	1.0	-	0.0	0.8			
BEDFORD	*sativa*	-	0.0	0.8	-	0.0	1.8	-	-	0.0	0.0	0.8			-	0.0	0.0	-	0.0	0.0	-	0.0	0.1	-	0.0	0.8	-	0.0	0.8			
BELLISSIMO	*sativa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.6	0.8			-	0.0	0.1	-	0.0	0.0	-	0.0	0.0	-	0.0	0.8	-	0.0	0.0			
CAPITAN	*sativa*	-	0.0	0.5	-	0.0	0.5	-	-	0.0	0.3	0.8			-	0.0	0.3	-	0.0	0.8	-	0.0	0.0	-	0.0	0.8	-	0.0	0.0			
CG DM16	*sativa*	-	0.0	0.0	-	0.0	0.5	-	-	0.0	0.5	0.0			-	0.0	0.3	-	0.0	0.5	-	0.0	0.3	-	0.0	0.5	-	0.0	0.5			
COBHAM GREEN	*sativa*	-	0.0	0.6	-	0.0	0.8	-	-	0.0	0.8	0.6			-	0.0	0.0	-	0.0	0.8	-	0.0	0.0	-	0.0	0.8	-	0.0	0.8			
COLORADO	*sativa*	-	0.0	0.8	-	0.0	-	-	-	0.0	0.5	0.8			-	0.0	0.3	-	0.0	-	-	0.0	0.5	-	0.0	0.0	-	0.0	0.8			
DANDIE	*sativa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.2			-	0.0	0.0	-	0.0	0.2	-	0.0	0.0	-	0.0	0.7	-	0.0	0.2			
DESIGN	*sativa*	-	0.0	0.0	-	0.0	2.7	-	-	0.0	0.2	0.4			-	0.0	0.0	-	0.0	0.2	-	0.0	0.0	-	0.0	0.0	-	0.0	0.4			

Germplasm set		Effector response [1]																								
		BLR32			*BLR33*			*BLR35*			*BLR36*			*BLR37*			*BLR38*			*YFP*			*PsojNIP*		
Accession/ line	*Lactuca* species	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
CGN09357	*aculeata*	-	0.0	0.0	-	0.0	0.3	-	0.0	0.0	-	0.0	0.0	-	0.0	0.6	-	0.0	0.0	-	0.0	0.2	3.0	2.6	-
CGN15692	*aculeata*	-	0.0	0.1	-	0.0	0.0	-	0.0	0.0	-	0.5	0.8	-	0.0	0.8	-	0.0	0.1	-	-	0.0	3.0	2.8	-
CGN04664	*altaica*	-	0.0	0.0	-	0.0	0.0	-	-	0.3	-	-	0.0	-	-	0.0	-	-	0.0	-	0.0	0.3	3.0	3.0	-
CGN15711	*altaica*	-	0.0	0.0	-	0.0	0.0	-	0.3	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	3.0	3.0	-
R201		-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	-	0.0	3.0	-	-
ANGIE (8002RZ)	*sativa*	-	-	0.0	-	-	0.0	-	-	0.5	-	-	0.0	-	-	0.0	-	-	0.0	-	0.0	0.3	-	2.0	-
CAROLUS 4166RZ		-	-	0.7	-	-	0.7	-	-	0.2	-	-	0.0	-	-	0.2	-	-	0.0	-	0.5	0.5	-	1.7	-
LUCIUS 4155RZ		-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.3	-	-	0.1	-	0.0	0.0	-	1.3	-
MARKIES 4287RZ		-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.5	-	-	0.5	-	0.0	0.0	-	2.0	-
PENELOPE 42175R		-	-	0.3	-	-	0.3	-	-	0.0	-	-	0.0	-	-	0.3	-	-	0.5	-	0.0	0.0	-	3.0	-
PI 491204		-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.5	-	-	0.0	-	0.0	0.5	-	3.0	-
PI 491226		-	-	0.6	-	-	0.6	-	-	0.6	-	-	0.0	-	-	0.1	-	-	0.1	-	-	0.2	-	1.6	-
WILBUR 8542RZ		-	-	0.0	-	-	0.1	-	-	0.0	-	-	-	-	-	0.8	-	-	-	-	-	0.2	-	2.8	-
CGN17444	*saligna*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.3	3.0	-	-
CGN04662	*saligna*	0.0	-	0.3	0.0	-	0.3	0.0	-	0.8	0.0	-	0.3	0.0	-	0.3	0.3	-	0.3	0.0	-	-	-	2.8	3.0
CGN05147	*saligna*	0.5	0.0	0.4	0.0	0.0	0.4	0.0	0.0	0.7	0.3	0.0	0.4	0.0	0.0	0.4	0.0	0.0	0.4	0.0	-	0.2	2.0	2.7	2.5
CGN05157	*saligna*	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	-	0.2	3.0	1.7	3.0
CGN05265	*saligna*	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	-	2.0	3.0	2.5
CGN05267	*saligna*	0.3	0.0	0.3	0.3	0.0	0.3	0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0	0.3	0.3	0.0	0.3	0.0	0.0	-	3.0	2.8	2.0
CGN05271	*saligna*	0.0	0.0	0.4	0.0	0.0	0.2	0.0	0.0	0.4	0.0	0.0	0.4	0.0	0.0	0.4	0.0	0.0	0.2	0.0	0.3	-	3.0	2.7	2.0
CGN05282	*saligna*	0.0	-	0.0	0.0	-	0.0	0.0	-	0.2	0.0	-	0.2	0.0	-	1.7	0.0	-	0.0	0.0	0.5	0.3	-	2.7	2.0
CGN05301	*saligna*	0.0	0.0	0.2	0.0	0.0	0.2	0.0	0.0	0.2	0.0	0.0	0.2	0.0	0.0	0.2	0.0	0.0	0.0	0.0	-	0.0	2.0	2.7	2.5
CGN05304	*saligna*	0.0	0.3	0.4	0.0	0.3	2.4	0.0	0.0	1.4	0.0	0.5	0.0	0.0	0.0	0.4	0.0	0.0	0.4	0.0	0.0	0.5	3.0	2.4	2.5
CGN05306	*saligna*	0.0	0.5	0.0	0.0	0.0	0.0	1.0	0.0	-	0.0	0.5	0.5	0.0	0.0	0.5	0.0	0.0	1.5	0.0	-	-	3.0	2.5	2.0
CGN05308	*saligna*	0.0	-	0.0	0.0	-	0.0	0.0	-	0.3	0.0	-	0.3	0.0	-	0.3	0.0	-	0.0	0.0	-	0.3	-	2.8	2.5
CGN05309	*saligna*	0.0	0.0	0.8	0.0	0.0	1.6	0.0	0.0	0.6	0.0	0.0	1.3	0.0	0.0	0.8	0.0	0.0	1.8	0.0	-	0.0	2.5	1.8	2.5
CGN05310	*saligna*	1.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.5	0.0	-	0.0	2.0	0.3	2.5
CGN05311	*saligna*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	-	0.0	2.0	0.5	2.0
CGN05313	*saligna*	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	1.0	3.0	2.5	1.5
CGN05314	*saligna*	0.0	0.3	0.0	0.0	0.5	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	-	3.0	2.5	2.0
CGN05315	*saligna*	-	0.0	0.1	-	0.0	0.1	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	0.3	-	3.0	1.3	2.5
CGN05317	*saligna*	0.0	0.3	0.0	0.0	0.3	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.5	0.8	0.0	0.0	0.6	3.0	1.5	2.0
CGN05318	*saligna*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.4	0.0	0.0	1.4	0.0	0.5	0.4	0.3	0.5	0.0	0.0	0.0	-	3.0	1.7	2.0
CGN05320	*saligna*	0.0	0.3	0.0	0.3	0.3	0.2	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	-	0.0	3.0	1.7	2.5
CGN05321	*saligna*	0.5	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.5	-	0.3	1.0	1.6	1.8
CGN05322	*saligna*	0.0	-	-	0.3	-	-	0.0	0.0	0.4	0.0	0.0	0.4	0.3	0.0	0.4	0.0	0.0	0.4	0.0	0.0	-	3.0	1.7	2.0
CGN05323	*saligna*	0.0	0.0	0.1	0.0	0.0	0.1	1.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	-	3.0	1.6	1.5
CGN05324	*saligna*	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.2	1.0	1.5	2.0
CGN05325	*saligna*	0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.0	0.3	1.0	0.0	-	2.0	2.8	1.5
CGN05327	*saligna*	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	2.0	2.3	2.5
CGN05329	*saligna*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	3.0	2.5	2.0
CGN05330	*saligna*	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	-	3.0	1.5	2.0
CGN05796	*saligna*	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0	-	2.0	2.8	2.0
CGN05882	*saligna*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	3.0	2.5	2.5
CGN05895	*saligna*	0.5	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.3	0.0	0.5	0.0	0.0	0.0	0.0	0.0	-	0.0	3.0	0.8	1.5
CGN05947	*saligna*	0.0	0.0	-	0.0	0.0	-	0.0	2.0	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	3.0	2.5	2.0
CGN09311	*saligna*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	0.3	3.0	2.5	1.5
CGN09313	*saligna*	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.2	0.0	0.0	0.2	0.0	0.5	0.2	0.0	0.0	0.7	0.0	0.0	0.0	2.0	2.7	3.0
CGN09314	*saligna*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	2.0	2.5	1.5
CGN10888	*saligna*	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.3	0.3	-	0.3	-	-	0.3	0.0	0.0	-	-	2.8	2.0
CGN11341	*saligna*	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-	2.0	2.8	2.5
CGN13326	*saligna*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	3.0	2.5	2.0
CGN13327	*saligna*	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.0	0.0	-	-	-	1.0
CGN13330	*saligna*	0.0	-	0.3	0.0	-	0.3	0.0	-	0.3	0.0	-	0.0	0.0	-	0.3	0.0	-	0.3	0.0	0.0	0.2	-	1.8	2.5
CGN15705	*saligna*	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	-	0.0	3.0	-	2.5
CGN15726	*saligna*	0.0	-	0.5	0.0	-	0.5	0.0	-	0.0	0.0	-	0.5	0.0	-	0.5	0.0	-	0.5	0.0	-	0.2	-	3.0	3.0
CGN20697	*saligna*	0.0	-	0.5	0.0	-	0.5	0.0	-	0.5	0.0	-	0.0	0.0	-	0.5	0.0	-	0.5	0.0	-	0.2	-	3.0	3.0
HRI11140	*saligna*	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	0.0	1.8	-	2.5	-
LJ85314	*saligna*	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.3	-	-	0.0	-	0.0	0.1	-	2.8	-
LJ85314	*saligna*	-	-	0.0	-	-	0.0	-	-	0.8	-	-	0.0	-	-	0.0	-	-	0.0	-	0.0	0.3	-	3.0	-
PI491000	*saligna*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	3.0	3.0	-
PI491207	*saligna*	0.0	0.0	0.2	0.0	0.5	0.0	0.0	0.0	0.2	0.0	0.0	0.2	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	-	3.0	1.7	2.5
PI491208	*saligna*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	0.0	0.0	0.5	2.0	2.5	2.5
PI503623	*saligna*	-	0.0	0.2	-	0.0	0.2	-	0.5	0.2	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	-	0.5	3.0	2.7	2.5
PI509523	*saligna*	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	2.0	-	1.5	2.0
PI509524	*saligna*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.0	0.0	3.0	3.0	2.0
UC94ISR1	*saligna*	0.0	-	-	0.0	-	-	0.0	-	-	0.5	-	-	0.0	-	-	0.0	-	-	0.0	0.0	0.5	-	-	2.0
UC94US104	*saligna*	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	-	-	0.0	0.0	-	1.0	-	3.0	2.0
AMPLUS	*sativa*	-	0.0	0.8	-	0.0	0.8	-	0.0	0.8	-	0.0	0.8	-	0.0	0.3	-	0.0	0.0	-	0.5	0.3	3.0	2.8	-
ARGELES	*sativa*	-	0.0	0.8	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	-	-	0.5	0.5	3.0	2.8	-
BALESTA	*sativa*	-	0.0	0.0	-	0.0	1.0	-	0.0	1.0	-	0.0	1.0	-	0.0	0.3	-	0.0	0.3	-	0.0	0.5	3.0	3.0	-
BEDFORD	*sativa*	-	0.0	0.1	-	0.0	0.3	-	0.0	0.3	-	0.0	0.8	-	0.0	0.8	-	0.0	0.8	-	0.0	0.5	3.0	1.3	-
BELLISSIMO	*sativa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.3	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.7	3.0	2.8	-
CAPITAN	*sativa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.8	-	0.0	0.0	-	0.0	0.8	-	0.0	0.0	-	0.0	0.2	3.0	2.8	-
CG DM16	*sativa*	-	0.0	0.5	-	0.0	0.5	-	0.0	0.5	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	3.0	1.5	-
COBHAM GREEN	*sativa*	-	0.0	0.8	-	0.0	0.8	-	0.0	0.6	-	0.0	0.3	-	0.0	0.6	-	0.0	0.3	-	0.0	0.2	3.0	2.8	-
COLORADO	*sativa*	-	0.0	0.3	-	0.0	0.0	-	0.0	0.8	-	0.0	0.8	-	0.0	0.8	-	0.0	0.8	-	0.5	0.5	3.0	1.3	-
DANDIE	*sativa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	3.0	2.9	-
DESIGN	*sativa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.2	-	0.0	0.5	3.0	2.7	-

Germplasm set		Effector response [1]																									
		BLC01			*BLR40*			*BLN01*					*BLN03*			*BLN04*			*BLN05*			*BLN06*			*BLQ04*		
Accession/ line	*Lactuca* species	1	2	3	1	2	3	1A	1B	2	3A	3B	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
DISCOVERY	*sativa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.3	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.8	-	0.0	-
HILDE	*sativa*	-	0.0	0.5	-	0.0	0.0	-	-	0.0	-	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	0.0
ICEBERG	*sativa*	-	-	0.0	-	-	0.0	-	-	-	0.8	0.0	-	-	0.0	-	-	0.0	-	-	0.8	-	-	0.0	-	-	0.0
KIGALIE	*sativa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.3	-	-	0.0	0.0	-	0.0	0.8	-	0.0	0.0	-	0.0	1.0	-	0.0	0.0
LEDNICKY	*sativa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	1.5	-	0.0	0.0	-	0.0	0.5	-	0.0	0.0	-	0.0	1.5	-	0.0	0.0
LJ85289	*sativa*	0.0	-	0.0	0.0	-	0.0	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0
NINJA	*sativa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.8	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	1.0	-	0.0	0.0
NORDEN	*sativa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5
NUN Dm15	*sativa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.3	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	0.0
NUN DM17	*sativa*	-	-	0.0	-	-	0.0	-	-	-	0.8	1.5	-	-	0.0	-	-	0.5	-	-	0.0	-	-	1.3	-	-	0.0
OLOF	*sativa*	-	-	0.0	-	-	0.0	-	-	-	0.0	0.3	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.3	-	-	0.0
PENNLAKE	*sativa*	-	0.0	1.0	-	0.0	1.0	-	-	0.0	0.3	1.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	1.0	-	0.0	2.0
PI491226	*sativa*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
R4T57D	*sativa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	1.3	-	0.0	0.0	-	0.0	1.3	-	0.0	0.0	-	0.0	0.3	-	0.0	0.0
RYZ2164	*sativa*	-	0.0	1.0	-	0.0	-	-	-	0.0	0.3	1.0	-	0.0	-	-	0.0	0.0	-	0.0	0.5	-	0.0	1.0	-	0.0	-
RYZ910457	*sativa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.8	-	0.0	1.6	-	0.0	0.8	-	0.0	0.1	-	0.0	0.3	-	0.0	0.0
SABINE	*sativa*	-	0.0	0.3	-	0.0	-	-	-	0.0	0.0	1.0	-	0.0	1.0	-	0.0	1.0	-	0.0	0.0	-	0.0	0.8	-	0.0	-
SALADIN	*sativa*	-	0.0	0.5	-	0.0	0.5	-	-	0.0	0.5	0.0	-	0.0	0.5	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	0.5
SALADIN	*sativa*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
UC2202	*sativa*	-	0.0	0.0	-	0.0	0.5	-	-	0.0	0.0	0.5	-	0.0	0.0	-	0.0	0.3	-	0.0	0.0	-	0.0	1.5	-	0.0	0.0
UC2203	*sativa*	-	0.5	0.0	-	0.0	0.0	-	-	0.0	0.3	0.5	-	0.0	0.0	-	0.0	0.8	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5
UC2204	*sativa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	0.0
UC2205	*sativa*	-	0.0	-	-	0.0	0.8	-	-	0.0	-	-	-	0.0	0.8	-	0.0	0.0	-	0.0	0.8	-	0.0	-	-	0.0	0.8
UC2206	*sativa*	-	0.0	0.5	-	1.0	-	-	-	0.0	-	0.0	-	0.0	1.0	-	0.0	1.0	-	0.0	0.0	-	0.0	1.0	-	0.0	-
UCDM 10	*sativa*	-	0.0	0.3	-	0.0	1.0	-	-	0.0	0.0	1.0	-	0.0	-	-	0.0	-	-	0.0	0.8	-	0.0	1.0	-	0.0	0.5
UCDM 14	*sativa*	-	0.0	0.4	-	0.0	0.2	-	-	0.0	0.4	0.4	-	0.0	0.0	-	0.0	0.4	-	0.0	0.0	-	0.0	0.4	-	0.0	0.0
UCDM 2	*sativa*	-	0.0	0.0	-	0.0	0.5	-	-	0.0	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	0.5	-	0.0	0.0
VALMAINE	*sativa*	-	0.0	0.0	-	0.0	0.5	-	-	0.0	0.0	0.3	-	0.0	-	-	0.0	-	-	0.0	0.0	-	0.0	0.5	-	0.0	0.3
Fenston DmV05	*sativa?*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN05091	*serriola*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN05096	*serriola*	-	0.0	-	-	0.3	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN09290	*serriola*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN10939	*serriola*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN11334	*serriola*	-	0.0	-	-	0.3	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN11335	*serriola*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN14257	*serriola*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN14258	*serriola*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN14270	*serriola*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN14296	*serriola*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN15685	*serriola*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN17387	*serriola*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN20706	*serriola*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN22747	*serriola*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN05913	*serriola*	0.0	-	-	0.0	-	-	0.0	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.5	-	-
CGN05916	*serriola*	-	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0
CGN10887	*serriola*	0.0	-	0.1	0.5	-	2.3	0.0	0.0	-	0.1	0.0	0.0	-	0.0	0.0	-	0.1	0.0	-	0.0	0.0	-	0.3	0.0	-	0.3
CGN14255	*serriola*	0.0	-	0.0	0.0	-	0.0	0.0	0.0	-	0.0	0.0	0.0	-	0.0	0.3	-	0.0	0.0	-	0.0	0.0	-	-	0.0	-	0.0
CGN14263	*serriola*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.0	0.0	0.4	0.0	0.0	0.4	0.0	0.0	0.0
CGN14271	*serriola*	0.3	-	0.3	0.0	-	0.0	0.5	0.0	-	0.0	0.0	-	-	0.0	0.0	-	0.3	0.3	-	1.0	0.3	-	1.0	0.0	-	0.0
CGN14278	*serriola*	0.0	-	-	0.0	-	0.7	0.0	0.0	-	0.0	0.0	0.8	-	0.0	0.0	-	0.2	0.0	-	0.0	0.0	-	1.7	0.0	-	0.0
LJ85314	*serriola*	-	-	1.0	-	-	0.0	-	-	-	0.0	0.0	-	-	0.1	-	-	0.4	-	-	0.3	-	-	0.8	-	-	0.0
LJ85314	*serriola*	-	-	0.0	-	-	0.0	-	-	-	0.0	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.3	-	-	0.0
LS 102	*serriola*	-	0.0	0.0	-	0.0	0.4	-	-	0.0	0.0	0.0	-	0.0	0.0	-	0.0	0.2	-	0.0	0.0	-	0.0	2.7	-	0.0	0.7
LSE 57/15	*serriola*	-	-	0.0	-	-	1.8	-	-	-	0.0	0.0	-	-	-	-	-	0.0	-	-	0.0	-	-	0.8	-	-	0.0
LSE/18	*serriola*	-	-	0.0	-	-	0.0	-	-	-	0.0	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.6	-	-	0.0
PI491108	*serriola*	-	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	1.0	0.3	0.0	0.0
PIVT 1309	*serriola*	-	-	0.0	-	-	-	-	-	-	0.0	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.8	-	-	0.0
UC96US23	*serriola*	-	0.0	0.5	0.0	0.0	0.0	-	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.3	1.0	0.0	1.0	0.0	0.0	0.0
W66331A	*serriola*	-	-	0.0	0.0	-	0.3	-	0.5	-	0.8	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.6	0.0	-	0.0
W66336A	*serriola*	-	-	0.0	-	-	0.5	-	-	-	0.3	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.5	-	-	0.0
CGN17434	*serriola?*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
Maximus bind RZ	*serriola?*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN17435	*virosa?*	-	0.0	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-
CGN05941	*virosa*	-	-	0.0	-	0.0	0.5	-	-	-	0.5	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	-	0.0	-	-	0.0
CGN04683	*virosa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	1.0	2.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0
CGN05148	*virosa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.5	0.0	-	0.0	0.0	-	0.0	0.0
CGN05332	*virosa*	-	0.0	0.5	-	0.0	0.0	-	-	0.0	0.0	0.5	-	0.0	0.5	-	0.0	0.5	-	0.0	0.5	-	0.0	0.0	-	0.0	0.0
CGN05333	*virosa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0
CGN05816	*virosa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.3	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0
CGN05978	*virosa*	-	-	0.0	-	0.0	0.5	-	-	-	0.5	0.0	-	0.0	0.0	-	1.0	0.0	-	0.0	0.0	-	-	0.0	-	-	0.0
CGN13302	*virosa*	-	-	0.0	-	-	0.0	-	-	-	0.0	0.5	-	-	0.0	-	-	0.0	-	-	0.5	-	-	0.5	-	-	0.0
CGN13339	*virosa*	-	-	0.0	-	-	0.5	-	-	-	0.0	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0
CGN13356	*virosa*	0.0	-	0.0	0.0	-	0.5	0.0	0.3	-	0.5	0.0	0.0	-	0.0	0.0	-	0.5	0.0	-	0.5	0.0	-	1.0	0.0	-	0.5
CGN13357	*virosa*	-	-	0.0	-	-	0.0	-	-	-	0.0	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.5	-	-	0.0
CGN17436	*virosa*	-	-	0.5	-	-	0.0	-	-	-	0.5	0.0	-	-	0.5	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.5
CGN18635	*virosa*	-	-	0.0	-	-	0.0	-	-	-	0.3	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0
CGN19040	*virosa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	0.0	0.0	-	0.0	0.5	-	0.0	0.5	-	0.0	0.0	-	0.0	0.0	-	0.5	0.0
CGN23887	*virosa*	-	0.0	0.5	-	0.0	0.5	-	-	0.0	0.0	0.0	-	0.0	0.5	-	0.0	0.0	-	1.0	0.0	-	0.0	0.0	-	0.0	0.0
CGN09364	*georgica*	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	0.5	-	0.0	0.5	0.0	0.0	0.0	1.0	0.0	0.0	-	0.0	0.0

Germplasm set		Effector response [1]																														
		BLR32			*BLR33*			*BLR35*			*BLR36*			*BLR37*			*BLR38*			*YFP*			*PsojNIP*		
Accession/ line	*Lactuca* species	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
DISCOVERY	*sativa*	-	0.0	-	-	0.0	-	-	0.0	0.5	-	0.0	0.3	-	0.0	0.5	-	0.0	0.0	-	0.0	0.3	3.0	3.0	-
HILDE	*sativa*	-	0.0	0.5	-	0.0	0.5	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	0.0	-	0.0	0.3	3.0	2.5	-
ICEBERG	*sativa*	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.8	-	0.5	1.3	-	1.0	-
KIGALIE	*sativa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.3	3.0	3.0	-
LEDNICKY	*sativa*	-	0.0	0.3	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.2	3.0	2.5	-
LJ85289	*sativa*	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	0.0	0.5	-	-	3.0
NINJA	*sativa*	-	0.0	0.0	-	0.0	0.5	-	0.0	0.3	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	3.0	1.3	-
NORDEN	*sativa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	-	0.3	3.0	3.0	-
NUN Dm15	*sativa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.3	-	0.0	0.0	-	0.0	0.6	3.0	1.5	-
NUN DM17	*sativa*	-	-	0.3	-	-	0.3	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.2	-	-	-
OLOF	*sativa*	-	-	0.0	-	-	0.3	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	0.0	0.2	-	2.3	-
PENNLAKE	*sativa*	-	0.0	0.0	-	0.0	1.0	-	0.0	0.5	-	0.0	0.0	-	0.0	1.0	-	0.0	0.0	-	-	0.3	3.0	-	-
PI491226	*sativa*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.0	2.0	-	-
R4T57D	*sativa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	1.0	-	0.0	0.0	-	0.0	0.0	3.0	3.0	-
RYZ2164	*sativa*	-	0.0	0.8	-	0.0	1.0	-	0.0	1.0	-	0.0	0.3	-	0.0	1.0	-	0.0	-	-	0.3	0.2	3.0	3.0	-
RYZ910457	*sativa*	-	0.0	0.8	-	0.0	0.8	-	0.0	0.1	-	0.0	0.0	-	0.0	0.8	-	0.0	0.3	-	0.0	0.3	3.0	2.3	-
SABINE	*sativa*	-	0.0	0.0	-	0.0	0.3	-	0.0	0.3	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	0.0	3.0	3.0	-
SALADIN	*sativa*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.5	0.3	3.0	0.5	-
SALADIN	*sativa*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	3.0	-	-
UC2202	*sativa*	-	0.0	0.0	-	0.0	0.0	-	0.5	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	0.0	-	1.0	0.4	3.0	1.0	-
UC2203	*sativa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.3	-	0.0	0.8	-	0.0	0.5	-	0.0	0.3	3.0	0.0	-
UC2204	*sativa*	-	0.0	0.8	-	0.0	0.5	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	0.4	3.0	3.0	-
UC2205	*sativa*	-	0.0	0.3	-	0.0	0.5	-	0.0	-	-	0.0	0.3	-	0.0	0.8	-	0.0	0.8	-	0.5	0.8	3.0	0.8	-
UC2206	*sativa*	-	0.0	-	-	0.0	-	-	0.5	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.5	3.0	2.0	-
UCDM 10	*sativa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	0.5	-	0.0	1.0	-	0.5	0.0	3.0	3.0	-
UCDM 14	*sativa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.4	-	0.0	0.4	-	0.5	0.3	3.0	2.4	-
UCDM 2	*sativa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	0.0	-	0.0	1.0	-	0.0	0.3	-	-	0.8	3.0	2.0	-
VALMAINE	*sativa*	-	0.0	0.3	-	0.0	0.5	-	0.0	0.0	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	3.0	2.5	-
Fenston DmV05	*sativa?*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	-	0.0	3.0	-	-
CGN05091	*serriola*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.5	-	3.0	-	-
CGN05096	*serriola*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.3	3.0	-	-
CGN09290	*serriola*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.0	3.0	-	-
CGN10939	*serriola*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	-	0.0	3.0	-	-
CGN11334	*serriola*	-	0.3	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	3.0	-	-
CGN11335	*serriola*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.0	2.5	-	-
CGN14257	*serriola*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.0	3.0	-	-
CGN14258	*serriola*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.2	2.5	-	-
CGN14270	*serriola*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.0	3.0	-	-
CGN14296	*serriola*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	3.0	-	-
CGN15685	*serriola*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.5	3.0	-	-
CGN17387	*serriola*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.5	3.0	-	-
CGN20706	*serriola*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.0	3.0	-	-
CGN22747	*serriola*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	-	-	3.0	-	-
CGN05913	*serriola*	0.0	-	-	0.5	-	-	0.3	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	-	-	3.0
CGN05916	*serriola*	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	3.0	3.0	2.5
CGN10887	*serriola*	0.0	-	0.1	0.5	-	0.3	0.0	-	0.3	0.0	-	0.3	0.5	-	0.8	0.0	-	0.0	0.0	0.0	0.0	-	2.8	2.0
CGN14255	*serriola*	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	0.5	0.3	-	2.0	2.5
CGN14263	*serriola*	0.0	0.0	1.4	0.0	0.0	0.0	2.0	0.0	0.0	1.5	0.0	0.0	0.8	0.0	0.4	2.0	0.0	0.0	0.0	0.0	0.2	3.0	2.4	1.0
CGN14271	*serriola*	0.5	-	0.0	0.0	-	0.0	0.5	-	1.0	0.0	-	0.0	0.5	-	1.0	0.5	-	0.8	0.0	0.0	0.5	-	3.0	3.0
CGN14278	*serriola*	0.0	-	1.4	0.0	-	0.4	0.0	-	0.0	0.0	-	0.7	0.5	-	0.0	0.0	-	0.7	1.0	0.0	0.2	-	2.7	2.5
LJ85314	*serriola*	-	-	0.0	-	-	0.0	-	-	0.8	-	-	0.0	-	-	0.0	-	-	0.0	-	0.0	0.0	-	3.0	-
LJ85314	*serriola*	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.3	-	-	0.0	-	-	-	-	2.8	-
LS 102	*serriola*	-	0.0	1.7	-	0.0	1.4	-	0.0	1.4	-	0.0	0.7	-	0.0	0.7	-	3.0	2.7	-	0.0	0.5	3.0	2.7	-
LSE 57/15	*serriola*	-	-	-	-	-	0.5	-	-	0.5	-	-	-	-	-	1.8	-	-	0.0	-	0.0	0.0	-	2.8	-
LSE/18	*serriola*	-	-	0.0	-	-	0.0	-	-	0.6	-	-	0.0	-	-	0.6	-	-	0.8	-	0.0	-	-	2.8	-
PI491108	*serriola*	0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.5	0.0	0.5	3.0	3.0	1.8
PIVT 1309	*serriola*	-	-	0.3	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	0.5	0.3	-	3.0	-
UC96US23	*serriola*	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.5	0.5	3.0	3.0	1.5
W66331A	*serriola*	0.0	-	0.3	0.0	-	0.3	0.0	-	0.0	0.5	-	0.0	0.5	-	0.0	0.0	-	0.0	0.5	0.0	0.4	-	2.8	1.8
W66336A	*serriola*	-	-	0.5	-	-	0.5	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.5	-	0.3	0.0	-	3.0	-
CGN17434	*serriola?*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.3	3.0	-	-
Maximus bind RZ	*serriola?*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.6	3.0	-	-
CGN17435	*virosa?*	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	0.0	3.0	-	-
CGN05941	*virosa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.5	3.0	2.0	-
CGN04683	*virosa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	-	0.0	3.0	2.0	-
CGN05148	*virosa*	-	0.0	2.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.3	3.0	0.0	-
CGN05332	*virosa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	-	0.3	0.5	0.5	-
CGN05333	*virosa*	-	0.0	0.0	-	0.0	0.5	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	-	0.2	1.0	3.0	-
CGN05816	*virosa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	-	0.5	3.0	2.8	-
CGN05978	*virosa*	-	0.0	0.0	-	0.0	0.0	-	-	0.0	-	-	0.0	-	-	0.5	-	-	0.0	-	0.0	0.0	3.0	1.0	-
CGN13302	*virosa*	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.5	-	-	0.5	-	-	0.0	-	0.0	0.5	-	2.0	-
CGN13339	*virosa*	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.5	-	-	0.5	-	-	0.0	-	-	0.3	-	3.0	-
CGN13356	*virosa*	0.3	-	0.0	0.0	-	0.5	0.0	-	0.5	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.8	-	0.5	2.0
CGN13357	*virosa*	-	-	-	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	-	0.0	-	1.0	-
CGN17436	*virosa*	-	-	0.0	-	-	0.0	-	-	0.5	-	-	0.0	-	-	0.5	-	-	0.0	0.0	0.0	-	-	1.0	-
CGN18635	*virosa*	-	-	0.0	-	-	0.3	-	-	0.3	-	-	0.3	-	-	0.0	-	-	0.3	-	-	0.0	-	1.8	-
CGN19040	*virosa*	-	0.0	0.0	-	0.0	0.5	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	0.0	-	-	0.4	3.0	1.0	-
CGN23887	*virosa*	-	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0	0.5	-	0.0	0.5	-	0.0	0.0	-	0.0	0.0	1.0	1.0	-
CGN09364	*georgica*	0.0	0.0	0.0	0.0	0.0	0.5	0.0	-	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	2.5	1.0	2.0

[1] effector score minus YFP score. Scores range from 0 = no response to 3 = severe necrosis. '-' indicates this accession/line was not tested in this experiment. Results of three independent experiments are shown.

Supplemental table 4, Overview of validated effector responses.

Accession/ line	*Lactuca species*	*Resistance genes*	*Effector*	*Effector response germplasm screening* [1]			*Effector response validation*
				1	*2*	*3*	
LS102	*serriola*	Dm17	BLR38	-	3	2.7	Necrotic response
LS102	*serriola*	Dm17	BLN06	-	0	2.7	Chlorotic response
NUNDM17	*sativa*	Dm17	BLN06	-	-	1.3	Necrotic response
RYZ2164	*sativa*	Dm17	BLN06	-	0	1	Chlorotic response
CGN05318	*saligna*		BLR35	0	2	0.4	No response
PI491000	*saligna*		BLN05	-	3	0	No response
CGN14263	*serriola*	Dm43/dm44	BLR35	2	0	0	No response
CGN14263	*serriola*	Dm43/dm44	BLR36	1.5	0	0	No response
CGN14263	*serriola*	Dm43/dm44	BLR38	2	0	0	No response
Design	*sativa*		BLG04	-	0	2.7	Necrotic response
Colorado	*sativa*	Dm18	BLR31	-	0	1.3	No response

[1] effector score minus YFP score. Scores range from 0 = no response to 3 = severe necrosis. '-' indicates this accession/line was not tested with this effector in this experiment

Supplemental table 5, Genes with NLR associated domains in *L. sativa* cv. Salinas that are located within the mapping intervals associated with BLR38 recognition.

Locus chromosome 4[1]

Alias[3]	*Domains*[4]	*Protein length (aa)*
Lsa017025.1	TIR	333
Lsa005833.1	TIR, Protein tyrosine kinase	499
Lsa007832.3	TIR, NB-ARC, LRR	1302
Lsa031407.1	TIR	103
Lsa031413.1	NB-ARC, LRR	934
Lsa022931.1	LRR	421

Locus chromosome 8[2]

Alias[3]	*Domains*[4]	*Protein length (aa)*
Lsa013198.1	LRR	596
Lsa016543.1	LRR, Ubiquitin elongating factor core	552
Lsa008487.1	LRR, Protein kinase	624
Lsa043030.1	TIR, NB-ARC, LRR	1157

[1] defined as the smallest mapping interval positioned at 274,187,931 to 292,881,001bp in *L. sativa* Salinas genome version 8

[2] defined as the smallest mapping interval positioned at 47,352,309 to 55,420,000 bp in *L. sativa* Salinas genome version 8

[3] according to the Lettuce Genome Resource http://lgr.genomecenter.ucdavis.edu

[4] identified with Pfam

Chapter 3

Lettuce protein targets of downy mildew effectors

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Abstract

Plant pathogenic bacteria, fungi and oomycetes secrete effectors to manipulate host cell processes. Over the last decade the genomes and transcriptomes of many agriculturally important plant pathogens have been sequenced and vast candidate effector repertoires were identified using bioinformatic analyses. Elucidating the contribution of individual effectors to pathogenicity is the next major hurdle. To advance our understanding of the molecular mechanisms underlying lettuce susceptibility to the downy mildew *Bremia lactucae,* we mapped a network of physical interactions between *B. lactucae* effectors and lettuce target proteins. Using a lettuce cDNA library-based yeast-two-hybrid system, 61 protein-protein interactions were identified involving 21 *B. lactucae* effectors and 46 unique lettuce proteins. The subcellular localization of twelve fluorescent protein tagged effector – target pairs was determined using confocal microscopy. Relocalization of effector or target to the nucleus was observed for four effector-target pairs upon co-expression.

Introduction

Plant pathogenic bacteria, fungi and oomycetes deploy effector proteins to manipulate host cell processes. Importantly, effectors serve to suppress and circumvent plant immune responses. Basal host defense responses are activated upon recognition of ubiquitously present pathogen-associated molecular patterns (PAMPs) by plant pattern recognition receptors (PRRs). Evolutionary adapted pathogens release effectors to suppress this pattern-triggered immunity (PTI). Specialized intracellular nucleotide- binding and leucine-rich repeat receptors (NLRs) recognize host translocated effectors or effector-induced perturbations on host proteins and activate effector-triggered immunity (ETI). In turn, ETI can be counteracted by effectors leading to a state of effector-triggered susceptibility (ETS) (Jones & Dangl, 2006).

Fungi and oomycetes secrete apoplastic effectors that operate at the host-pathogen interface and host-translocated effectors that act intracellularly in the host. Fungal genomes encode extensive candidate effector sets, e.g. small apoplastic cysteine-rich proteins (Sperschneider *et al.*, 2016). Plant pathogenic downy mildews and *Phytophthora* species express host-translocated Crinklers and RXLR effectors (Jiang *et al.*, 2008; Schornack *et al.*, 2010; Stassen *et al.*, 2012; Anderson *et al.*, 2015). Plant pathogenic Gram-negative bacteria such as *Pseudomonas syringae,* inject type III effectors into host cells (Schechter *et al.*, 2006). A major challenge now lies in elucidating the contribution of individual effectors to the infection process through the identification of plant targets and analysis of the underlying molecular mechanisms leading to disease susceptibility (also see **Chapter 1**).

To systematically identify effector targets in *Arabidopsis thaliana* physical interactions between Arabidopsis proteins and effector proteins of the bacterium *P. syringae*, the obligate biotrophic oomycete *Hyaloperonospora arabidopsidis* and the obligate biotrophic ascomycete *Golovinomyces orontii* were mapped using a yeast-two-hybrid (Y2H) based pipeline (Mukhtar *et al.*, 2011; Weßling *et al.*, 2014). Interactions between 123 effectors and 178 Arabidopsis proteins were found. Nine Arabidopsis proteins interacted with effectors from all three pathogens, whereas another 24 proteins interacted with effectors from two of the three pathogens. Arabidopsis proteins that interacted with effectors from multiple pathogens are proposed to function as cellular hubs on which pathogen effectors converge to effectively undermine plant immune responses (Mukhtar *et al.*, 2011). Disease assays with one or more pathogens were performed on Arabidopsis insertion mutants corresponding to 124 targets and an altered susceptibility phenotype was observed in mutant lines for 63 targets. Susceptibility phenotypes were more frequently observed in mutant lines corresponding to targets that interacted with multiple effectors (Weßling *et al.*, 2014).

The obligate biotrophic oomycete *Bremia lactucae,* the causal agent of downy mildew disease in lettuce, is a major problem in lettuce production worldwide. Consequently, a multitude of genetic studies have been carried out resulting in the identification of over 50 genes mediating resistance to *B. lactucae* in lettuce (Parra *et al.*, 2016). On the pathogen side, research efforts have led to the identification and cloning of one Crinkler and 49 *B. lactucae* RXLR-like effectors (Stassen *et al.*, 2013) (**Chapter 2**). In this study, interactions between 21 *B. lactucae* effectors and 46 unique lettuce proteins were uncovered using the Y2H system to gain insight into the molecular mechanisms underlying lettuce susceptibility to *B. lactucae* infection. The subcellular localization of a selection of targets and interacting effectors was visualized by confocal fluorescence microscopy. Upon co-expression of selected effectors

and their lettuce targets in *Nicotiana benthamiana*, relocalization of the effector or target to the nucleus was observed in four instances, providing additional evidence of interaction. Furthermore, stable transformed lines with hairpinRNA constructs were generated of three effector targets, but these failed to show altered susceptibility phenotypes.

Materials & methods

Generation of the Y2H prey library and bait constructs

A *Lactuca sativa* cv. Olof cDNA library was constructed using Invitrogen Custom Services (Invitrogen, Carlsbad, CA). Briefly, RNA was isolated using phenol/ chloroform extraction from mock treated seedlings, *B. lactucae* race 24 (compatible interaction) and isolate F703 (incompatible interaction) infected seedlings at 3 days after inoculation, and benzothiadiazole (0.1 mg/ml) treated seedlings 24h after spraying. RNA originating from the differentially treated seedlings, was mixed in equal amounts. A three-frame uncut cDNA library in pENTR222 was created from 2 mg RNA using Gateway cloning technology. The library was transferred into yeast-two-hybrid destination vector pDEST22 to generate GAL4 activation domain (AD) lettuce fusion proteins. The pDEST22 library in *E. coli* strain DH10B originated from 22 x 10^6 colony forming units (cfu) with an average insert size of 1.1 kb.

B. lactucae effectors were amplified from the predicted signal peptide cleavage site of their coding sequences and new start codons were introduced. Gateway entry clones of *B. lactucae* effectors were recombined with the pDEST32 yeast-two-hybrid destination vector using LR clonase to generate GAL4 DNA binding domain (DBD) effector fusion proteins.

Yeast strains and transformation

To create competent yeast, cells were grown o/n in 250 ml YEPD at 28 °C and 200 rpm to an OD_{600} of 0.2-0.8. Cells were spun down at 1800 rpm for 5 min, washed with 50 ml sterile ddH_2O, spun down again and washed with 50 ml TE/LiAc (100 mM LiAc, 10 mM Tris, 1 mM EDTA, pH 8.0). After a final centrifugation step, the yeast was resuspended in TE/LiAc to an OD_{600} of 50. For single construct transformation, 20 µl of competent yeast was gently mixed with 11 µl 10xTE (100 mM Tris, 10 mM EDTA, pH 8.0), 13 µl 1 M LiAc, 82 µl 60% PEG (MW 3,350), 20 µl salmon sperm DNA (Sigma #D1626; 2 mg/ml in TE, heated at 95 °C for 5 min and transferred to ice) and 200 ng plasmid. Reactions were incubated at 30 °C for 30 min, and then transferred to a water bath at 42 °C for 15 min. To each transformation reaction 1 ml ddH_2O was added, the tubes were spun down at 5000 rpm for 30 sec and the pellet was resuspended in ddH_2O. Bait strains were plated on synthetic complete (Sc) –Leu medium and prey strains were plated on Sc –Trp medium. Colonies appeared after two to three days at 30 C. For library transformation the protocol was scaled up to 3200 µl competent yeast cells and 90 µg plasmid DNA. Yeast colonies were harvested in YEPD medium + 20% (v/v) glycerol and 1 ml aliquots with an OD_{600} of 40 were frozen at -80 °C. The yeast prey library consisted of 1.1 x 10^6 – 1.5 x 10^6 individual colonies. Yeast strain Y8800 (genotype MATa trp1−901 leu2−3,112 ura3−52 his3− 200 gal4Δ gal80Δ cyh2R GAL1::HIS3@LYS2 GAL2::ADE2 GAL7::LacZ@met2) was used for prey and yeast strain Y8930 (genotype MATα trp1−901 leu2−3,112 ura3−52 his3−200 gal4Δ gal80Δ cyh2R GAL1::HIS3@LYS2 GAL2::ADE2 GAL7::LacZ@met2) was used for bait. Bait strains that grew on Sc –Leu –His plates in the absence of prey were considered auto-activating and discarded.

Library screening

The mating method was used for library screening (Fromont-Racine *et al.*, 2002). A 1 ml yeast library aliquot was thawed on ice and used to inoculate 100 ml YEPD medium. After incubation with shaking at 28 °C for 1 hour, the yeast cells were spun down at 1800 rpm for 5 min and washed twice with ddH_2O, before resuspension in YEPD medium to an OD_{600} of 1. 6 ml of yeast library was mixed with an equal amount of overnight cultured bait strain cells and yeast cells were collected by centrifugation. The pellet was resuspended in 300 µl ddH_2O and plated on YEPD medium + 100 µg/ml ampicillin. After incubation at 30 °C for 4 hours, 2 ml ddH_2O was added to the plate and yeast was scraped off. Yeast was collected by centrifugation and the pellet was resuspended in 600 µl ddH_2O. Yeast was plated on Sc –Leu –Trp –His + amp medium and incubated at 30 °C for four days. A 1:10,000 dilution was plated on Sc –Leu –Trp + amp medium to determine the number of diploid yeast screened. Per bait a minimum of one million diploids was screened.

Up to 96 colonies per Sc –Leu –Trp –His + amp plate were picked, resuspended in 25 µl ddH_2O and spotted on fresh Sc –Leu –Trp –His + amp medium in duplo. After incubation at 30 °C for two days, one plate was used for replica plating and the other for colony PCR. Yeast was replica plated on Sc –Leu –Trp –His + 2 mM 3-amino-1,2,4-triazole (3AT) (Formedium) + amp medium and Sc –Leu –Trp –His +5 mM 3AT + amp medium followed by incubation at 30 °C for two days, and plated on Sc –Leu –Trp –Ade + amp medium followed by incubation at 20 °C for five days.

For yeast colony PCR, yeast patches from a Sc –Leu –Trp –His + amp plate were lightly touched with a pipette tip, resuspended in 30 µl 0.02 M NaOH and heated at 99 °C for 10 min. Lysates were spun down briefly and 1 µl of the supernatant was used for a 10 µl PCR reaction with DreamTaq DNA polymerase (ThermoScientific) and primers pDEST22 and AP22 (Supplemental table 1) to detect a DNA fragment that recurred as unspecific 'interactor' with almost all bait screened. The prey fragment from colonies negative for the unspecific 'interactor' was amplified with primers pDEST22 and pDEST22/32 (Supplemental table 1). Prey PCR products were purified using Agencourt AMPure XP beads according to the manufacturer's protocol and Sanger sequenced. Prey sequences were used in a BLASTn search against a previously described lettuce transcriptome (**Chapter 2**) to identify the corresponding scaffold/ contig on which the lettuce cDNA fragment was located. A representative colony per identified scaffold/ contig was grown o/n in 4 ml YEPD and 1-3 ml was used for plasmid isolation. Yeast was spun down at 2000 rpm for two minutes and the pellet was resuspended in 1 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The centrifugation step was repeated and the pellet was resuspended in 200 µl Resuspension Buffer with RNase A supplemented with 15 U Zymolyase-20T (Amsbio #120491-1) and 5 µl 2-mercaptoethanol. After incubation at 37 °C for 60 minutes, plasmid DNA was purified using the GenElute Plasmid miniprep kit (Sigma). Due to the low plasmid DNA yield of yeast, prey plasmid was transformed in *E. coli* DH5α cells and plasmid DNA isolated from a single *E. coli* colony was used for sequencing and retransformation.

To confirm the interaction found in the library screen, bait and prey plasmid were cotransformed in yeast strain Y8930, selected on Sc –Leu –Trp + amp medium and replica plated on Sc –Leu –Trp –His + amp, Sc –Leu –Trp –His + 2 mM 3AT + amp, Sc –Leu –Trp –His + 5 mM 3AT + amp and Sc –Leu –Trp –Ade + amp medium.

Bioinformatic analysis of lettuce targets
Lettuce prey gene models were extracted using a BLASTn search against the lettuce genome (Reyes-Chin-Wo *et al.*, 2017). Where necessary, incomplete gene models were corrected using the lettuce transcriptome and prey sequencing data. Prey sequences with a stop codon within the first 50 aa after the GAL4-AD sequence were considered not in frame. Presence of signal peptides was predicted using SignalP4.1 (Bendtsen *et al.*, 2004) and transmembrane domains were identified with TMHMM (Sonnhammer *et al.*, 1998; Krogh *et al.*, 2001). Domain prediction was performed using InterProScan5. Subcellular localization was predicted using TargetP 1.1 (Emanuelsson *et al.*, 2000) and YLoc (Briesemeister *et al.*, 2010a,b). The presence of importin-α dependent nuclear localization signals was predicted using cNLS Mapper (Kosugi *et al.*, 2009). The transcriptional regulation of Arabidopsis homologs identified by reciprocal BLAST, in response to biotic stress was determined via GeneVestigator (Zimmermann *et al.*, 2004).

Transient expression in *N. benthamiana*
Full-length prey sequences were amplified from *L. sativa* cv. Olof cDNA or prey plasmid using primers listed in Supplemental table 1 and recombined in a modified pGemTEasy vector containing the pDONR201 Gateway recombination site (pGemTEasy^{mod}) using BP clonase. Bait and prey entry clones were further recombined in pUBN-YFP-DEST and pUBN-CFP-DEST (kind gift from dr. Christopher Grefen; (Grefen *et al.*, 2010)) respectively using LR clonase and transformed in *Agrobacterium tumefaciens* strain C58C1 (pGV2260) with selection on rifampicin (50 µg/ml), carbenicillin (50 µg/ml) and spectinomycin (100 µg/ml). Leaves of four to five-week-old *N. benthamiana* plants were co-infiltrated with an *A. tumefaciens* strain carrying the P19 silencing suppressor (Scholthof, 2006) in combination with strains harboring bait or prey fusion constructs resuspended in infiltration buffer (10 mM MES, 10 mM MgCl2, and 150 µM acetosyringone, pH 5.6) to an OD of 0.3 per *A. tumefaciens* strain.

Confocal fluorescence microscopy
Microscopy was performed at 2-3 days after *Agrobacterium*-infiltration using a Zeiss LSM 700 laser scanning microscope. Leaf sections were incubated in propidium iodide (PI) solution (5 mg/ml) for 7-10 min prior to imaging to stain the cell wall. Excitation of Cyan Fluorescent Protein (CFP), Yellow Fluorescent Protein (YFP) and PI was done at 405 nm, 488 nm and 555 nm respectively. Emitted light of CFP and YFP was captured using a 490-555 nm band-pass filter, whereas emitted light of PI was captured using a 560 nm long pass filter.

HairpinRNA mediated silencing in lettuce
Gene fragments of approximately 300 bp were amplified from *Lsa022944.1, Lsa044405.1* and *Lsa021294.1* using primers listed in Supplemental table 1 and cloned in pGemTEasy^{mod} using BP clonase. Entry clones were recombined with the pHELLSGATE12 destination vector (Wesley *et al.*, 2001; Helliwell & Waterhouse, 2003) to generate a construct with the gene fragments in the sense and anti-sense orientation separated by *PDK* and *Catalase-1* introns. pHELLSGATE12 vectors with *Lsa022944.1, Lsa044405.1* and *Lsa021294.1* gene fragments were transformed in *A. tumefaciens* strain GV2260MPI with selection on rifampicin (20 µg/ml), streptomycin (250 µg/ml) and spectinomycin (100 µg/ml). *L. sativa* cv. Wendell and CobhamGreen were transformed essentially as described before (Pileggi *et al.*, 2001).

Quantitative RT-PCR

The silencing efficiency and specificity of hpRNA constructs in T2 families was determined using qRT-PCR. 100 mg of leaf material was flash-frozen in liquid nitrogen and ground to a fine powder using the TissueLyser (Qiagen). RNA was extracted using the Spectrum Plant Total RNA kit (Sigma-Aldrich) followed by DNase I (Thermo Fisher Scientific) treatment. cDNA was synthesized using RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific). Gene expression levels were determined in two technical replicates with SYBR Green as reporter dye (Thermo Fisher Scientific). Expression levels were calculated relative to *L. sativa* cv. Salinas *ACTIN* (Lsa015946.1).

Transcript abundance of effectors during infection of lettuce seedlings with *B. lactucae* race 24 was determined as described in **Chapter 2**. The sequences of all primers used for qRT-PCR are listed in Supplemental table 1.

***B. lactucae* disease assays**

Leaf discs were punched from four to five-weeks-old lettuce plants, placed upside down in a tray containing water-soaked filter paper and sprayed with a Bl:24 spore suspension (10-100 spores/µl). Leaf disc assays were performed at 15°C, 80% relative humidity and 15 hrs of light/day. The infection severity was scored at 13 dpi.

Results

Effector interaction screening identifies new and known target candidates

To identify lettuce proteins that are targeted by *B. lactucae* effectors a yeast-two-hybrid screen was performed. Initially, 46 previously described *B. lactucae* effectors were selected and the coding sequence lacking the signal peptide-encoding part was cloned in frame with the GAL4-DBD in the bait vector. Of the 46 bait constructs three showed auto-activation in the Y2H system. The remaining 43 effectors were used as baits in Y2H screens with a prey library composed of *L. sativa* cv. Olof complementary DNA (cDNA). To eliminate false positives, candidate effector targets were carefully scrutinized. Firstly, lettuce gene fragments that were out of frame with the GAL4 activation domain coding sequence were discarded. Secondly, prey constructs that tested positive for autoactivation of the *HIS3* reporter in the absence of the corresponding bait construct were removed. Thirdly, bait and prey plasmids were retransformed in yeast to test if the interaction could be confirmed. These selection steps resulted, predominantly, in the elimination of candidate effector targets that were identified only once. In contrast, most candidate effector targets identified in two or more yeast colonies proved reliable interactors. Therefore, candidate effector targets identified only once were omitted from further analysis. Ultimately, the library screens and subsequent validation steps resulted in interactors for 21 effectors (46%) (Table 1) and a set of 46 unique interacting lettuce proteins (Table 2).

Interactions between effectors and lettuce proteins were further classified according to the number of colonies corresponding to the same lettuce gene and the interaction strength based on reporter gene activation (Figure 1). Due to the stringent selection criteria, only two weak (activation of *HIS3* reporter only) interactions (3%) were identified, 25 interactions (41%) were classified as intermediate (activation of *HIS3* reporter in the presence of 2 mM 3AT) and 34 interactions (56%) were strong (activation of both the *HIS3* and *ADE2* reporters). The vast majority of interactions between effectors and lettuce targets

were highly specific: only eight preys (17%) interacted with multiple effectors. Lsa002122.1, a reticulon-like protein, stood out from other lettuce targets due to its interaction with five effectors and a combined total of 104 yeast colonies (Table 2). The most yeast colonies, 50, within a single effector screen were identified with effector BLR18 for Lsa011822.1 that encodes COP9 signalosome subunit 5 (CSN5). However, Lsa011822.1 also displayed weak activation of the *HIS3* reporter in the presence of empty bait vector.

The most represented family of proteins in the screen is the prenylated rab acceptor (PRA1) family for which fragments of six members were identified and three members passed all selection criteria. *PRA1* genes encode small transmembrane proteins that localize to the secretory pathway in Arabidopsis and are proposed to play a role in vesicular trafficking in plants (Alvim Kamei *et al.*, 2008). Also, they have previously been described as targets of *G. orontii* effector candidates in Arabidopsis (Weßling *et al.*, 2014). In lettuce the PRA1 protein family is composed of 17 members. The six Y2H identified PRA1 proteins were found in three clades of the phylogenetic tree (Figure 2a). To determine the specificity of the interactions between *B. lactucae* effectors and PRA1 proteins, a targeted Y2H assay was performed. All six identified prey constructs containing lettuce *PRA1* gene fragments were cotransformed with three interacting and one non-interacting *B. lactucae* effector in yeast and plated on selective medium of increasing stringency. The negative control BLR09 did not interact with any of the PRA1 members. The interactions between effectors BLR27, BLR32 and BLN01 with individual PRA1 proteins as identified in the library screens were confirmed. Furthermore, BLR27 and BLR32 interacted with more PRA1 proteins than determined by the library screen, indicating that the Y2H screening was not exhaustive and not all possible interactions were captured. In contrast, BLN01 interacted robustly with Lsa013759.1 but only weakly with other PRA1 proteins (Figure 2b).

Table 1, Number of interacting plant proteins identified for individual effectors by Y2H screening.

Effector	*Plant proteins*	*Effector*	*Plant proteins*	*Effector*	*Plant proteins*
BLC01	-	BLR08	4	BLR25	-
BLG01	-	BLR09	7	BLR26	2
BLG02	2	BLR11	9	BLR27	4
BLG03	1	BLR12	1	BLR28	1
BLN01	7	BLR13	-	BLR29	-
BLN03	1	BLR14	-	BLR30	-
BLN04	3	BLR15	-	BLR31	-
BLN05	-	BLR16	-	BLR32	1
BLN06	-	BLR17	-	BLR33	-
BLN08	-	BLR18	2	BLR35	2
BLQ01	-	BLR19	AB	BLR36	-
BLQ04	2	BLR20	1	BLR37	AB
BLR03	2	BLR21	1	BLR38	2
BLR04	-	BLR22	AB	BLR40	-
BLR05	6	BLR23	-		
BLR07	-	BLR24	-		

AB: Autoactivating bait

Fourteen plant targets were selected for validation of protein-protein interactions *in planta*, including the top ten ranked effector targets by total number of yeast colonies. In addition, two membrane steroid-binding proteins (MSBPs), Lsa010116.1 and Lsa044405.1, were selected based on gene expression data of the orthologous gene in Arabidopsis. This *MSBP2* gene (*AT3G48890*) is of interest because it is strongly upregulated in Col-0 plants infected with *P. infestans*, and the fungal pathogens *Sclerotinia sclerotiorum* and *Alternaria brassicicola* (Supplemental table 2). Finally, Lsa022944.1 and Lsa006137.1 were chosen because of their predicted functions in targeting proteins for degradation and gene transcription respectively, which will be described in more detail later in this chapter.

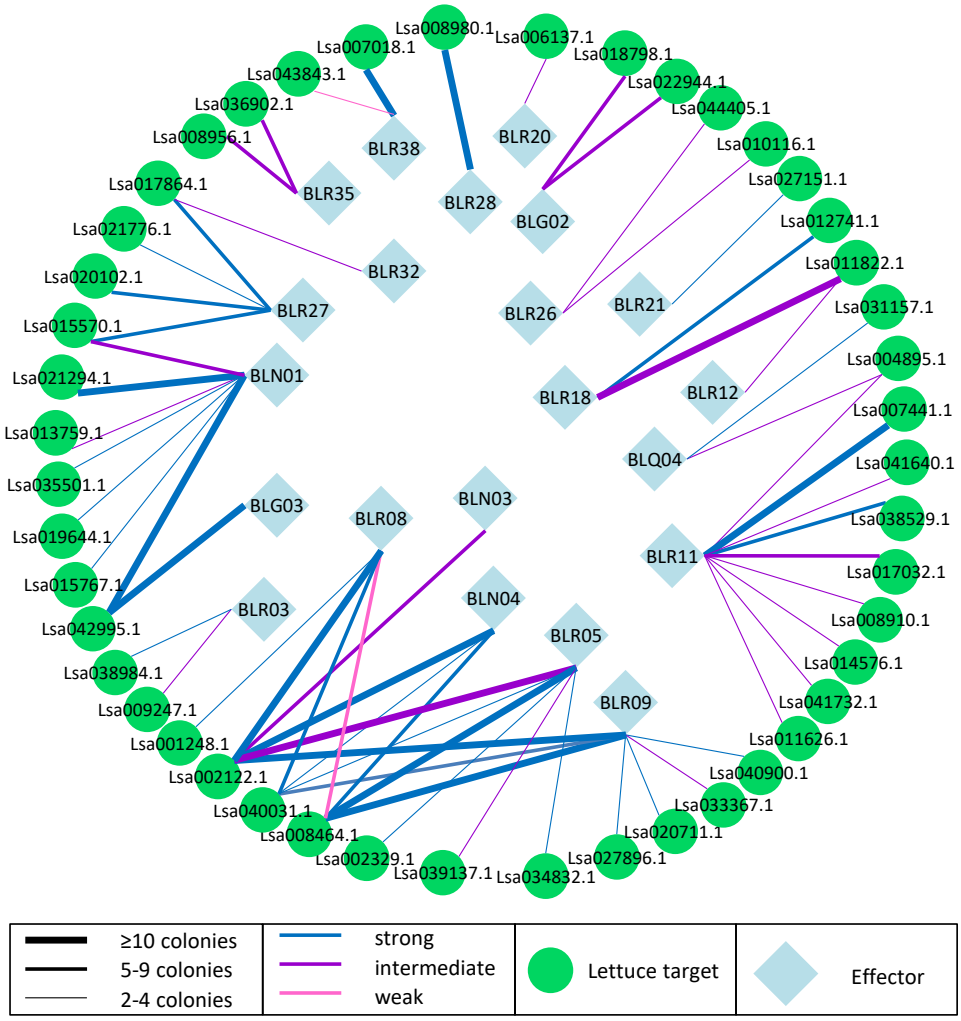

Figure 1, Yeast-two-hybrid identified *B. lactucae* effector – lettuce protein network. Interactions between *B. lactucae* effectors (blue diamonds) and lettuce proteins (green circles) are depicted according to the number of independent colonies representing a lettuce target and the level of reporter gene activation.

Table 2, Lettuce proteins identified in yeast-two-hybrid screens

Lactuca ID[1]	*Interacting effectors (# colonies)*	*Length (aa)*	*Signal peptide* [2]	*Transmembrane domains* [3]	*Domains/ family* [4]
Lsa018798.1	BLG02 (7)	812	-	-	AAA ATPase, CDC48 family
Lsa022944.1	BLG02 (9)	421	-	-	MATH/TRAF domain; BTB/POZ domain
Lsa042995.1	BLG03 (23), BLN01 (42)	270	-	-	Ferritin-like domain
Lsa013759.1	BLN01 (4)	219	-	79-98; 102-119; 139-161; 195-217	Prenylated rab acceptor PRA1 family
Lsa015767.1	BLN01 (3)	381	-	-	Zinc finger, RING/FYVE/PHD-type
Lsa019644.1	BLN01 (2)	408	-	372-394	-
Lsa021294.1	BLN01 (11)	347	-	-	WAT1-related protein
Lsa035501.1	BLN01 (3)	388	-	-	SANT/Myb domain
Lsa015570.1	BLN01 (8), BLR27 (6)	249	-	120-142; 152-174; 181-215	-
Lsa002122.1	BLN03 (6), BLN04 (13), BLR05 (29), BLR08 (25), BLR09 (31)	296	-	129-147; 151-173; 217-239	Reticulon domain
Lsa008464.1	BLN04 (9), BLR05 (23), BLR08 (9), BLR09 (15)	191	1-24	114-136	-
Lsa040031.1	BLN04 (4), BLR05 (14), BLR08 (6), BLR09 (7)	497	-	467-486	NAC domain

Table 2 continued.

Lactuca ID[1]	*Interacting effectors (# colonies)*	*Length (aa)*	*Signal peptide* [2]	*Transmembrane domains* [3]	*Domains/ family* [4]
Lsa031157.1	BLQ04 (2)	886	-	-	TATA element modulatory factor 1 DNA binding domain
Lsa004895.1	BLQ04 (4), BLR11 (4)	963	-	-	TATA element modulatory factor 1 DNA binding domain
Lsa009247.1	BLR03 (3)	418	-	-	CBS domain
Lsa038984.1	BLR03 (4)	338	-	-	E3 ubiquitin-protein ligase BOI-like family
Lsa002329.1	BLR05 (2)	137	-	111-133	Cytochrome b5-like heme/steroid binding domain
Lsa034832.1	BLR05 (2)	171	-	135-157	-
Lsa039137.1	BLR05 (3)	279	-	111-133; 159-181; 248-270	Uncharacterised protein family UPF0114
Lsa001248.1	BLR08 (2)	682	-	522-544; 614-636	Protein of unknown function DUF639
Lsa020711.1	BLR09 (2)	596	-	568-590	NAC domain
Lsa027896.1	BLR09 (3)	341	-	-	AIG1-type guanine nucleotide-binding (G) domain
Lsa033367.1	BLR09 (2)	552	-	530-547	CBS domain
Lsa040900.1	BLR09 (2)	576	-	-	Pentatricopeptide repeat domain

Table 2 continued.

Lactuca ID[1]	*Interacting effectors (# colonies)*	*Length (aa)*	*Signal peptide* [2]	*Transmembrane domains* [3]	*Domains/ family* [4]
Lsa007441.1	BLR11 (13)	698	-	-	Heat shock protein Hsp90 family
Lsa008910.1	BLR11 (2)	729	-	-	-
Lsa011626.1	BLR11 (2)	148	-	-	-
Lsa014576.1	BLR11 (3)	531	-	-	Ubiquitin system component Cue domain
Lsa017032.1	BLR11 (5)	671	-	-	-
Lsa038529.1	BLR11 (5)	561	-	-	Protein OBERON family
Lsa041640.1	BLR11 (3)	535	-	-	Serine/threonine/dual specificity protein kinase, catalytic domain
Lsa041732.1	BLR11 (4)	352	-	-	SH3 domain
Lsa011822.1	BLR12 (3), BLR18 (50)	361	-	-	JAB1/MPN/MOV34 metalloenzyme domain
Lsa012741.1	BLR18 (7)	316	-	-	Thylakoid formation protein
Lsa006137.1	BLR20 (3)	290	-	-	AP2/ERF domain
Lsa027151.1	BLR21 (2)	207	-	-	Mitotic spindle checkpoint protein Mad2 family
Lsa010116.1	BLR26 (2)	177	-	18-40	Cytochrome b5-like heme/steroid binding domain

Table 2 continued.

Lactuca ID[1]	*Interacting effectors (# colonies)*	*Length (aa)*	*Signal peptide* [2]	*Transmembrane domains* [3]	*Domains/ family* [4]
Lsa044405.1	BLR26 (4)	214	-	23-45	Cytochrome b5-like heme/steroid binding domain
Lsa020102.1	BLR27 (7)	185	-	71-102; 122-156	Prenylated rab acceptor PRA1 family
Lsa021776.1	BLR27 (2)	274	-	-	RNA recognition motif domain
Lsa017864.1	BLR27 (7), BLR32 (3)	181	-	70-102; 117-136; 143-160	Prenylated rab acceptor PRA1 family
Lsa008980.1	BLR28 (18)	420	-	-	Chaperone DnaJ domain
Lsa008956.1	BLR35 (5)	865	-	-	Kinesin motor domain; P-loop containing nucleoside triphosphate hydrolase domain
Lsa036902.1	BLR35 (9)	375	-	338-360	-
Lsa007018.1	BLR38 (11)	382	-	-	-
Lsa043843.1	BLR38 (4)	429	-	-	Prephenate dehydratase domain

[1] according to the Lettuce Genome Resource http://lgr.genomecenter.ucdavis.edu/

[2] predicted using SignalP 4.1

[3] predicted using TMHMM Server v.2.0

[4] predicted using InterProScan5

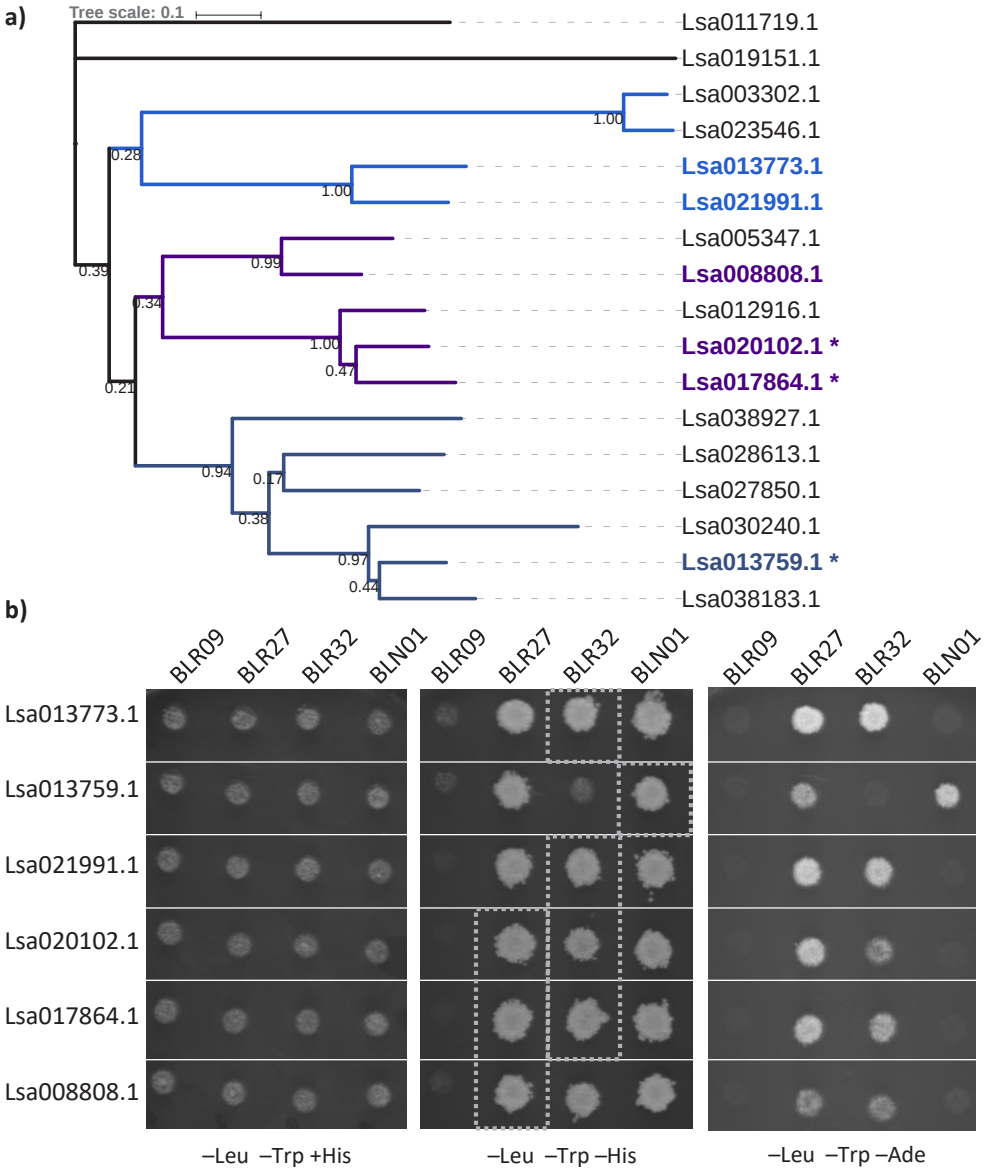

Figure 2, Three *B. lactucae* effectors interact with multiple PRA1 family members. a) Phylogenetic analysis on 17 PRA1 domain-containing proteins. Sequences were aligned using Clustal Omega. Tree construction was performed in MEGA 7.0 using Neighbor-Joining with 1000 bootstrap replicates. The bootstrap values are indicated at each node. Proteins with an asterisk (*) passed the stringent selection criteria set for Y2H-identified effector targets and were divided over two clades. Proteins in bold were used for targeted Y2H assays. b) A targeted Y2H was performed with yeast isolated prey plasmids containing gene fragments of six PRA1 family members using *B. lactucae* effectors BLR27, BLR32 and BLN01 as bait. BLR09 was included as a negative control bait. Left: permissive plate containing histidine. Middle: moderately selective plate lacking histidine. Right: strongly selective plate lacking adenine. The positions of previously identified interactions in the library screens are indicated with grey dotted blocks for selection on selective medium lacking histidine.

To further explore the selected effector targets, a closer look was taken at the prey plasmid-encoded gene fragments. DNA sequences obtained by Sanger sequencing were aligned to the corresponding lettuce coding sequences. Lettuce gene fragments that included the start codon of the lettuce coding sequence (Figure 3) were detected for eight preys. Yeast clones of the remaining six preys only contained fragments that started downstream of the predicted start codon. The use of a cDNA library that contains both full-length coding sequences and gene fragments likely increased the number of identified protein-protein interactions. For example, the fragments encoding the two MSBP proteins did not include the N-terminal predicted transmembrane domain. Membrane-associated proteins are frequently not identified as interactors in conventional Y2H assays because they cannot fold correctly in the nucleus or are unable to enter the nucleus (Thaminy *et al.*, 2004).

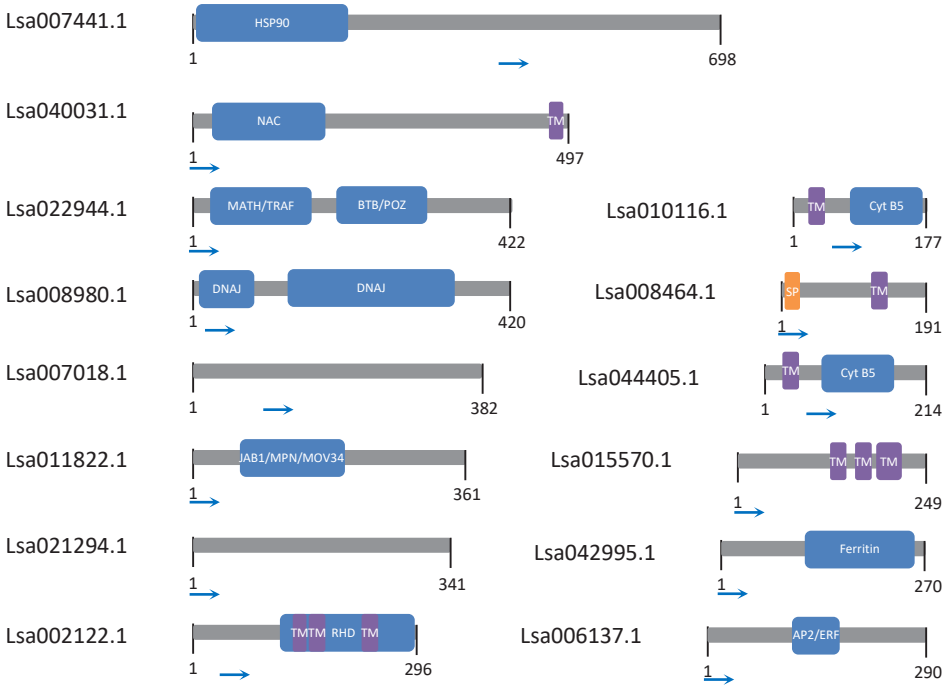

Figure 3, Graphical representation of Y2H-identified effector targets. The start position of the longest identified gene fragment is indicated with a blue arrow. Domains were predicted using InterProScan5. TM = transmembrane domain, SP = signal peptide

Colocalization of effectors and their plant targets

As proteins that are localized in the same subcellular compartment are more likely to be true interactors *in planta*, the subcellular localization of *B. lactucae* effectors and their plant targets was determined using confocal fluorescence microscopy. The full-length coding sequence of the selected lettuce genes was cloned and fused downstream of *CFP*. Effectors were fused to *YFP*. The fusion proteins were produced in *Nicotiana benthamiana* leaves using *Agrobacterium*-mediated transient expression. The localization of effectors BLN03, BLN04, BLR05, BLR08 and BLR09 with the interacting lettuce proteins Lsa040031.1 (LsNAC069), Lsa008464.1 (LsPUF001) and Lsa002122.1 (LsRTNLB05) is described in **Chapter 4**.

Of the 11 effectors described here, four localized exclusively to the cytoplasm and three showed both cytoplasmic and nuclear localization (Figure 4 and Table 3). YFP-BLR28 and BLR38-YFP contain predicted nuclear localization signals (Supplemental table 3) and localized solely to the nucleus (also see **Chapter 2**). BLG03 is recognized in *Dm2* lettuce lines (Stassen *et al.*, 2013) and YFP-BLG03 was associated with the plasma membrane similarly to BLN06-YFP and BLR40-YFP (**Chapter 2**). YFP-BLR12 appeared as punctate structures and upon prolonged overexpression also labeled the endoplasmic reticulum suggesting that the punctate structures represent components of the secretory pathway. However, this requires confirmation using appropriate markers.

Subsequently, the lettuce proteins were expressed in the absence of interacting effectors since co-expression may affect the localization. Seven of the eleven tested proteins localized to the cytoplasm and/or nucleus (Figure 5 and Table 3). Lsa007018.1 and Lsa021294.1 localization was restricted to small, punctate structures in the cytoplasm. In contrast to BLR12, no ER localization was observed for Lsa007018.1 and Lsa021294.1. The structures were neither associated with the plasma membrane, therefore their origin remains undetermined. Expression of MSBPs CFP-Lsa010116.1 and CFP-Lsa044405.1 induced severe stress (visible as collapse of the ER) in *N. benthamiana.* Therefore, these proteins could not be accurately visualized.

Table 3, Subcelullar localization of fluorescent protein fusions of individually expressed *B. lactucae* effectors and interacting lettuce proteins

Effector	*Subcellular localization*	*Lettuce protein*	*Subcellular localization*
BLG02	Cytoplasm and occasionally nucleus	Lsa006137.1	Nucleus
BLG03	Plasma membrane	Lsa007018.1	Punctate structures
BLN01	Cytoplasm	Lsa007441.1	Cytoplasm
BLR11	Cytoplasm	Lsa008980.1	Cytoplasm
BLR12	Punctate structures	Lsa010116.1	ND
BLR18	Cytoplasm and nucleus	Lsa011822.1	Cytoplasm and nucleus
BLR20	Cytoplasm	Lsa015570.1	Cytoplasm
BLR26	Cytoplasm and (weakly) nucleus	Lsa021294.1	Punctate structures
BLR27	Cytoplasm	Lsa022944.1	Nucleus
BLR28	Nucleus	Lsa042995.1	Cytoplasm
BLR38	Nucleus	Lsa044405.1	ND

ND: Not determined

Upon co-expression of the effectors with lettuce proteins, (partial) colocalization was observed for 9 out of 12 combinations (Figure 6 and Table 4). As expected, the cytoplasmic localized effectors YFP-BLR11, YFP-BLR27 and YFP-BLN01 colocalized upon co-expression with their cytoplasmic targets CFP-Lsa007441.1, CFP-Lsa015570.1 and CFP-Lsa042995.1. Interestingly, in several cases co-expression induced a relocalization, specifically, to the nucleus.

Relocalization of effector BLG02 occurred in the presence of the nuclear localized protein CFP-Lsa022944.1: YFP-BLG02 was mainly cytoplasmic and, in some cells, nuclear localized, but showed a consistent nuclear and cytoplasmic (nucleocytoplasmic) signal in cells that also expressed CFP-Lsa022944.1 (Figure 6). The cytoplasmic effector YFP-BLR20 relocalized to the nucleus (Figure 6) upon co-expression of nuclear localized CFP-Lsa006137.1 and the intensity of the YFP nuclear signal was dependent on CFP-Lsa006137.1 signal intensity.

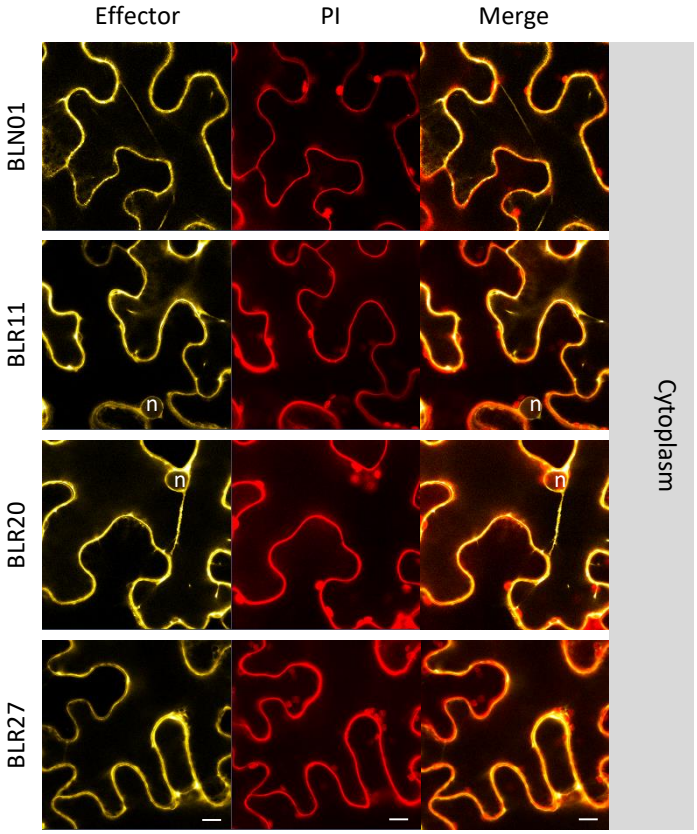

Figure 4, Subcellular localization of *B. lactucae* effectors in *N. benthamiana*. Effectors were fused N-terminally to YFP at the predicted signal peptide cleavage site, with the exception of BLR38 that was cloned as a C-terminal fusion protein, and transiently expressed in *N. benthamiana* using *Agrobacterium*. Images were taken 2-3 days after infiltration. Leaf sections were incubated in propidium iodide (PI) to stain the cell wall. Bars indicate 10 µm. n = nucleus

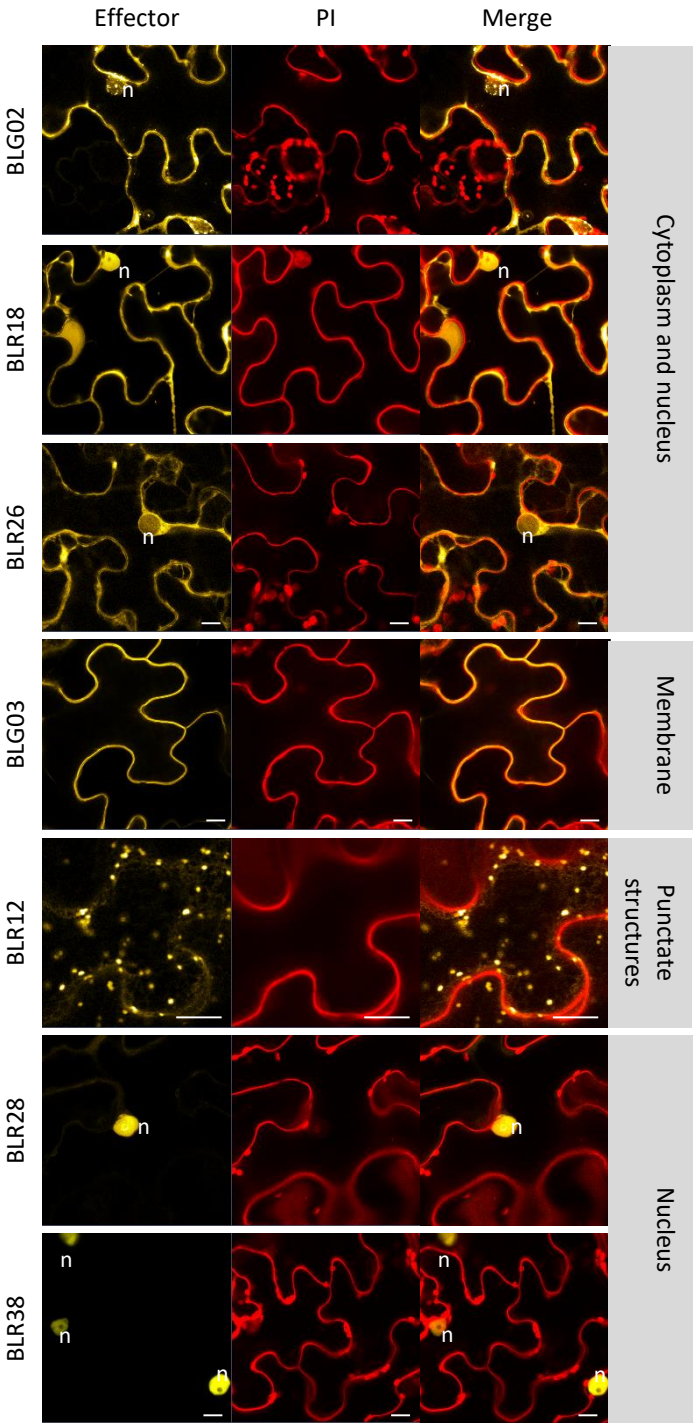

Figure 4 continued.

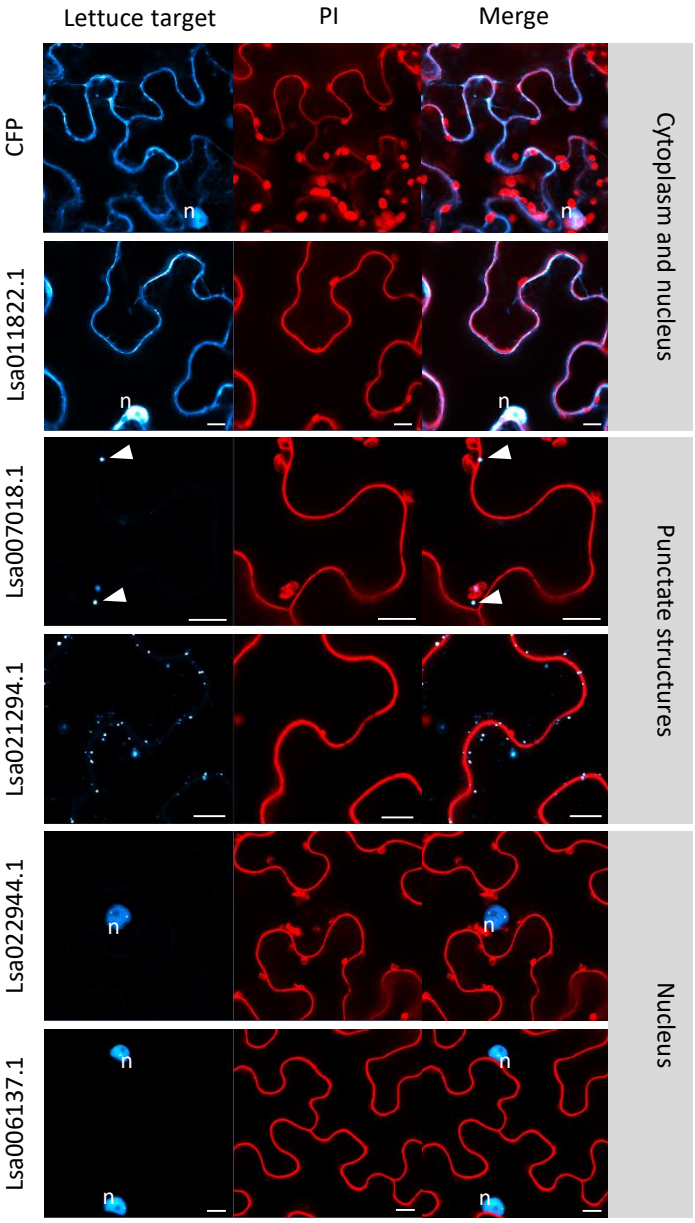

Figure 5, Subcellular localization of effector targets in *N. benthamiana*. Lettuce proteins were fused N-terminally to CFP and transiently expressed in *N. benthamiana* using *Agrobacterium*. Images were taken 2-3 days after infiltration. Leaf sections were incubated in propidium iodide (PI) to stain the cell wall. Bars indicate 10 µm. n = nucleus. Arrowheads indicate the position of punctate structures.

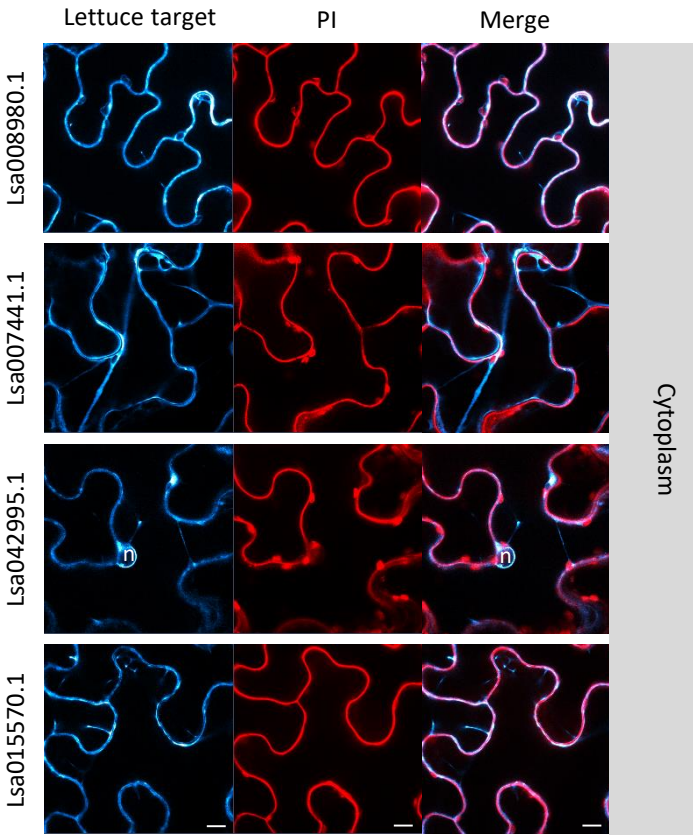

Figure 5 continued.

Table 4, Subcellular localization of *B. lactucae* effectors and interacting lettuce proteins upon co-expression in *N. benthamiana.*

Effector	*Lettuce protein*	*Subcellular localization effector*	*Subcellular localization lettuce protein*	*Colocalization*
BLG02	Lsa022944.1	Cytoplasm and nucleus	Nucleus	Yes
BLG03	Lsa042995.1	Plasma membrane	Cytoplasm	No
BLN01	Lsa021294.1	Cytoplasm and punctate structures	Punctate structures	No
BLN01	Lsa042995.1	Cytoplasm	Cytoplasm	Yes
BLN01	Lsa015570.1	Cytoplasm	Cytoplasm	Yes
BLR11	Lsa007441.1	Cytoplasm	Cytoplasm	Yes
BLR12	Lsa011822.1	Punctate structures	Cytoplasm and nucleus	No
BLR18	Lsa011822.1	Cytoplasm and nucleus	Cytoplasm and nucleus	Yes
BLR20	Lsa006137.1	Cytoplasm and nucleus	Nucleus	Yes
BLR27	Lsa015570.1	Cytoplasm	Cytoplasm	Yes
BLR28	Lsa008980.1	Nucleus	Cytoplasm and nucleus	Yes
BLR38	Lsa007018.1	Nucleus	Nucleus	Yes

In other cases, effector expression led to target relocalization. Effector BLR38-YFP induced a relocalization of its target CFP-Lsa007018.1 from punctate cytoplasmic structures to the nucleus (Figure 6). Also, nuclear-localized effector YFP-BLR28 induced relocalization of CFP-Lsa008980.1 to the nucleus (Figure 6). In summary, nine of the twelve effector-lettuce protein pairs colocalized in *N. benthamiana*. Furthermore, relocalization occurred upon expression of four effector-lettuce protein pairs providing evidence that the Y2H-identified interactions also occur *in planta*.

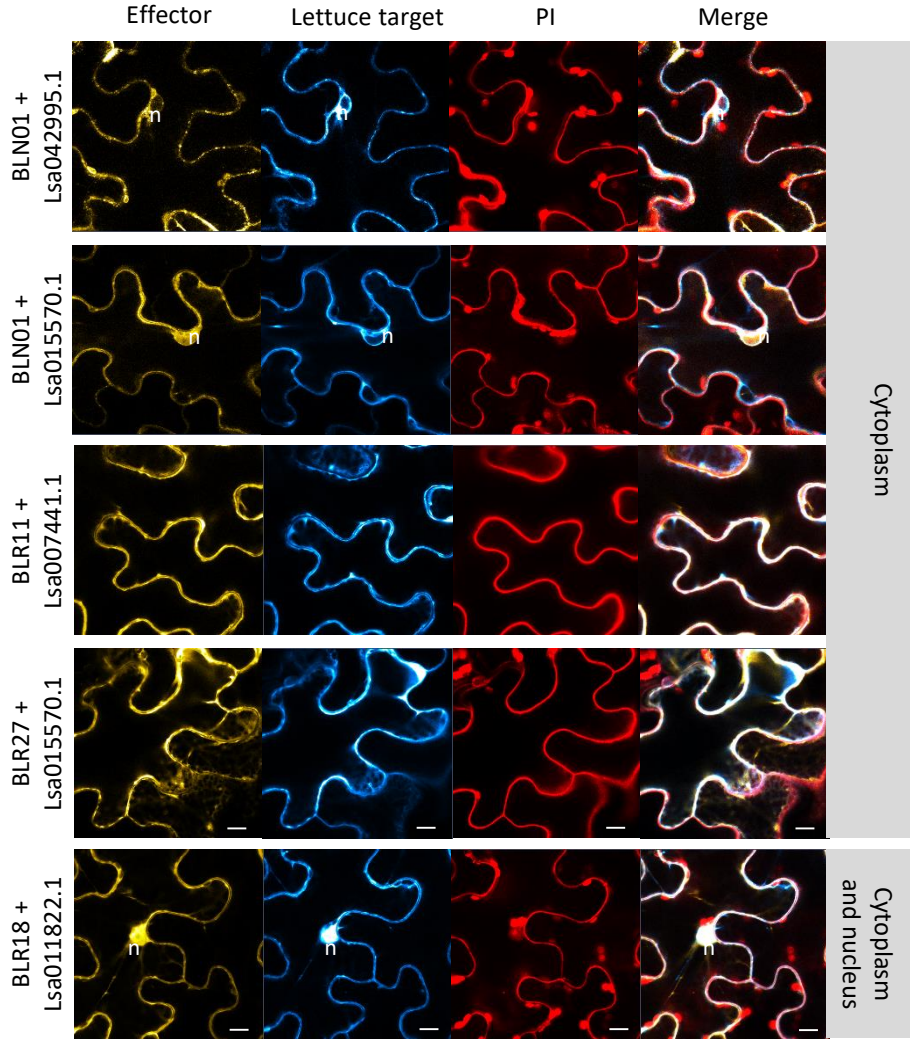

Figure 6, Colocalization of *B. lactucae* effectors and their targets in *N. benthamiana*. *B. lactucae* effectors and lettuce proteins were transiently co-expressed in *N. benthamiana* using *Agrobacterium*. Images were taken 2-3 days after infiltration. Leaf sections were incubated in propidium iodide (PI) to stain the cell wall. Bars indicate 10 µm. n = nucleus.

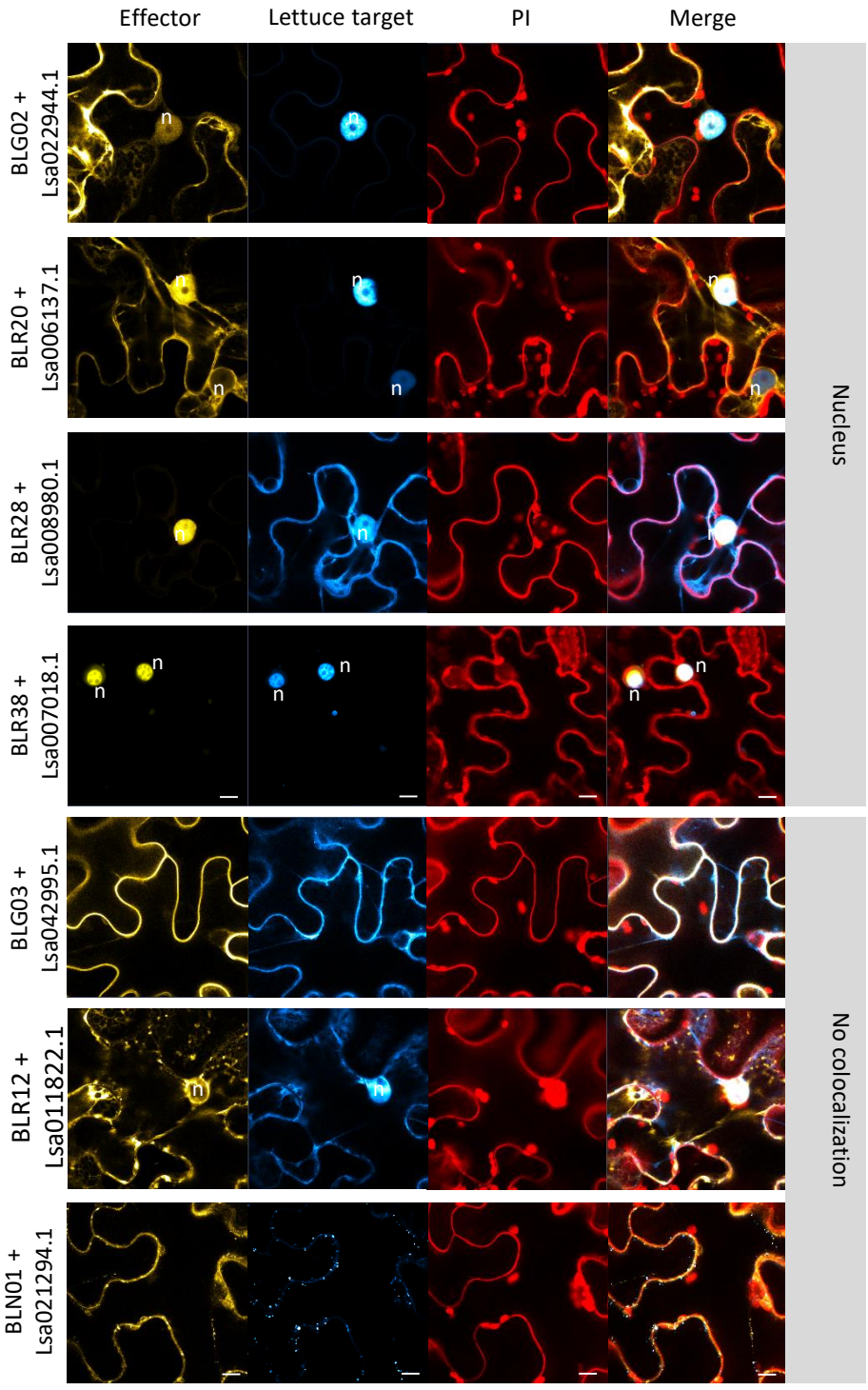

Figure 6 continued.

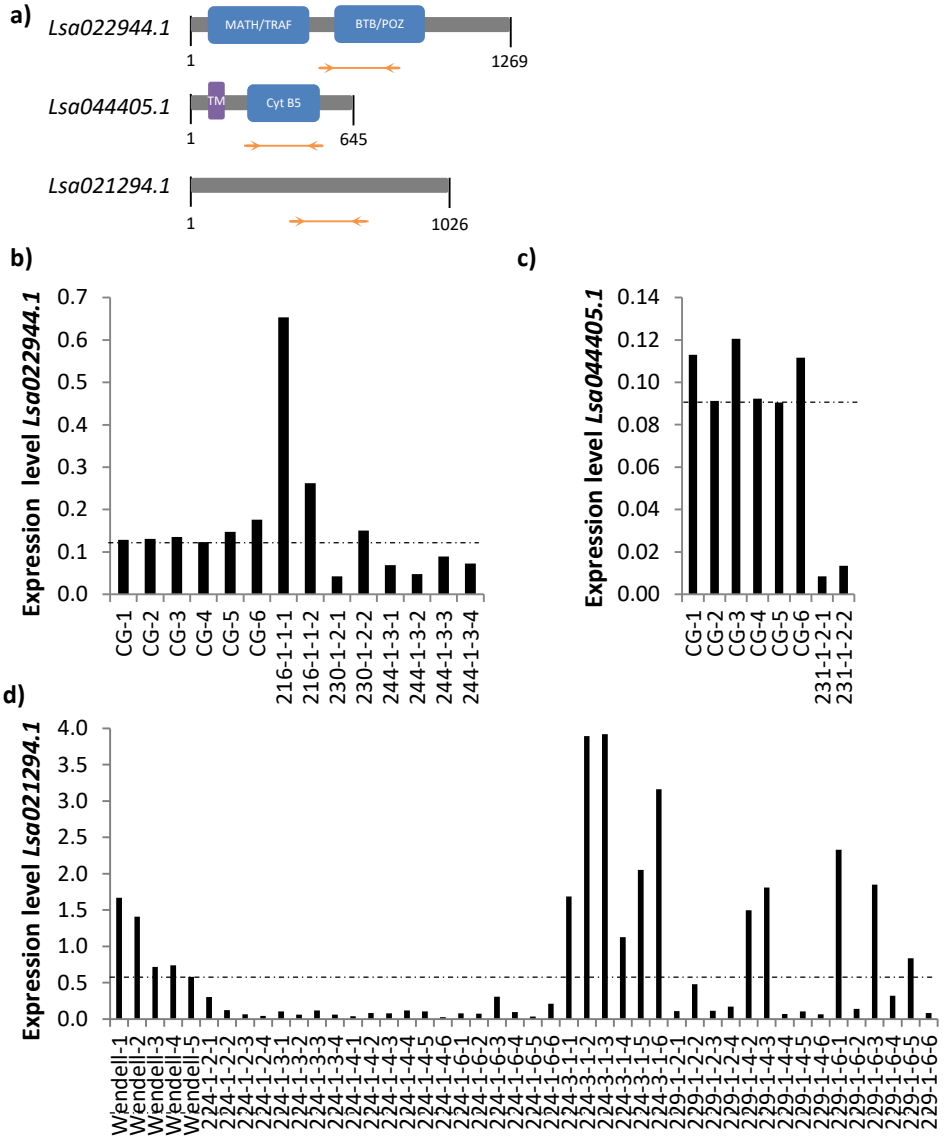

Figure 7, Expression of target genes in hpRNA-silenced T2 transformants. a) Schematic representation of effector target genes. Double orange arrows represent the region of the lettuce genes targeted by the hpRNA constructs. hpRNA constructs were transformed in *L. sativa* cv. CobhamGreen (b & c) or Wendell (d). When available, multiple homozygous and segregating T2 families were tested for silencing and the transcript level of individual T2 plants was determined using qRT-PCR. The name of each T2 plant is composed of the T0 transformant number (XXX-X), followed by the T1 specifier and the T2 specifier. The dashed line indicates the lowest transcript level observed in control plants. Expression is relative to lettuce *ACTIN*.

Silencing of effector targets

To determine if the identified effector targets contribute to susceptibility or resistance to *B. lactucae* infection, lettuce lines were stably transformed with hairpinRNA (hpRNA) constructs targeting *Lsa021294.1*, *Lsa022944.1* and *Lsa044405.1*. It should be noted that the choice for these targets was made before the localization experiments were conducted. The regions selected to form the hairpins were approximately 300 bp long (Figure 7a).

Genes for which the coding sequence matched with the hpRNA region over a length of >20 bp formed potential off-targets. Per hpRNA construct, off-targets were identified using BLASTn of the hpRNA region against the *L. sativa* cv. Salinas reference genome. The hpRNA regions of *Lsa021294.1* and *Lsa022944.1* were both highly specific (Supplemental figure 1), however, due to the short length of *Lsa044405.1* (645 bp) and the central position of the steroid binding domain, the designed hpRNA construct was less specific and two potential off-targets *Lsa008140.1* and *Lsa040789.1* were identified (Supplemental figure 1).

The silencing efficiency and specificity were determined in T2 transformants using quantitative RT-PCR (Figure 7b and c). *Lsa022944.1* transcript levels were not consistently reduced in T2 transformants with maximum reductions of transcript levels of up to 50%, which may be caused by variation between plants. In contrast the two T2 transformants with an *Lsa044405.1*-silencing construct were efficiently silenced showing decreases in transcript levels of 90%. Although the *Lsa044405.1* hpRNA was likely to result in silencing of potential off-targets *Lsa008140.1* and *Lsa040789.1*, transcript levels of *Lsa008140.1* were similar to

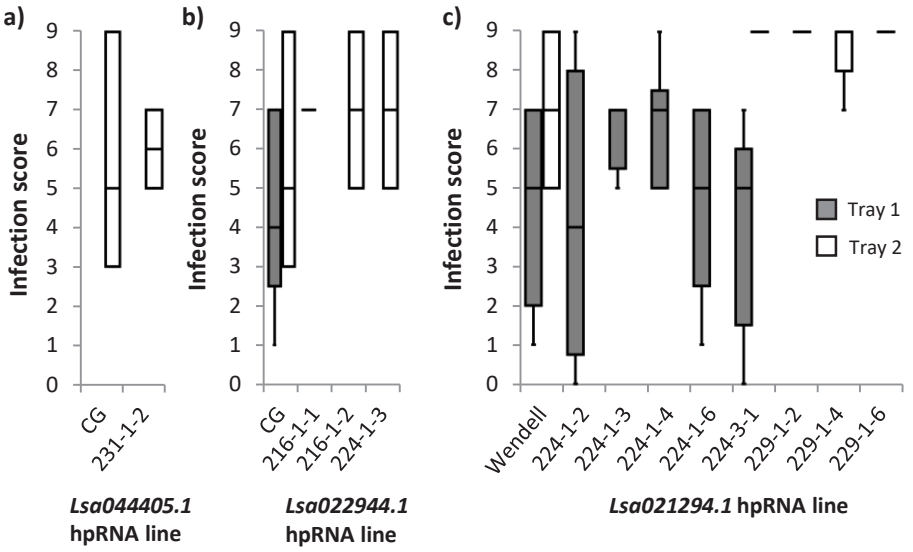

Figure 8, Lettuce hpRNA lines do not show altered susceptibility to *B. lactucae*. Susceptibility to Bl:24 in a) *Lsa022944.1*, b) *Lsa044405.1* and c) *Lsa021294.1* hpRNA T2 families. Leaf discs were inoculated with Bl:24 and infection levels were scored at 13 days after inoculation. Infection classes were defined by the leaf disc surface area covered in sporangiophores with 0 = no sporulation and 9 = heavy sporulation. Boxplots represent data of two to six T2 plants per line for which silencing was determined in Figure 7. The name of each T2 family is composed of the T0 transformant number (XXX-X), followed by the T1 specifier. Due to differences in the infection level of untransformed *L. sativa* Wendell and CobhamGreen (CG) plants between trays with leaf discs, the data is depicted per tray.

untransformed plants and *Lsa040789.1* transcript levels were reduced by only 50% in T2 transformants (Supplemental figure 2a). The *Lsa021294.1* hpRNA reduced transcript levels by 90-95% in multiple T2 families whereas transcript levels of *WALLS ARE THIN1*-related family member *Lsa007403.1* (Supplemental figure 2b) were not affected.

To assess if lines with reduced effector target expression showed altered susceptibility to *B. lactucae*, the hpRNA lines were inoculated with race Bl:24. The *L. sativa* cv. Wendell and CobhamGreen parental lines in which the hpRNA constructs had been transformed, were moderately to highly susceptible to infection. The hpRNA lines were also moderately to highly susceptible and no altered susceptibility phenotypes were observed (Figure 8). In conclusion, these data suggest that silencing of *Lsa021294.1* and *Lsa044405.1* does not affect susceptibility of lettuce to *B. lactucae* infection.

Discussion

Specificity of Y2H identified effector targets

To shed light on the molecular mechanisms underlying susceptibility of lettuce to *B. lactucae* infection, lettuce targets of *B. lactucae* effectors were identified using Y2H screens based on a lettuce cDNA library. Twenty-one (46% of screened) effectors interacted with 46 unique effector targets. These effectors interacted on average with 3 lettuce proteins and the vast majority (83%) of lettuce proteins interacted specifically with a single *B. lactucae* effector. Previously published screenings with *C. elegans* and human fragment libraries resulted in interactors for 37% and 31% of bait proteins respectively corresponding to on average 2.2 and 2 interactors per bait (Boxem *et al.*, 2008; Waaijers *et al.*, 2013). Yet, an average of 3.4 interactors per effector was obtained in a screen with effectors from *G. orontii, H. arabidopsidis* and *P. syringae* against a library of ~8000 immune-related full-length Arabidopsis proteins (Weßling *et al.*, 2014).

Based on the identification of Arabidopsis proteins that interacted with effectors from three pathogens (Weßling *et al.*, 2014), effectors have been proposed to converge on conserved host proteins. This proposition fits with insights into the mechanisms by which independent bacterial type III effectors converge on immune-related proteins such as MAPK proteins (Bi & Zhou, 2017), SERK3/BAK1 (Göhre *et al.*, 2008; Shan *et al.*, 2008; Gimenez-Ibanez *et al.*, 2009; Xiang *et al.*, 2011; Li *et al.*, 2016) and RIN4 (Block & Alfano, 2011).

We were interested to determine if those proteins identified as major hubs, i.e. interacting with effectors from multiple pathogens, would also emerge in our screens with *B. lactucae* effectors. The list of Arabidopsis hubs was dominated by TCP transcription factors (Weßling *et al.*, 2014). Specifically, TCP13, TCP14 and TCP15 interacted with effectors originating from all three pathogens, and TCP19 and TCP21 interacted with effectors from at least one pathogen (Weßling *et al.*, 2014). TCPs operate as transcriptional activators or repressors in plant growth and development (Martín-Trillo & Cubas, 2010; Li, 2015). Lettuce TCP family members were also identified as targets of *B. lactucae* effectors. However, these TCPs were weak activators of the *HIS3* reporter, frequently found as single colonies in Y2H screens and autoactivating. Weßling and colleagues also classified Arabidopsis CSN5A as a hub due to its interaction with 12 *P. syringae* effectors, 11 *H. arabidopsidis* effectors and 9 *G. orontii* effectors in Y2H screens (Weßling *et al.*, 2014). In our library screens, lettuce CSN5 was identified with two *B. lactucae* effectors, but also showed weak activation of the *HIS3* reporter in the presence of an empty bait vector. Interaction between CSN5 and the GAL4 DNA binding domain was previously reported using multiple GAL4-DBD based vectors and yeast strains (Nordgård *et al.*, 2001; Tucker *et al.*, 2009). Rejecting CSN5 as false positive because of stronger reporter gene activation in the presence of *B. lactucae* effectors than empty vector is only partly defendable. Effectors may have unforeseen additive effects that enhance reporter gene activation but do not reflect biological relevance. Thus, though some of the previously reported hubs may represent host proteins that play a prominent role in disease susceptibility, others may be false positives.

The nucleus and membrane as locations of effector activities

The subcellular localization of a subset of effectors and their targets was investigated using confocal microscopy in *N. benthamiana* since proteins that localize to the same compartment are more likely to represent true interactors *in planta*. Fusion proteins may be subject to post-translational modifications and/ or proteolysis that can affect their localization. In the absence of immunoblotting data to determine fusion protein size and stability, we cannot distinguish free CFP/YFP, which is evenly distributed over the nucleus and cytoplasm, from intact fusion proteins with the same localization pattern. In our study, only YFP- BLR18 and CFP-Lsa011822.1 displayed an even nucleocytoplasmic distribution pattern; effector fusion proteins YFP-BLG02 and YFP-BLR26 were also nucleocytoplasmic localized but there were clear intensity differences that are not typically associated with free YFP. Therefore, we expect that the observed localization of the vast majority of tested effectors and targets is valuable.

A study on *H. arabidopsidis* RXLR effector localization revealed a preference for nuclear (including nucleocytoplasmic) localization (66%) (Caillaud *et al.*, 2012). Strikingly, *B. lactucae* RXLR effector BLR38 that is recognized in *L. serriola* LS102 targeted the nucleus in *N. benthamiana* and induced a relocalization of its target Lsa007018.1 to the nucleus. Lsa007018.1 shows homology to the Arabidopsis FLOWERING LOCUS C EXPRESSOR (FLX) protein family of which two members are involved in flowering time control in Arabidopsis (Lee & Amasino, 2013). The punctate structures of CFP-Lsa007018.1 resemble the structures formed by cytoplasmic FLX homodimers in Arabidopsis (Choi *et al.*, 2011). Heterodimers of FLX with FRIGIDA or FRIGIDA ESSENTIAL 1 were restricted to the nucleus (Choi *et al.*, 2011), suggesting that the observed relocalization of CFP-Lsa007018.1 upon co-expression with BLR38-YFP may reflect a different interaction state. BLR28 also induced relocalization of its target, DnaJ protein Lsa008980.1, to the nucleus. DnaJ proteins function as co-chaperones to 70 kDa heat shock proteins to aid in protein folding, relocalization and degradation (Rajan & D'Silva, 2009).

Furthermore, two predominantly cytoplasmic effectors, YFP-BLG02 and YFP-BLR20, displayed a nucleocytoplasmic localization pattern when co-expressed with their targets, CFP-Lsa022944.1 and CFP-Lsa006137.1. Lsa006137.1 contains an AP2/ERF domain that is typically found in members of the AP2/ERF transcription factor family. These proteins were shown to be involved in responses to biotic and abiotic stress in Arabidopsis, tomato and rice (Gutterson & Reuber, 2004; Xu *et al.*, 2011). Lsa022944.1 contains a BTB/POZ (broad complex, tram track, bric-a-brac /POX virus and zinc finger) domain that is also found in 80 Arabidopsis proteins and is known to mediate protein-protein interactions. Specifically, the BTB/POZ domain acts as a substrate-specific adaptor for Cullin-RING E3 ubiquitin Ligases (CRLs) that ubiquitinate proteins targeted for degradation by the proteasome. Recruitment of substrates may occur via a Meprin and TRAF homology (MATH) domain (Xu *et al.*, 2003; Krek, 2003). The Arabidopsis MATH-BTB/POZ protein BPM3 localizes to the nucleus and promotes degradation of transcription factor ATHB6 that negatively regulates abscisic acid signaling (Lechner *et al.*, 2011). Furthermore, BPMs interact with APETALA 2/ethylene-responsive element binding factor (AP2/ERF) transcription factors (Weber & Hellmann, 2009).

These relocalization events suggest that physical interactions between RXLR effectors and targets occur *in planta*, which may affect their activity and consequently, disease development. Functional analysis of these targets may provide further insights into the mechanisms underlying *B. lactucae* susceptibility.

B. lactucae effectors BLG03, BLN06 and BLR40 that are recognized in specific lettuce lines (**Chapter 2**; Stassen *et al.*, 2013) localized to the plasma membrane (**Chapter 2;** this chapter). The plant membrane network was also targeted by 26% of tested *H. arabidopsidis* effectors (Caillaud *et al.*, 2012). During infection, vast intracellular rearrangements take place to position the nucleus, ER membranes and Golgi bodies close to specialized pathogen feeding structures, the haustoria. Haustoria are surrounded by a host-derived extrahaustorial membrane of which the biogenesis is still poorly understood. Plant plasma membrane proteins differentially localize to the extrahaustorial membrane (Koh *et al.*, 2005; Lu *et al.*, 2012), suggesting that the extrahaustorial membrane does not originate from the plasma membrane. Recent research indicates that the extrahaustorial membrane has endoplasmic reticulum (Kwaaitaal *et al.*, 2017) and late endocytic vesicle (Bozkurt *et al.*, 2015) properties keeping extrahaustorial membrane formation an elusive process. Remarkably, the *H. arabidopsidis* effector RxL17 localized to the tonoplast in uninfected Arabidopsis cells, but was localized at the extrahaustorial membrane in infected cells (Caillaud *et al.*, 2012). Thus, it will be interesting to see how *B. lactucae* effectors behave in infected lettuce cells and if some of the plasma membrane-associated effectors are found at the extrahaustorial membrane.

Functional analysis

To investigate the biological relevance of the identified effector targets, hpRNA constructs of three targets were transformed in lettuce to generate silenced transgenic lines: Lsa022944.1 is a MATH/TRAF and BTB/POZ domain containing protein, Lsa044405.1 is a membrane steroid-binding domain containing protein and Lsa021294.1 is a WALLS ARE THIN1-related protein family member. The *Lsa022944.1* hpRNA construct failed to reduce transcript levels effectively. StNRL1 is a potato BTB/POZ domain protein that interacts with *P. infestans* effector Pi02860 in yeast and *in planta.* Silencing of *StNRL1* reduced *P. infestans* colonization suggesting that *StNRL1* acts as a susceptibility factor (Yang *et al.*, 2016). The hpRNA constructs targeting *Lsa044405.1* and *Lsa021294.1* reduced transcript levels by 90-95%. However, silenced plants did not show altered susceptibility phenotypes. The Arabidopsis membrane steroid-binding protein 1 (MSBP1) binds the plant hormone brassinolide (BR) *in vitro* that promotes cell elongation in plants. Plants overexpressing MSBP1 displayed reduced cell elongation indicating that MSBP1 acts as a negative regulator of brassinosteroid signaling (Yang *et al.*, 2005). Lsa021294.1 is related to the tonoplast localized protein WALLS ARE THIN 1 (WAT1). WAT1 facilitates export of auxin from vacuoles to regulate secondary cell wall formation (Ranocha *et al.*, 2013) and *wat1* mutants display enhanced resistance to vascular pathogens (Denancé et al., 2013).

It is currently unknown what the biological functions are of Lsa04405.1, Lsa021294.1 and Lsa022944.1 and to what extent these resemble the functions of MSBP1, WAT1 and StNRL1. The effector targets are all part of larger gene families and we cannot exclude that there is functional redundancy. Thus, further characterization of the effector targets and their interaction with *B. lactucae* effectors is required to determine their role during *B. lactucae* infection of lettuce.

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Supplemental data

Lsa021294.1* hpRNA fragment alignment with *Lsa007403.1
Score = 93.3 bits (102), Expect = 9E-19
Identities = 102/135 (75%), Gaps = 3/135 (2%)
Strand = Plus/Plus

```
Lsa021294.1  164  ATGAACCTGTGAAGGAAGCCTCAAG---GCCTCGCATACCACCAAGCCTTTTGTTAGCAT  220
                  |||| |||||||  || || || ||    | | | |||  ||||| || | || |||| ||
Lsa007403.1  518  ATGAGCCTGTGACAGATGCATCGAGTAGGGCCCACATTTCACCAGGCATGTTTTTAGTAT  577

Lsa021294.1  221  CCCAAACAACCACACCTCGGCTTACACCTCCTGGGTCCCCACCAACTCTCTCAGCATCTG  280
                  | ||||||| |||||||||| | |||||||||| || || ||||    | ||||||||||
Lsa007403.1  578  CGCAAACAAGCACACCTCGGTTAACACCTCCTGGATCTCCTCCAAGCTTATCAGCATCTG  637

Lsa021294.1  281  CTTCCCCAACAAGAA  295
                    || ||||||||||
Lsa007403.1  638  GATCTCCAACAAGAA  652
```

Lsa022944.1* hpRNA fragment alignment with *Lsa038672.1
Score = 46.4 bits (50), Expect = 1E-04
Identities = 98/140 (70%), Gaps = 5/140 (4%)
Strand = Plus/Plus

```
Lsa022944.1  122  CTTGCAGCCCGATCTCCAGTCTTCAGAGCCCAATTCTTCGGCCTCGTTGGAA-ACCCCAA  180
                  |||||||||||| || || || ||||| || ||| | || || | |  || || | |  ||
Lsa038672.1  658  CTTGCAGCCCGCTCACCTGTTTTCAGGGCTCAACTTTTTGGTC-CAATGAAAGATCAAAA  716

Lsa022944.1  181  TA-TGGATCAAGTAGAGTTAAAAGACATCGAACCCTCAATTTTCAAGGCTATGCTTGTGT  239
                  ||   |||  | || ||||  | ||||| || ||| |  ||||||||||  |||||  |
Lsa038672.1  717  TACCCGATGTATTATAGTT-GAGGACATAGAGCCCCCTGTTTTCAAGGCATTGCTTCATT  775

Lsa022944.1  240  TTATATACTCGGATACGTTT  259
                  |||| || | ||||| ||||
Lsa038672.1  776  TTATGTATTGGGATA-GTTT  794
```

Lsa044405.1* hpRNA fragment alignment with *Lsa008140.1
Score = 421.5 bits (466), Expect = 2E-117
Identities = 283/316 (89%), Gaps = 0/316 (0%)
Strand = Plus/Plus

```
Lsa044405.1  1    GGCGAGATTTCCGAGGAGGAGCTGAAAGCCTACGACGGCAACGATCCCGAAAAGCCTCTT  60
                  |||||||||||||||||||| |||||||||||||||||||||||||  |   | ||||||||
Lsa008140.1  214  GGCGAGATTTCCGAGGAGGAGTTGAAAGCCTACGACGGCAACGATTCTAACAAGCCTCTC  273

Lsa044405.1  61   CTTATGGCCATCAAGGGTCAGATCTACGATGTCTCACAAAGCAGGATGTTCTATGGGCCA  120
                  |||||||| |||||||||| |||||||||||||||||||||||||||||||||||||||||| |||
Lsa008140.1  274  CTTATGGCGATCAAGGGTCAAATCTACGATGTCTCACAAAGCAGGATGTTCTATGGACCA  333

Lsa044405.1  121  GGTGGGCCATACGCACTGTTTGCAGGAAAAGATGCTAGCAGAGCTCTTGCAAAAATGTCA  180
                  ||||| ||||| ||  |||||||||| || |||||||| |||||||||| |||||||||
Lsa008140.1  334  GGTGGACCATATGCGTTGTTTGCAGGGAAGGATGCTAGTAGAGCTCTTGCGAAAATGTCA  393

Lsa044405.1  181  TTTGAAGATAAAGATTTGAATGGTGATCTCACCGGTCTTGGTGCATTTGAGCTTGATGCA  240
                  |||||||||||||||| ||||||||||||| |||||||||| ||| |||||||| ||
Lsa008140.1  394  TTTGAAGATAAAGATTTAAATGGTGATCTCACTGGTCTTGGTGTATTCGAGCTTGAAGCC  453

Lsa044405.1  241  TTGCAAGATTGGGAATATAAGTTCATGAGCAAGTATGTTAAGGTTGGATCCATTAAGAAC  300
                  || ||||||||||||||||||||||||||||||||||||||| || |||||  | |||||||||
Lsa008140.1  454  TTACAAGATTGGGAATATAAGTTCATGAGCAAGTATGTCAAAGTTGGTACAATTAAGAAT  513

Lsa044405.1  301  TCAGAAGCTCCACCCG  316
                   ||||| || ||||||
Lsa008140.1  514  CCAGAATCTGCACCCG  529
```

Lsa044405.1* hpRNA fragment alignment with *Lsa040789.1
Score = 300.6 bits (332), Expect = 4E-81
Identities = 238/286 (83%), Gaps = 0/286 (0%)
Strand = Plus/Plus

```
Lsa044405.1  11   CCGAGGAGGAGCTGAAAGCCTACGACGGCAACGATCCCGAAAAGCCTCTTCTTATGGCCA  70
                  |||| |||||| |||| || |||||||||||||||  | || ||||| ||||||||||
Lsa040789.1  218  CCGAAGAGGAGTTGAAGGCTTACGACGGCAACGATCCTAAGAAACCTCTGCTTATGGCCA  277

Lsa044405.1  71   TCAAGGGTCAGATCTACGATGTCTCACAAAGCAGGATGTTCTATGGGCCAGGTGGGCCAT  130
                  | || |||||||||||||||||| |||||||| ||||| || || |||||||| || |
Lsa040789.1  278  TAAAAGGTCAGATCTACGATGTCTCGCAAAGCAGAATGTTTTACGGACCAGGTGGTCCCT  337

Lsa044405.1  131  ACGCACTGTTTGCAGGAAAAGATGCTAGCAGAGCTCTTGCAAAAATGTCATTTGAAGATA  190
                  | |||||||| || |||||||||||||||||| |||||||||||||||||||| || |
Lsa040789.1  338  ATGCACTGTTCGCCGGAAAAAGATGCTAGCAGAGCACTTGCAAAAATGTCATTTGATGAGA  397

Lsa044405.1  191  AAGATTTGAATGGTGATCTCACCGGTCTTGGTGCATTTGAGCTTGATGCATTGCAAGATT  250
                  |||||||||  |||||| |||  |||||||||   || ||| | || || || |  ||||
Lsa040789.1  398  AAGATTTGACAGGTGATATCAGTGGTCTTGGTATGTTCGAGATGGACGCCTTACGTGATT  457

Lsa044405.1  251  GGGAATATAAGTTCATGAGCAAGTATGTTAAGGTTGGATCCATTAA  296
                  ||||||| || |||||||||||||||| ||| || ||| | || ||
Lsa040789.1  458  GGGAATACAAATTCATGAGCAAGTATGCTAAAGTAGGAACTATCAA  503
```

Lsa044405.1* hpRNA fragment alignment with *Lsa002359.1
Score = 179.8 bits (198), Expect = 8E-45
Identities = 210/284 (73%), Gaps = 0/284 (0%)
Strand = Plus/Plus

```
Lsa044405.1 13    GAGGAGGAGCTGAAAGCCTACGACGGCAACGATCCCGAAAAGCCTCTTCTTATGGCCATC  72
                  || |||||| ||||| | ||||| || |  |||||  | |||||| |||||||| |||
Lsa002359.1 1054  GATGAGGAGTTGAAAACATACGATGGTACAGATCCTAAGAAGCCTGTTCTTATGGCGATC 1113

Lsa044405.1 73    AAGGGTCAGATCTACGATGTCTCACAAAGCAGGATGTTCTATGGGCCAGGTGGGCCATAC  132
                  || |||||||| || ||||| |||   |  |||||||| |||||  |||||||  | ||
Lsa002359.1 1114  AAAGGTCAGATATATGATGTATCATCATCCAGGATGTTTTATGGAGCAGGTGGTACTTAT 1173

Lsa044405.1 133   GCACTGTTTGCAGGAAAAGATGCTAGCAGAGCTCTTGCAAAAATGTCATTTGAAGATAAA  192
                  |    ||      || |||||||| || |||||  |||||||| |    ||||||||  ||
Lsa002359.1 1174  GGGGAGTGGAGTGGGAAAGATGCCAGTAGAGCCATTGCAAAACTCAGCTTTGAAGAGGAA 1233

Lsa044405.1 193   GATTTGAATGGTGATCTCACCGGTCTTGGTGCATTTGAGCTTGATGCATTGCAAGATTGG  252
                  ||||||||  | |||||||| ||| | ||       |||||| || || ||  | ||||||
Lsa002359.1 1234  GATTTGAACAGCGATCTCACTGGTTTAGGGAAGGCTGAGCTGGAGGCCTTAGATGATTGG 1293

Lsa044405.1 253   GAATATAAGTTCATGAGCAAGTATGTTAAGGTTGGATCCATTAA  296
                  |||   | ||||| ||| ||||||||||||||||||||| ||
Lsa002359.1 1294  GAAATCATGTTCAGGAGTAAGTATGTTAAGGTTGGATCCATCAA  1337
```

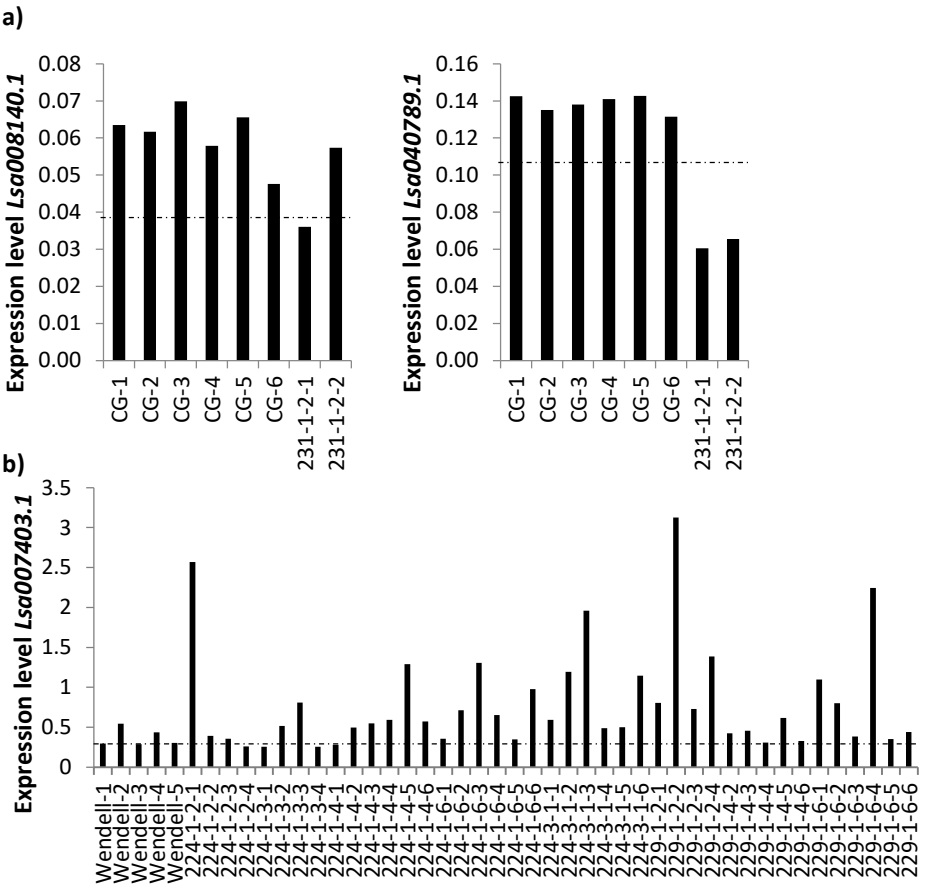
Lsa044405.1* hpRNA fragment alignment with *Lsa002356.1
Score = 176.2 bits (194), Expect = 1E-43
Identities = 213/290 (73%), Gaps = 0/290 (0%)
Strand = Plus/Plus

```
Lsa044405.1 4     GAGATTTCCGAGGAGGAGCTGAAAGCCTACGACGGCAACGATCCCGAAAAGCCTCTTCTT  63
                  || || || || |||||| ||||||| || || || |  |||||    |||||| |||||
Lsa002356.1 1054  GACATGTCGGAAGAGGAGTTGAAAGCATATGATGGTACAGATCCTAGGAAGCCTGTTCTT 1113

Lsa044405.1 64    ATGGCCATCAAGGGTCAGATCTACGATGTCTCACAAAGCAGGATGTTCTATGGGCCAGGT  123
                    |||| ||||| |||||||||| |||||||||     |||||||| || ||  |||||
Lsa002356.1 1114  TTGGCGATCAAAGGTCAGATCTATGATGTCTCATCGGCCAGGATGTTTTACGGAGCAGGT 1173

Lsa044405.1 124   GGGCCATACGCACTGTTTGCAGGAAAAGATGCTAGCAGAGCTCTTGCAAAAATGTCATTT  183
                  ||  | || | |  ||      || |||||||| || |||||  |||||||| |    |||
Lsa002356.1 1174  GGTACTTATGGAGAGTGGAGTGGGAAAGATGCCAGTAGAGCCATTGCAAAATTCAGCTTT 1233

Lsa044405.1 184   GAAGATAAAGATTTGAATGGTGATCTCACCGGTCTTGGTGCATTTGAGCTTGATGCATTG  243
                  |||||  ||||||||||  | |||||||| ||| | ||     ||||||| || |||
Lsa002356.1 1234  GAAGAGGAAGATTTGAACCGCGATCTCACTGGTTTAGGGAAGGTTGAGCTGGAGGCCTTA 1293

Lsa044405.1 244   CAAGATTGGGAATATAAGTTCATGAGCAAGTATGTTAAGGTTGGATCCAT  293
                    | ||||||||     | ||||| ||| ||||||||||||||||||||
Lsa002356.1 1294  GATGATTGGGACATCATGTTCAGGAGTAAGTATGTTAAGGTTGGATCCAT  1343
```

Lsa044405.1* hpRNA fragment alignment with *Lsa010116.1
Score = 134.7 bits (148), Expect = 3E-31
Identities = 199/277 (71%), Gaps = 4/277 (1%)
Strand = Plus/Plus

```
Lsa044405.1 13    GAGGAGGAGCTGAAAGCCTACGACGGCAACGATCCCGAAAAGCCTCTTCTT-ATGGCCAT  71
                  ||||| ||  ||| | | ||| |||   |||||   || || ||| || ||||||||
Lsa010116.1 196   GAGGACGAATTGAGACCTTACAACGGTTCTGATCCTAGTAAACC-CTTATTGATGGCCAT  254

Lsa044405.1 72    CAAGGGTCAGATCTACGATGTCTCACAAAGCAGGATGTTCTATGGGCCAGGTGGGCCATA  131
                    || ||  |||||||||||||| || |     |||||||||||||| || || || ||||
Lsa010116.1 255   TAAAGGAAAGATCTACGATGTTTCTCGCTCCAGGATGTTCTATGGTCCTGGAGGACCATA  314

Lsa044405.1 132   CGCACTGTTTGCAGGAAAAGATGCTAGCAGAGCTCTTGCAAAAATGTCATTTGAAGATAA  191
                  |||  ||||||| || |  |||||||| ||||| | ||    |||||||||| |
Lsa010116.1 315   CGCGTTGTTTGCTGGTAGGGATGCTAGCCGAGCTTTAGCTCTCATGTCATTTGAGGCATC  374

Lsa044405.1 192   AGATTTGAATGGTGATCTCACCGGTCTTGGTGCATTTGAGCTTGATGCATTGCAAGATTG  251
                    ||| | | |||  |  |    |||||  |||||| ||||||||| |   || |||||||
Lsa010116.1 375   TGATCTTACTGGGAACATTGAGGGTCTGAGTGCATCTGAGCTTGAAGTTCTGGAAGATTG  434

Lsa044405.1 252   GGAATATAAG-TTCATGAGCAAGTATGTTAAGGTTGG  287
                  ||||  |||| || | ||| |||||||| ||||||||
Lsa010116.1 435   GGAAGCTAAGTTTGAGGAG-AAGTATGTGAAGGTTGG  470
```

Supplemental figure 1, Alignment of hpRNA fragments of effector targets with possible off-targets. Off-targets were identified using BLASTn of hpRNA sequences against *L. sativa* cv. Salinas CDS with an E-value cut-off of 0.001 and max. hits = 10 in CLC Main.



Supplemental figure 2, Transcript levels of genes closely related to effector targets in hpRNA-silenced lettuce lines. The transcript level of a) the two highest scoring hits for the *Lsa044405.1* hpRNA fragment and b) the best hit for the *Lsa022944* hpRNA fragment in individual T2 plants. The name of each T2 plant is composed of the T0 transformant number (XXX-X), followed by the T1 specifier and the T2 specifier. The dashed line indicates the lowest transcript level observed in control plants. Expression is relative to lettuce *ACTIN*.

Supplemental table 1, Primers used in this study.

Primers with BP cloning sites for full-length gene amplification

Gene	*Orientation*	*Sequence*[1]
Lsa022944.1	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGCTTGTCAATAATTTCGATCA
Lsa022944.1	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTT*TACAACCGTCTACGCATACG
Lsa021294.1	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGTTGGCGAAAGAATCGCTT
Lsa021294.1	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTT*CAATGGAAGTTACGAGTGATT
Lsa015570.1	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGGCTGTGATGAACGGTGG
Lsa015570.1	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTC*TAGTTGACTACAACCGGTG
Lsa042995.1	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGTCGATGAACACAGCCATT
Lsa042995.1	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTT*CAAACACCCTCCTCAAGAAG
Lsa007441.1	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGGGGGACACAGAGACGT
Lsa007441.1	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTT*TAGTCGACCTCTTCCATCTT
Lsa011822.1	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGGATCCCTACTCCTTCTC
Lsa011822.1	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTT*CAAGATTGAACCATAGGCTC
Lsa006137.1	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGTGTGGTGGTGCGATCAT
Lsa006137.1	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTT*TAGAAAGATCCATCCATAATGA
Lsa010116.1	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGTATACAATGGTAACGGATG
Lsa010116.1	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTC*TACTCTACTTTAGTCTGATCC
Lsa044405.1	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGGCTGTTGAATTGTTGGAG
Lsa044405.1	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTT*CACTCATCTTTCACTTCCTTA
Lsa008980.1	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGTTTGGAAGACAGCCGAAG
Lsa008980.1	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTT*CATTGCTGCGCACATTGCA
Lsa007018.1	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGGGAAGCAAAGGTAGACTT
Lsa007018.1	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTC*TATCTCCGGGCAGGGTG

Primers with BP cloning sites for hairpinRNA construct amplification

Gene	*Orientation*	*Sequence*[1]
Lsa022944.1	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*CACCATTTCCATTCCACCAT
Lsa022944.1	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTT*CATGTTTGTTGATGCCGTGT
Lsa044405.1	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*GGCGAGATTTCCGAGGAGGA
Lsa044405.1	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTT*CGGGTGGAGCTTCTGAGTT
Lsa021294.1	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*GAAGGAAGTTCGACAACAGG
Lsa021294.1	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTT*GGAGAAACTGGCTTTGGTGT

[1] Sequences in italic represent attB cloning sites.

Primers for sequencing of hairpinRNA constructs in the destination vector

Name	*Orientation*	*Sequence*
pHellsgate12 promoter	Fwd	GGGATGACGCACAATCC
pHellsgate12 terminator	Rev	GAGCTACACATGCTCAGG
pHellsgate12 intron	Rev	CCGAATTCCTCGAGACCAC
pHellsgate12 intron	Fwd	AGGGTCCTAACCAAGAAAATG

Primers for qRT-PCR

Gene	*Orientation*	*Sequence*
Lsa022944.1	Fwd	GCGGTGAGGGGTTGAGTAG
Lsa022944.1	Rev	GCCGCCGATGTCAAATCCA
Lsa044405.1	Fwd	GCCGGTGTTGCCATCTA
Lsa044405.1	Rev	GGGCCTATGTTGTGGTTGA
Lsa021294.1	Fwd	ATTACATCCTCACGACCATT
Lsa021294.1	Rev	TTCCTGACATCGAGCCAT
Lettuce *ACTIN*	Fwd	CTATCCAGGCTGTGCTTTCC
Lettuce *ACTIN*	Rev	ACCCTTCGTAGATCGGGACT
Lsa008140.1	Fwd	GTCGTCTGCTACACCTGT T
Lsa008140.1	Rev	CCGGCTGCTAATTCTGTACT
Lsa040789.1	Fwd	AAAACCCCTGAACCCGCA
Lsa040789.1	Rev	GATCAGCAGCAACCACAGT
Lsa007403.1	Fwd	AGACAAGTGAGGGGCAGA
Lsa007403.1	Rev	AGATCCTTGTTTTCCGGGC

Primers for amplification of lettuce gene fragments in Y2H vectors

Name	*Orientation*	*Sequence*
pDEST22	Fwd	TATAACGCGTTTGGAATCACT
AP22	Rev	GCTTTCGTGTGATGAACCCA
pDEST22/32	Rev	AGCCGACAACCTTGATTGGAGAC

Supplemental table 2, List of perturbations inducing a >5-fold change in transcript levels of the Arabidopsis *AT3G48890* gene[1].

Perturbation	*Experiment*	*Study description*	*# samples experiment*	*# samples control*	*p-value*	*Fold change*
Biotic stress	AT-00681	*S. sclerotiorum* study 2 (Col-0) / mock inoculated rosette leaf samples (Col-0)	3	3	0	17.56
Biotic stress	AT-00681	*S. sclerotiorum* study 2 (*coi1-2*) / mock inoculated rosette leaf samples (*coi1-2*)	3	3	0	10.2
Biotic stress	AT-00108	*P. infestans* (6h) / mock treated leaf samples (6h)	2	3	0	8.02
Biotic stress	AT-00681	*S. sclerotiorum* (Col-0) / mock inoculated rosette leaf samples (Col-0)	3	3	0	7.16
Biotic stress	AT-00661	*A. brassicicola* study 3 (Col-0) / mock treated leaf samples (Col-0)	2	3	0.001	6
Biotic stress	AT-00661	*A. brassicicola* study 2 (Col-0) / mock treated leaf samples (Col-0)	3	3	0.009	5.96
Chemical treatment	AT-00684	ozone study 4 (*abi1td*) / untreated rosette leaf samples (*abi1td*)	3	3	0	5.95
Elicitor treatment	AT-00391	FLG22 + GA (2h) / untreated leaf disc samples (Ler)	3	3	0.001	5.74
Elicitor treatment	AT-00391	FLG22 study 7 (Ler) / untreated leaf disc samples (Ler)	3	3	0.008	5.69
Temperature shift	AT-00665	shift 28°C to 19°C study 2 (35S:RPS4-HS) / 28°C (35S:RPS4-HS)	3	3	0	5.47
Chemical treatment	AT-00684	ozone study 5 (*abi1td*) / untreated rosette leaf samples (*abi1td*)	2	3	0.002	5.39
Biotic stress	AT-00648	*P. cucumerina* study 2 (*agb1-1*) / mock inoculated rosette samples (*agb1-1*)	3	3	0	5.37
Biotic stress	AT-00425	*P. parasitica* (6h) / non-infected root samples (Col-0)	2	2	0.011	5.1

[1] Data obtained from Genevestigator

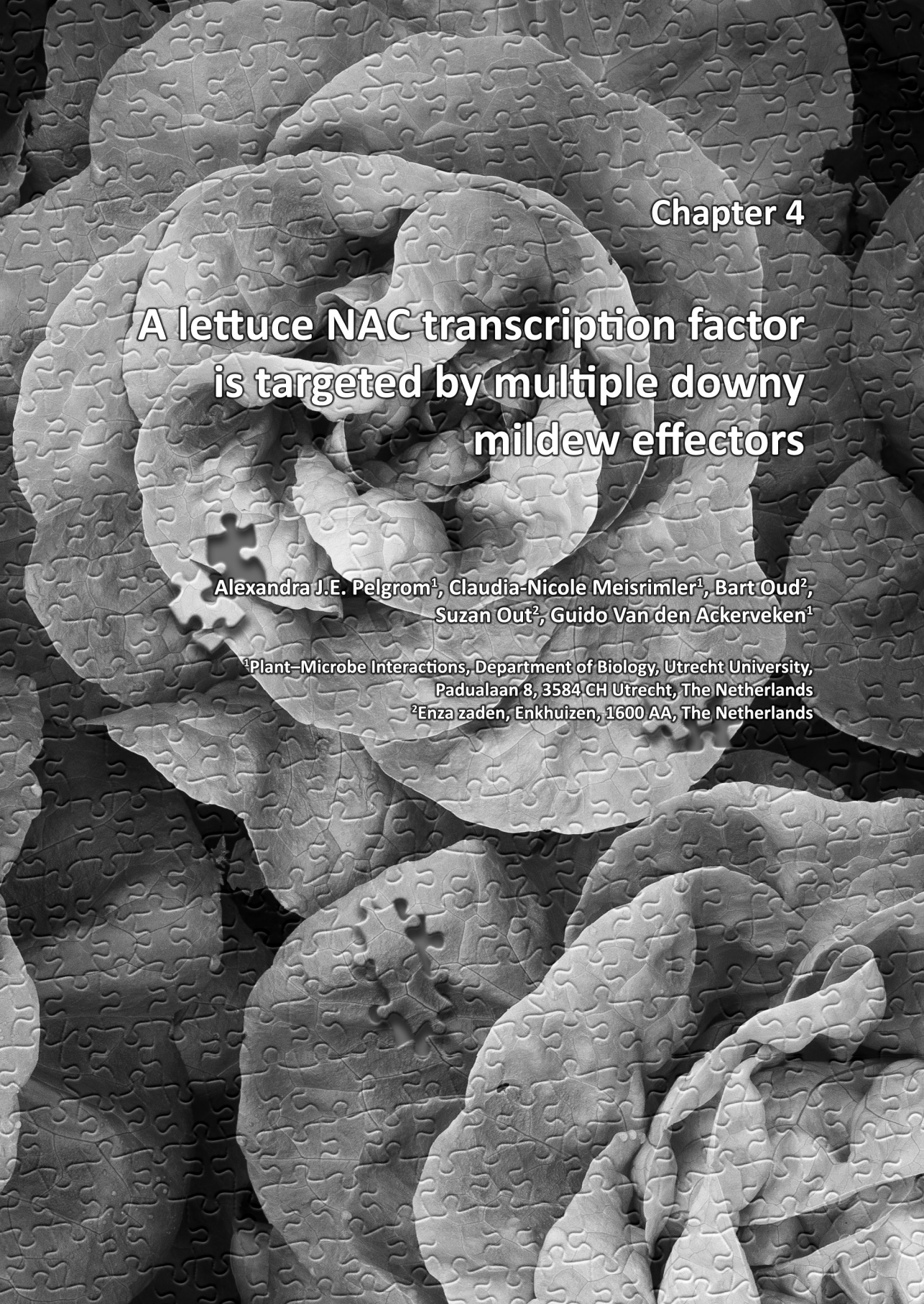
Supplemental table 3, Prediction of importin-α-dependent nuclear localization signals[1].

Protein	*NLS start position[2]*	*NLS sequence*	*Score[3]*
BLG02	95	KQTKRKRPKD	10
BLG03	12	RPQGKLRVNAATNVESDERFLDGLKALVRGFY	3.3
BLN01	-	-	-
BLR11	390	KTAKKRGTFDVVTPEAKKINH	4.5
BLR12	22	RIARRYLRETAAMEGQELEKNLGFDQTIKNVKKP	5.2
BLR18	-	-	-
BLR20	-	-	-
BLR26	122	RWSKQKLGQNLHLDGISREDLNALIGLKTRLEK	3.4
BLR27	13	LGKSRQLRSSVELVEGLLLTSDKLAKVI	3
BLR28	220	RFVLDNFKECRATIRYGTVEDWYKHPMLNKLLRV	4.3
BLR38	136	MPSSRKRPRALDE	9.5
Lsa006137.1	100	SSRKRKNQYR	8
Lsa007018.1	6	RLPPPHHLRRPLPGPGIGHHDSIPPEIHPQHGRFPP	4.3
Lsa007441.1	-	-	-
Lsa008980.1	265	PKFKRKGDDL	5
Lsa011822.1	57	DPHYFKRVKVS	5
Lsa015570.1	216	GVSKSASFAKRSLSVDTLHNTPRNYKEPAP	3.2
Lsa021294.1	281	FRQVRSRLSYEQFAAFLANVKELNSQKIYIYI	3.9
Lsa022944.1	-	-	-
Lsa042995.1	11	SRVLLKKPDVDLDSVPKNTIGSVKIPCFS	3.7

[1] Predicted using cNLS mapper

[2] If multiple NLSs were detected, the highest scoring NLS is given

[3] Higher scores are associated with a stronger nuclear localization



Chapter 4

A lettuce NAC transcription factor is targeted by multiple downy mildew effectors

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Abstract

The lettuce downy mildew pathogen *Bremia lactucae* produces RXLR(-like) effectors during infection. Although recognition of multiple effectors in specific lettuce lines has been reported, the effector targets in lettuce remain obscure. In this study, a protein-protein interaction network consisting of five *B. lactucae* effectors and three lettuce proteins was uncovered using the yeast-two-hybrid (Y2H) system. Transient expression of RXLR effectors BLR05, BLR08 and BLR09 and their targets *in planta* revealed that these proteins predominantly localize to the endoplasmic reticulum. Interaction of the lettuce membrane-associated NAC transcription factor (NAC MTF) LsNAC069 with *B. lactucae* effectors in the Y2H system did not require the N-terminal NAC domain but was dependent on the C-terminal region including the transmembrane domain. *B. lactucae* effectors also interacted with NAC MTFs from Arabidopsis and potato suggesting that NAC MTFs are conserved effector targets. Transcript levels of *LsNAC069* were not significantly affected during *B. lactucae* infection of lettuce seedlings. However, at the post-translational level, CFP-LsNAC069$^{\Delta NAC}$ was found to relocalize to the nucleus in plant cells treated with *Phytophthora capsici* culture filtrate. Relocalization was reduced in the presence of the serine and cysteine protease inhibitor TPCK indicating that proteolytic cleavage is required for relocalization. Furthermore, co-expression of effectors HA-BLR05, HA-BLR08 and HA-BLR09 with CFP-LsNAC069$^{\Delta NAC}$ reduced accumulation of CFP-LsNAC069$^{\Delta NAC}$ in the nucleus. Silencing of *LsNAC069* in lettuce with two distinct hairpinRNA constructs effectively decreased *LsNAC069* transcript levels but did not reproducibly affect susceptibility to *B. lactucae* infection, possibly because of genetic redundancy.

Introduction

Plant pathogenic downy mildews and *Phytophthora* spp. form a major threat to numerous economically important crops in agriculture. These filamentous oomycetes penetrate a variety of plant tissues and spread intercellularly through hyphal growth. Specialized feeding structures, the haustoria, are formed from hyphae, invade host cells, but remain separated from the plant cell cytoplasm by the plant-derived extrahaustorial membrane. Critically, haustoria also form the main secretion site of effector molecules that act extracellularly (apoplastic effectors) or intracellularly (host-translocated effectors) (Whisson *et al.*, 2007, 2016). In the co-evolutionary arms race between plants and pathogens, effectors are deployed to suppress plant immune responses. Immune responses are triggered by detection of pathogen-associated molecular patterns (PAMPs) resulting in pattern-triggered immunity (PTI), or of effectors leading to effector-triggered immunity (ETI). Host-adapted pathogens have evolved effectors to counter both layers of the plant immune system and induce a state of susceptibility (Jones & Dangl, 2006).

RXLR effectors constitute the main class of host-translocated effectors in downy mildews and *Phytophthora* spp. This class of effectors is characterized by an N-terminal signal peptide and conserved RXLR (Arg – any amino acid – Leu – Arg) motif followed by a sequence diverse C-terminal effector domain. Predicted RXLR effector repertoires range from dozens to several hundreds of proteins per species (Jiang *et al.*, 2008; Haas *et al.*, 2009; Fabro *et al.*, 2011; Stassen *et al.*, 2012). Yet the molecular function of most RXLR effectors remains unknown.

Recently, the *Phytophthora infestans* RXLR effector Pi03192 was reported to interact with potato (*Solanum tuberosum*) transcription factors StNTP1 and StNTP2 at the endoplasmic reticulum (McLellan *et al.*, 2013). The StNTPs belong to the NAC [no apical meristem (NAM), *Arabidopsis thaliana* transcription activation factor (ATAF1/2) and cup-shaped cotyledon (CUC2)] family of transcription factors. StNTP1 and StNTP2 were released from the membrane upon treatment of cells with *P. infestans* culture filtrate and then relocalized to the nucleus. Interestingly, relocalization of StNTP1 and StNTP2 was inhibited in the presence of effector Pi03192. Silencing of StNTP orthologs in *N. benthamiana* increased susceptibility to *P. infestans* infection, suggesting that these NAC transcription factors play a role in plant immunity and disease resistance (McLellan *et al.*, 2013).

StNTP1 and StNTP2 belong to a subgroup of the NAC family, whose members contain, in addition to the N-terminal NAC domain, also a C-terminal transmembrane domain. In Arabidopsis, fourteen of the 117 putative NAC genes (Nuruzzaman *et al.*, 2010) contain a functional transmembrane domain (Kim *et al.*, 2007a; Klein *et al.*, 2012; Yang *et al.*, 2014; Liang *et al.*, 2015; Zhao *et al.*, 2016) although putative transmembrane domains were predicted in four more NAC proteins (Kim *et al.*, 2010). These membrane-associated transcription factors (MTFs) localize predominantly to the endoplasmic reticulum (Klein *et al.*, 2012; Block *et al.*, 2014; Liang *et al.*, 2015) and a minority to the plasma membrane (Liang *et al.*, 2015; Zhao *et al.*, 2016).

MTFs enable plant cells to respond rapidly to developmental and environmental cues. MTFs switch from a membrane-tethered dormant state to a transcriptionally active form upon proteolytic cleavage. Proteolysis can be initiated by a variety of triggers including cold exposure, high salinity, mitochondrial stress and ER stress (Kim *et al.*, 2007b, 2008; Seo *et al.*, 2010; De Clercq *et al.*, 2013; Ng *et al.*, 2013). After cleavage, the transcription factor relocalizes to the nucleus to regulate gene expression.

In this study, we focus on effector targets of the lettuce downy mildew *Bremia lactucae.* The obligate biotrophic pathogen *B. lactucae* poses a major threat to lettuce cultivation worldwide. Analysis of the *B. lactucae* transcriptome resulted in the identification and cloning of 49 RXLR-like effectors and one Crinkler (Stassen *et al.*, 2012, 2013; Giesbers *et al.*, 2017). Multiple *B. lactucae* RXLR and RXLR-like effectors are also recognized in specific lettuce accessions (Stassen *et al.*, 2013; Giesbers *et al.*, 2017; **Chapter 2**) but no effector targets have been described yet. Here the identification of a NAC MTF, LsNAC069, as target of four *B. lactucae* RXLR(-like) effectors is described. LsNAC069 and its interacting effectors localize to the secretory pathway *in planta*. Interaction of LsNAC069 with *B. lactucae* effectors did not require the NAC domain, but was abolished in truncated variants missing the C-terminal region including the transmembrane domain. Relocalization of LsNAC069$^{\Delta NAC}$ was induced by treatment with *P. capsici* culture filtrate but was reduced upon co-expression of *B. lactucae* effectors.

Materials & methods

Plant growth conditions

Lettuce seed germination and *B. lactucae* disease assays were performed under short day growth conditions (9 h of light (100 µE/m2/s)) at 16°C. *Nicotiana benthamiana* plants and germinated lettuce seedlings were grown under long day conditions (16 h of light, 70% humidity) at 21°C.

Lettuce cDNA library construction

Lactuca sativa cv. Olof seedlings were sprayed with water, 0.1 mg/ml benzothiadiazole (BTH) solution, *B. lactucae* race Bl:24 (compatible interaction) or isolate F703 (incompatible interaction) spore suspension. RNA was extracted using phenol/ chloroform extraction at 24 hours after BTH treatment and 3 days after infection. 2 mg of RNA (0.5 mg of each of the treatments) was used to construct a three-frame uncut cDNA library in prey vector pDEST22 by Invitrogen Custom Services (Invitrogen, Carlsbad, California, USA).

Vector construction

Generation of pENTR/D-TOPO clones with *B. lactucae* effectors BLN03$^{22-169}$, BLN04$^{24-147}$, BLR05$^{22-97}$, BLR08$^{30-135}$ and BLR09$^{23-112}$ was previously described (**Chapter 2;** Stassen *et al.*, 2013). *B. lactucae* effector entry clones, pDONR221-Pi03192 and pDONR201-PiAVR2 (McLellan *et al.*, 2013) were recombined with bait destination vector pDEST32 using LR clonase to generate GAL4 DNA binding domain (DBD) fusion proteins.

LsNAC069, LsPUF001 and *LsRTNLB05* coding sequences were amplified from cDNA library plasmids or lettuce cDNA using primers listed in Supplemental table 1. The resulting PCR products were recombined using BP clonase in a modified pGemTEasy (pGemTEasy^{mod}) vector containing the pDONR201 Gateway recombination site. LsNAC069 truncations were generated using primers listed in Supplemental table 1, cloned in pGemTEasy^{mod} using BP clonase and transferred into pDEST22 using LR clonase. StNTP1, StNTP2 (McLellan *et al.*, 2013), ANAC013, ANAC017, ANAC053 and ANAC078 (De Clercq *et al.*, 2013) in pDONR221 were transferred to Y2H prey destination vector pDEST22 to generate GAL4 activation domain (AD) fusion proteins. pDEST22-ANAC016, pDEST22-ANAC086 and pDEST22-ANAC116 (also known as CBNAC or NTL9; (Nakano *et al.*, 2006)) plasmids were obtained

from an Arabidopsis transcription factor Y1H library (Pruneda-Paz *et al.*, 2014).

Entry clones of *B. lactucae* effectors and their targets were recombined using LR clonase with pUBN-Dest CFP and pUBN-Dest YFP (Grefen *et al.*, 2010) or with pB7WGY2, pB7WGC2 and pB7WGR2 (Karimi *et al.*, 2005) to create N-terminal YFP, CFP and RFP fusion proteins, and with pEarleyGate201 to generate HA fusion proteins.

Two *LsNAC069* gene fragments of 342 and 335 bp were amplified using primers listed in Supplemental table 1 and cloned in the pHELLSGATE12 vector (Helliwell & Waterhouse, 2003) to generate *LsNAC069* hairpin(hp)RNA constructs.

Y2H screens

All yeast transformations were performed using the TE/LiAc method (Schiestl & Gietz, 1989). The lettuce cDNA library was transformed in yeast strain Y8800 (genotype MATa trp1−901 leu2−3,112 ura3−52 his3− 200 gal4Δ gal80Δ cyh2R GAL1::HIS3@LYS2 GAL2::ADE2 GAL7::LacZ@met2) whereas *B. lactucae* effector constructs were transformed in yeast strain Y8930 (genotype MATα trp1−901 leu2−3,112 ura3−52 his3−200 gal4Δ gal80Δ cyh2R GAL1::HIS3@LYS2 GAL2::ADE2 GAL7::LacZ@met2). Effector strains that activated the *HIS3* reporter in the absence of prey vector were discarded.

Library Y2H screens were performed using the mating method (Fromont-Racine *et al.*, 2002). Successful bait-prey interactions were identified by selection of diploid yeast on Sc –Leu –Trp –His + 100 µg/ml ampicillin plates. Prey vector inserts were amplified from yeast by colony PCR. A small amount of yeast was resuspended in 30 µl 0.02 M NaOH, heated at 99 °C for 10 min and the debris pelleted by centrifugation. A 25 µl PCR reaction with DreamTAQ polymerase (ThermoScientific) was conducted using 2.5 µl of the yeast lysate supernatant as input. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter Genomics) according to the manufacturer's protocol. Purified PCR products were Sanger sequenced. The prey identity was determined by BLASTn against our lettuce transcriptome (**Chapter 2**) and the *L. sativa* cv. Salinas reference genome v8 (Reyes-Chin-Wo *et al.*, 2017). Out-of-frame GAL4 AD-fusion proteins and proteins with an early stop codon were discarded. To further verify interactions, bait plasmid and prey plasmid isolated from a representative yeast colony were cotransformed in yeast strain Y8930 and reporter gene activation was assessed. Targeted Y2H assays were performed by cotransformation of bait and prey plasmid in yeast strain Y8930.

Transient expression in *N. benthamiana* and lettuce

Constructs for transient expression in lettuce and *N. benthamiana* were transformed in *Agrobacterium tumefaciens* strain C58C1 (pGV2260). For expression in lettuce, freshly grown *A. tumefaciens* cells in LB medium with corresponding antibiotics were collected by centrifugation for 10 min at 4000 rpm and resuspended in induction medium (8.5 gr/L Na_2HPO_4 x $2H_2O$, 3 gr/L KH_2PO_4, 0.5 gr/L NaCl, 1 gr/L NH_4Cl, 1% (w/v) glucose, 50 µM acetosyringone) supplemented with antibiotics to an OD_{600}<1. Cell suspensions were incubated for 3-4 hours at 28°C and 200 rpm. The centrifugation step was repeated and cells were resuspended in infiltration medium (0.5x MS salts, 10 mM MES, 0.5 % (w/v) sucrose, 0.5 % (w/v) fructose, 150 µM acetosyringone, pH 5.6) to an OD_{600} of 0.4.

For expression in *N. benthamiana*, freshly grown *A. tumefaciens* cells were collected by centrifugation, resuspended in infiltration buffer (10mM MES, 10mM $MgCl_2$, and 150µM acetosyringone, pH 5.6) and incubated in the dark at RT for 1-4 hrs before infiltration. *Agrobacterium* strains were diluted to an OD_{600} of 0.3 each for colocalization experiments.

For relocalization experiments, *Agrobacterium* containing LsNAC069$^{\Delta NAC}$ construct at OD_{600} of 0.4 was mixed with *Agrobacterium* strains containing empty vector or three effectors (OD_{600} of 0.2 each) to a final OD_{600} of 1. For pUBN-Dest constructs, equal amounts of *Agrobacterium* strains containing LsNAC069 construct, the silencing suppressor P19, effectors or YFP were mixed to a final OD_{600} of 1.5. Leaves of lettuce and *N. benthamiana* plants were infiltrated with bacterial suspensions using a needleless syringe.

Relocalization assay

One day after infiltration of *N. benthamiana* leaves with *Agrobacterium* suspensions, the infiltrated leaf sections (with a diameter of ~15 mm) were cut out and incubated in 12-wells plates in tap water o/n in the dark at RT. Proteasome inhibitor MG132 (#474790, Bio-Connect) and N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK; #T4376, Sigma) were prepared in DMSO and added to a final concentration of 50 µM each. Culture filtrate for treatment was prepared by cutting V8 plates (20% (v/v) V8 juice, 1.5% (w/v) agar, 35mM CaCO3) containing *P. capsici* mycelium in squares and submerging these in water. After two days, water was refreshed and the plate was transferred to 4 °C for one hour. Supernatant was filtered through a double layer of Miracloth. CFP-LsNAC069 $^{\Delta NAC}$ localization was observed after 16 h of treatment.

Confocal imaging

For observation of fluorophore-tagged proteins confocal imaging was performed on the Zeiss 700 (Zeiss, Germany) using the 63x objective with oil emersion (Objective Plan-Apochromat 63x/1.40 Oil DIC). CFP, YFP and RFP were excited at 405 nm, 488 nm and 555 nm respectively. Emitted light of both CFP and YFP was captured using a 490-555 nm band-pass filter, and emitted light of RFP was captured using a 560 nm long pass filter. For quantification of relocalization experiments the same settings were used for all images. Analysis of the images was performed with ZEN lite (blue edition) v2.3.

Immunoblotting

Leaves were harvested 48 hours after infiltration with *Agrobacterium* suspensions, frozen in liquid nitrogen and stored until further processing at -80°C. Leaves were ground with an ice-cold mortar and pestle and proteins were extracted with lysis buffer (10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.5 mM EDTA, 0.25% (v/v) Triton-X 100, 0.25% (v/v) Tween-20, 5 mM DTT, 4% (w/v) polyvinylpyrrolidone) supplemented with cOmplete EDTA-free protease inhibitor cocktail (Roche) for 1 h at 4°C. Lysates were centrifuged for 15 min at 10,000 g at 4°C. For direct analysis of samples on Western blot, supernatant was mixed with 4x Bolt LDS sample buffer (ThermoFisher Scientific) containing reducing agent and processed according to the manufacturer's instructions. Proteins were separated using Bolt pre-cast gradient gels 4-12% (#NW04125, ThermoFisher Scientific) with Bolt Mes-SDS running buffer (#B002, ThermoFisher Scientific). Proteins were transferred for 50 min at 100 V (max. 0.4 mA) to a nitrocellulose membrane in a Mini-Trans blot cell (Bio-Rad) using Towbin transfer buffer without SDS (25 mM Tris, 192 mM glycine, 20% (v/v) methanol). Proteins were detected with antibody against GFP conjugated to HRP (ChromoTek). Proteins were detected using SuperSignal West Pico chemiluminescent substrate (ThermoFisher Scientific) and documented with a CCD-camera (ChemiDoc, Bio-Rad).

Stable silencing of *LsNAC069* in lettuce

pHELLSGATE12 constructs were transformed in *A. tumefaciens* strain GV2260MPI by electroporation followed by selection on rifampicin (20 µg/ml), streptomycin (250 µg/ml) and spectinomycin (100 µg/ml). *L. sativa* cv. Wendell was transformed essentially as described before (Pileggi *et al.*, 2001) and successful transformants were selected on kanamycin. Silencing efficiency and specificity were determined using qRT-PCR in T2 plants.

Quantitative RT-PCR

To determine the expression level of *B. lactucae* effectors and *LsNAC069* during *B. lactucae* infection of lettuce qRT-PCR was performed on cDNA of a previously described time course experiment (**Chapter 2**). RNA isolation from hpRNA silenced lettuce plants and cDNA synthesis was performed as previously described (**Chapter 2**). Relative transcript levels were determined using SYBR Green PCR Master mix (ThermoFisher Scientific) and the ViiA7 Real-Time PCR system (ThermoFisher Scientific). Transcript levels of lettuce genes were normalized to *L. sativa ACTIN* transcript. Effector expression levels were normalized to *B. lactucae ACTIN*. The primers used for qRT-PCR are listed in Supplemental table 1.

***B. lactucae* disease assays**

To determine the disease susceptibility of transgenic lines, 14 mm Ø leaf discs were punched from 4-weeks old T2 plants and placed upside down on four layers of filter paper soaked with MilliQ water. Leaf discs were spray-inoculated with a spore suspension of *B. lactucae* race Bl:24 (40 spores/µl). Trays with leaf discs were closed with a transparent lid and sealed with tape to maintain maximum humidity. The infection severity was determined at 11 days after infection by scoring the area of the leaf disc covered in sporangiophores.

Bioinformatic analyses

L. sativa cv. Salinas coding sequences corresponding to genome v8 (Genome ID 28333) were downloaded from the CoGe platform (https:// genomevolution.org/). Translated CDSs were searched using Hidden Markov Models for the presence of NAM (PF02365) and reticulon (PF02453) domains with *E*-value $1e^{-4}$ as the cut-off. Transmembrane domains were predicted using TMHMM 2.0 (Sonnhammer *et al.*, 1998; Krogh *et al.*, 2001) and TOPCONS (Bernsel *et al.*, 2009; Tsirigos *et al.*, 2015).

All identified NAM and reticulon domain-containing genes were named. Gene names are composed of the prefix '*Ls*' for *L. sativa* followed by NAC or RTLNB and a number. NAC and reticulon genes were numbered according to their position on lettuce chromosomal linkage groups 1-9 (Supplemental table 2 & 3). The use of RTLNB for plant reticulon like-proteins is according to the reticulon nomenclature proposed by Oertle and coworkers (Oertle *et al.*, 2003).

Alignment of NAC proteins was performed using Clustal Omega (Sievers *et al.*, 2014) with default parameters. After visual inspection, poorly aligned sequences LsNAC003, LsNAC005, LsNAC008, LsNAC032 and LsNAC034 were removed from the lettuce NAC proteins alignment and sequences were realigned. Phylogenetic trees were constructed using MEGA 7.0 (Kumar *et al.*, 2016) using the Neighbor-Joining method and bootstrap values based on 1000 iterations. Pairwise gap deletion was used for construction of a phylogenetic tree from the lettuce NAC proteins alignment. Trees were visualized using iTOL software (Letunic & Bork, 2016).

Results

A lettuce NAC transcription factor interacts with four *B. lactucae* effectors in yeast

To identify putative host targets of *B. lactucae* effectors, Y2H screens (see **Chapter 3**) were performed using a lettuce cDNA prey library with BLN03$^{22\text{-}169}$, BLN04$^{24\text{-}147}$, BLR05$^{22\text{-}97}$, BLR08$^{30\text{-}135}$ and BLR09$^{23\text{-}112}$ (hereafter BLN03, BLN04, BLR05, BLR08 and BLR09) that contain a single C-terminal transmembrane domain (Figure 1a), as bait. Interestingly, a protein-protein interaction network emerged that is formed by these five effectors and three prey proteins (Figure 1a). The corresponding prey clones encoded a protein with a reticulon-like domain (Lsat_1_v5_gn_3_35581.1, hereafter LsRTNLB05), a protein of unknown function (Lsat_1_v5_gn_3_68821.1, hereafter LsPUF001) and a NAC transcription factor (Lsat_1_v5_gn_6_99960.1, hereafter LsNAC069). Whereas for BLN03 and BLN04 no additional targets were identified, three additional targets were found for BLR05, one for BLR08 and four for BLR09, bringing the total to eleven distinct interacting lettuce proteins for these five effectors (**Chapter 3** Table 2).

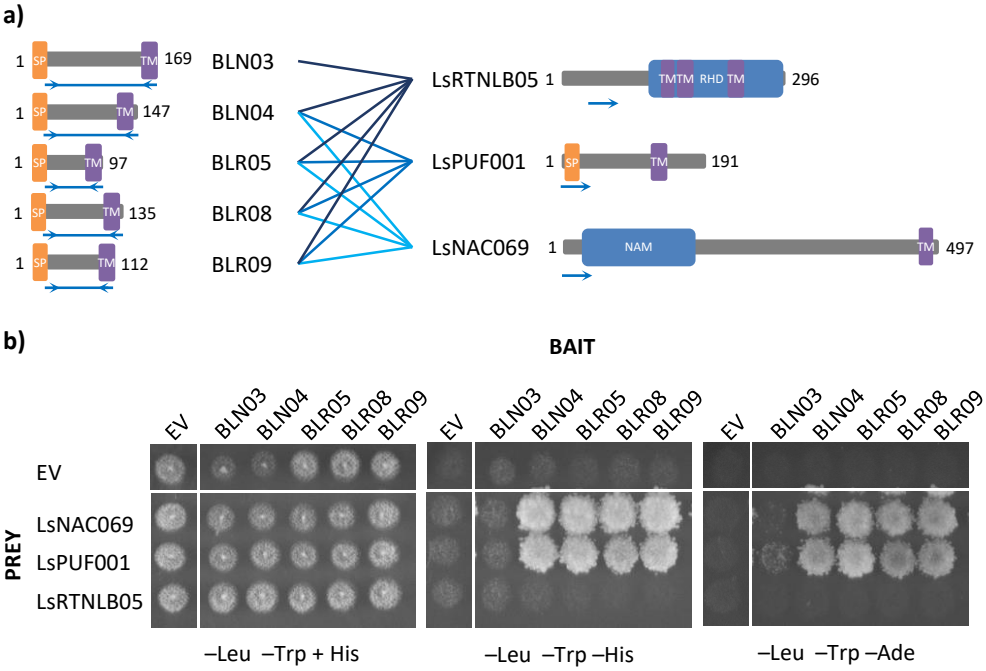

Figure 1, Five *B. lactucae* effectors converge on three lettuce proteins. a) Graphical representation of the five *B. lactucae* effectors and three effector targets that form an interaction network. Grey boxes represent full-length proteins. Predicted domains are shown as boxes of different colours. Double blue arrows underneath effectors indicate the region used as bait in Y2H. Single blue arrows underneath lettuce proteins indicate the start position of the longest identified gene fragment isolated from a prey clone. b) Y2H between *B. lactucae* effectors BLN03, BLN04, BLR05, BLR08 and BLR09 and full-length lettuce proteins. Left: permissive plate. Middle: moderately selective plate lacking histidine. Right: strongly selective plate lacking adenine. Preys LsNAC069 and LsPUF001 activate both reporter genes. Prey LsRTNLB05 fails to interact as full-length protein in Y2H. SP = signal peptide, RHD = reticulon homology domain, TM = transmembrane domain, EV = empty pDEST32 (bait) or pDEST22 (prey) vector

All 104 prey clones encoding LsRTNLB05 missed at least the N-terminal 37 amino acids, whereas LsNAC069 and LsPUF001 coding sequences were present from the start codon in multiple prey clones. To assess if the three effector targets still interact as full-length proteins, prey plasmids containing complete coding sequences, amplified from lettuce cDNA, were co-transformed with the effector bait plasmids in yeast. Full-length LsNAC069 and LsPUF001 consistently interacted with effectors BLN04, BLR05, BLR08 and BLR09, but not BLN03, confirming the observations from the Y2H library screen. However, no interaction was observed between full-length LsRTNLB05 and any of the five effectors (Figure 1b). Possibly, misfolding or inaccessibility of the interaction site hampers interaction of full-length LsRTNLB05 with effectors in the Y2H system.

Effectors and their targets localize to the secretory pathway *in planta*

To investigate the subcellular localization of the effectors and their targets, fluorophore-tagged proteins were transiently expressed in *N. benthamiana* using *A. tumefaciens*. Initially, effectors and their targets were cloned in pUBN-Dest CFP/ YFP vectors (Grefen *et al.*, 2010) which led to hypersensitive-like responses in *N. benthamiana* leaves transiently expressing CFP-LsNAC069 (Supplemental figure 1). This phenomenon was not observed with the pB7WGC/Y2 vector (Karimi *et al.*, 2005), therefore, all microscopy was performed with constructs in pB7WGC/Y2. N- and C-terminal fluorophore fusions of LsRTNLB05, BLN03 and BLN04 could not be detected in transient expression experiments. CFP-LsNAC069 and CFP-LsPUF001 labeled an intracellular reticulate compartment reminiscent of the endoplasmic reticulum. Subsequent co-expression with a luminal ER marker (fluorescent protein with N-terminal signal peptide and C-terminal ER-retention motif HDEL) (Nelson *et al.*, 2007) confirmed the ER localization for CFP-LsNAC069 and CFP-LsPUF001 (Figure 2). YFP-BLR05 and YFP-BLR09 also clearly localized to the ER (Figure 3a). Furthermore, mobile punctate structures closely associated with the ER in YFP-BLR05 expressing cells were identified as Golgi bodies (Figure 3b) using CFP fused to the cytoplasmic tail and transmembrane domain of soybean α-1,2-mannosidase I as Golgi marker (Nelson *et al.*, 2007). Strikingly, CFP-BLR08 labeled ring-like structures with varying sizes (up to 10 µm in diameter). The structures

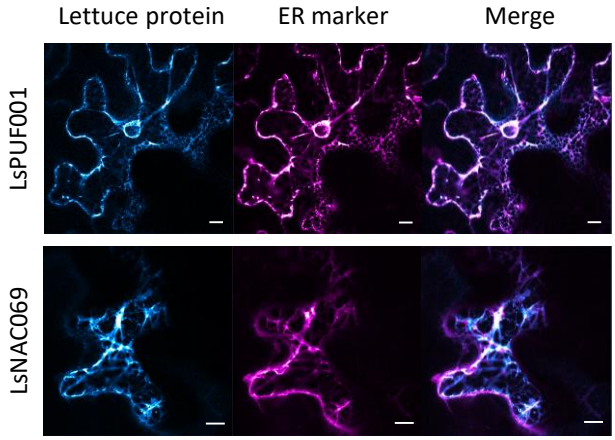

Figure 2, Effector targets LsNAC069 and LsPUF001 localize to the ER membrane. CFP-LsPUF001 and CFP-LsNAC069 colocalize with an RFP-tagged ER lumenal marker when transiently expressed in *N. benthamiana*. Scale bars indicate 10 µm.

were closely associated with the endoplasmic reticulum and weak labeling of the ER was observed in some cells (Figure 3a and Supplemental figure 2).

To determine if expression in *N. benthamiana* accurately reflects effector localization in lettuce, effector fusion proteins were transiently expressed in *L. sativa* cv. Olof. YFP-BLR05 and RFP-BLR09 also localized to the ER in lettuce (Supplemental figure 3) confirming the observations in *N. benthamiana*. YFP-BLR08 strongly labeled the ER in some lettuce cells, whereas small (≤1 µm), homogenously sized ring-like structures were more predominant in other cells (Supplemental figure 3). No large ring-like structures were observed, suggesting that these were specific for transient expression of CFP-BLR08 in *N. benthamiana*. Overall, these data indicate that BLR05, BLR08, BLR09 and their targets LsNAC069 and LsPUF001 localize to the secretory pathway *in planta*.

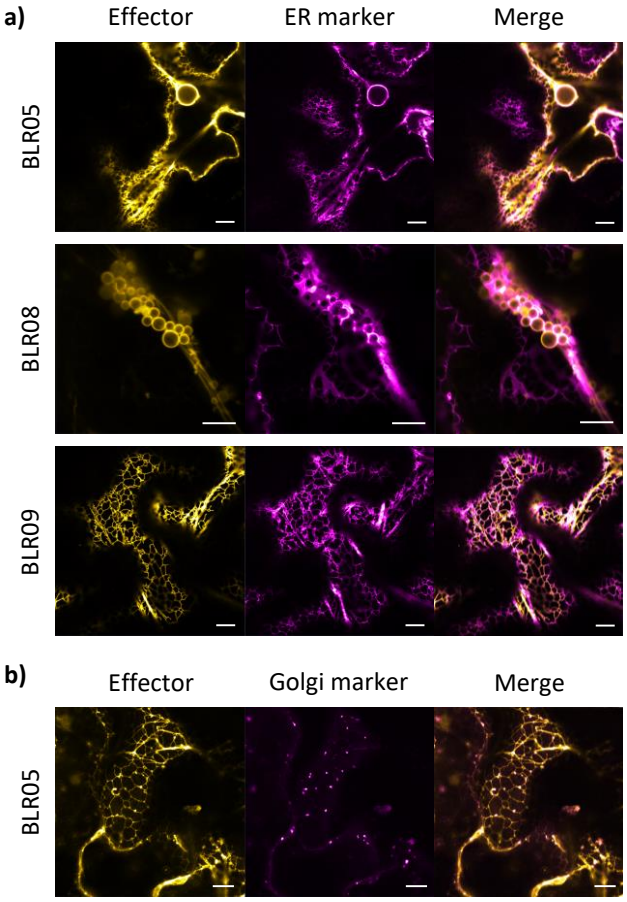

Figure 3, *B. lactucae* effectors BLR05, BLR08 and BLR09 are localized to the secretory pathway in *N. benthamiana*. a) YFP-BLR05 and YFP-BLR09 colocalize with an CFP-tagged ER lumenal marker. CFP-BLR08 was co-expressed with an RFP-tagged ER lumenal marker and forms ring-like structures that are closely associated with the ER. b) YFP-BLR05 also colocalizes with CFP-tagged Golgi marker. Scale bars indicate 10 µm.

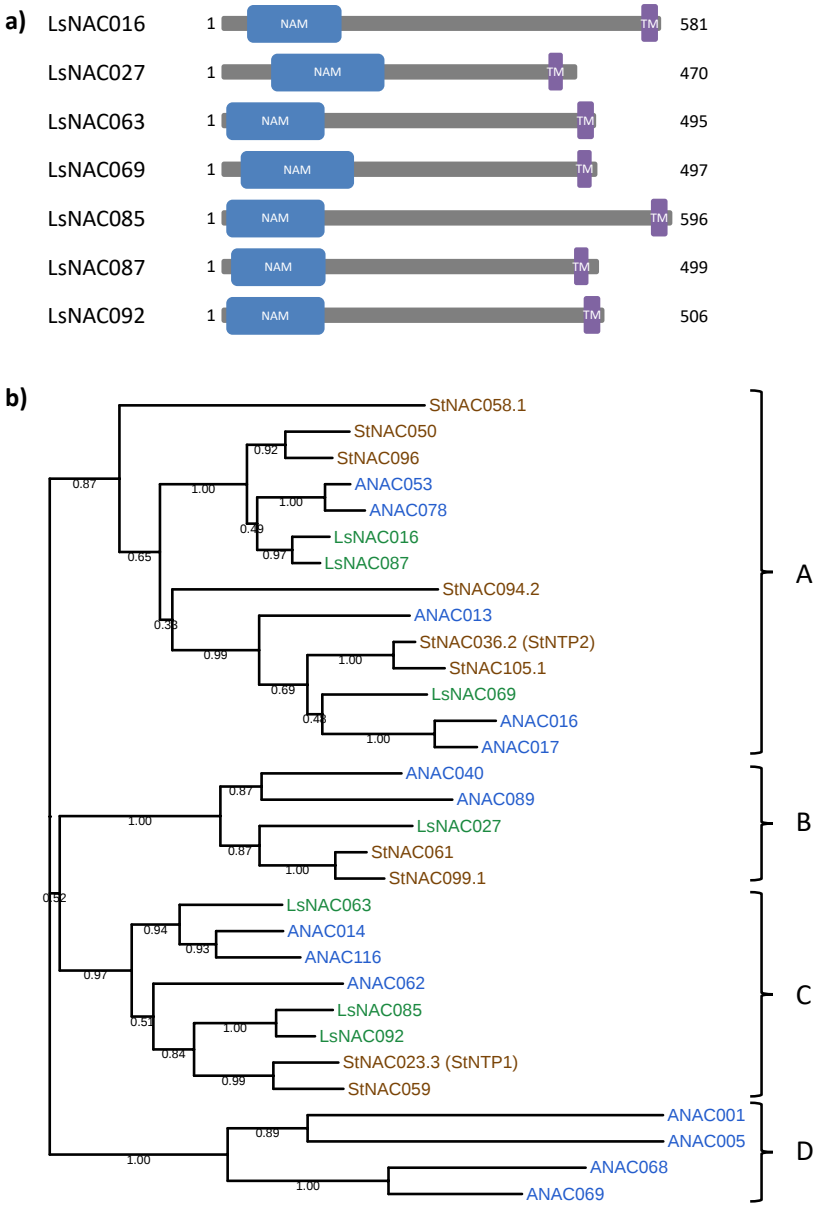

Figure 4, Tail-anchored NAC proteins in lettuce. a) Graphical representation of lettuce NAC proteins with a single putative C-terminal transmembrane domain. Grey boxes represent the full-length proteins. Position of the NAM domain is shown as a blue box, the predicted transmembrane domain (TM) is in purple. b) Phylogenetic relationship of tail-anchored NAC proteins from lettuce (green), potato (brown) and Arabidopsis (blue). Multiple alignments were generated using Clustal Omega. A Neighbor-Joining tree was constructed in MEGA 7.0 with 1000 bootstrap replicates. The bootstrap values are indicated at each node. The selected NAC MTFs are classified in four clades.

Phylogeny of the NAC gene family in lettuce

NAC transcription factors occur in vast gene families in plants with 117 genes identified in Arabidopsis, 74 in grape, 110 in potato, 151 in rice, and 152 in soybean and tobacco (Rushton *et al.*, 2008; Nuruzzaman *et al.*, 2010; Le *et al.*, 2011; Singh *et al.*, 2013; Wang *et al.*, 2013). The recent publication of the *L. sativa* cv. Salinas genome (Reyes-Chin-Wo *et al.*, 2017) provided an opportunity to characterize the NAC gene family in lettuce and the position of LsNAC069 herein. The lettuce NAC family is comprised of 99 members (Supplemental table 2). Phylogenetic analyses revealed that the NAC genes can be divided over nine subfamilies (labeled 1-9) (Supplemental figure 4).

Tail-anchored proteins are a class of transmembrane proteins that do not contain an N-terminal signal peptide but are targeted and anchored to the membrane via a single transmembrane domain in the last ~40 C-terminal amino acids (Pedrazzini, 2009; Borgese & Fasana, 2011). LsNAC069 meets the requirements for a tail-anchored protein due to the presence of a single transmembrane domain at the C-terminal end of the protein. Analysis of all lettuce NAC proteins for the presence of single C-terminal transmembrane domains resulted in the identification of six additional putative tail-anchored NAC proteins (Figure 4a and Supplemental figure 4). These membrane-associated NAC transcription factors (NAC MTFs) clustered together in subfamily 9 with LsNAC069.

The potato tail-anchored NAC transcription factors StNTP1 and StNTP2 were previously identified in a Y2H screen as targets of *P. infestans* effector Pi03192 (McLellan *et al.*, 2013). To explore the phylogenetic relationship of LsNAC069 to these and other tail-anchored NAC transcription factors, a phylogenetic tree was constructed that included 7 lettuce, 14 Arabidopsis and 10 potato NAC MTF proteins (Figure 4b). In the four distinct clades that were defined, LsNAC069 was found in clade A where it grouped with orthologs StNTP2, StNAC105, ANAC016 and ANAC017. StNTP1 was placed in clade C with StNAC059, three Arabidopsis NAC MTFs and three lettuce NAC MTFs.

NAC MTFs are conserved targets of pathogen effectors

The close phylogenetic relationship of LsNAC069 to StNTP2 raised the possibility that these NAC MTFs share a conserved effector interaction site. To determine if StNTP1 and StNTP2 can interact with *B. lactucae* effectors and LsNAC069 can interact with the *P. infestans* effector Pi03192, the corresponding coding sequences were cloned into Y2H vectors and tested for interaction. In agreement with previous findings (McLellan *et al.*, 2013), StNTP1 and StNTP2 interacted with Pi03192, but not with the unrelated *P. infestans* effector PiAvr2 (Figure 5a). LsNAC069 also interacted with Pi03192 but not with PiAvr2 (Figure 5a). StNTP1 interacted weakly with BLN03 whereas LsNAC069 and StNTP2 did not. Unexpectedly, both StNTP1 and StNTP2 interacted strongly with BLN04, BLR05, BLR08 and BLR09 (Figure 5a). Thus, LsNAC069, StNTP1 and STNTP2 appear to share a conserved effector interaction site.

To determine if this putative effector interaction site is also present in NAC MTFs from other plant species, six Arabidopsis NAC MTFs were tested (Figure 5b). Furthermore, ANAC086 was included that lacks a transmembrane domain but groups with the tested Arabidopsis NAC MTFs according to Shen and coworkers (Shen *et al.*, 2009). ANAC086 did not interact with any of the effectors. ANAC013, ANAC016 and ANAC017 cluster together with LsNAC069 and StNTP2 in clade A of the NAC MTFs tree, containing selected lettuce, potato and Arabidopsis proteins (Figure 4b). These three Arabidopsis NAC MTFs interacted strongly with effectors Pi03912, BLN04, BLR05, BLR08 and BLR09 similarly to LsNAC069, but interacted also weakly with BLN03. ANAC116 that grouped with StNTP1 in clade D, also

interacted with all five *B. lactucae* effectors in the Y2H assay. ANAC053 and ANAC078 belong to clade B (Figure 4b) and display a higher level of sequence similarity to LsNAC069 and StNTP2 than StNTP1 does. However, ANAC053 and ANAC078 were poor interactors. These findings support a model in which effectors require a specific interaction site in NAC MTFs.

The LsNAC069 C-terminal domain is required for effector interaction

To map the region of LsNAC069 that is required for interaction with the different effectors, multiple N- and C-terminal truncated constructs were generated (Figure 6a) and tested in the Y2H system. Removal of the NAC domain in LsNAC069$^{174\text{-}497}$ (hereafter LsNAC069$^{\Delta NAC}$) did not abolish interaction with the effectors (Figure 6b) indicating that the NAC domain is not required for interaction. This is corroborated by the fact that 14 of the 31 original Y2H identified prey clones with LsNAC069 fragments lacked the NAC domain (Supplemental figure 5). The shortest LsNAC069 fragment identified by Y2H that still interacted with effectors corresponded to the C-terminal 274 amino acid residues. Two constructs with further N-terminal truncations were tested. LsNAC069$^{267\text{-}497}$ still interacted similarly to the LsNAC069$^{\Delta NAC}$ construct. Only further truncation of the C-terminal region in LsNAC069$^{329\text{-}497}$ resulted in reduced reporter gene activation in the Y2H assay.

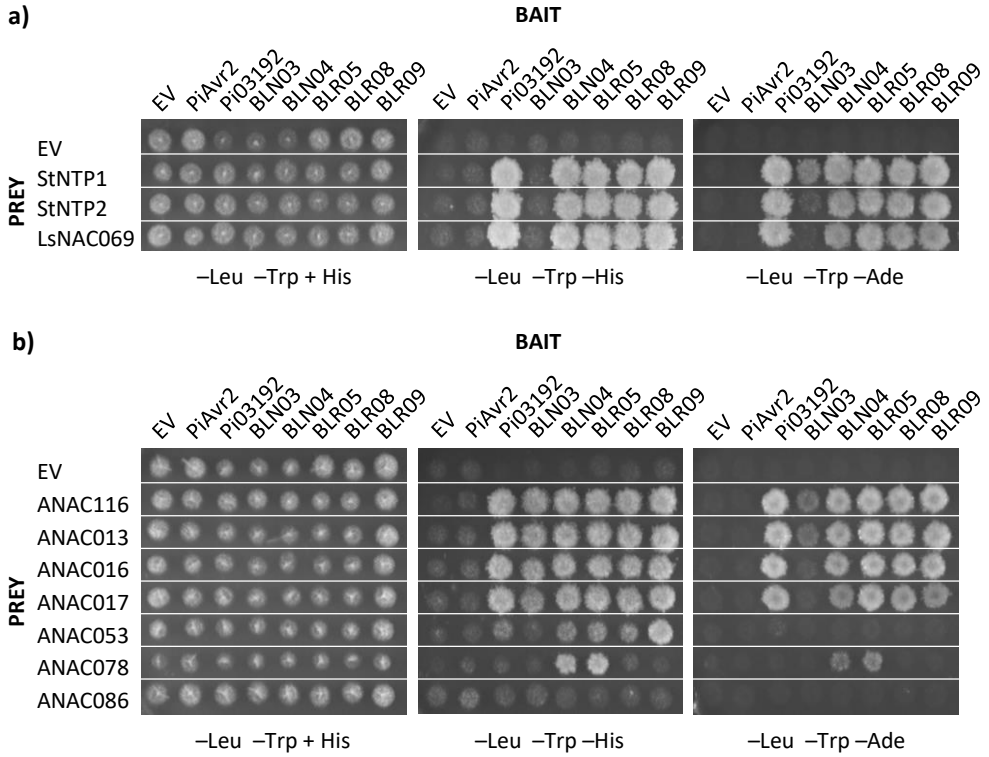

Figure 5, NAC MTFs are conserved effector targets. Y2H assay between *P. infestans* effectors PiAvr2, Pi03192, *B. lactucae* effectors BLN03, BLN04, BLR05, BLR08, BLR09 and a) *S. tuberosum* and *L. sativa* NAC transcription factors and b) Arabidopsis NAC transcription factors. Left: permissive plate. Middle: moderately selective plate lacking histidine. Right: strongly selective plate lacking adenine. EV = empty pDEST32 (bait) or pDEST22 (prey) vector

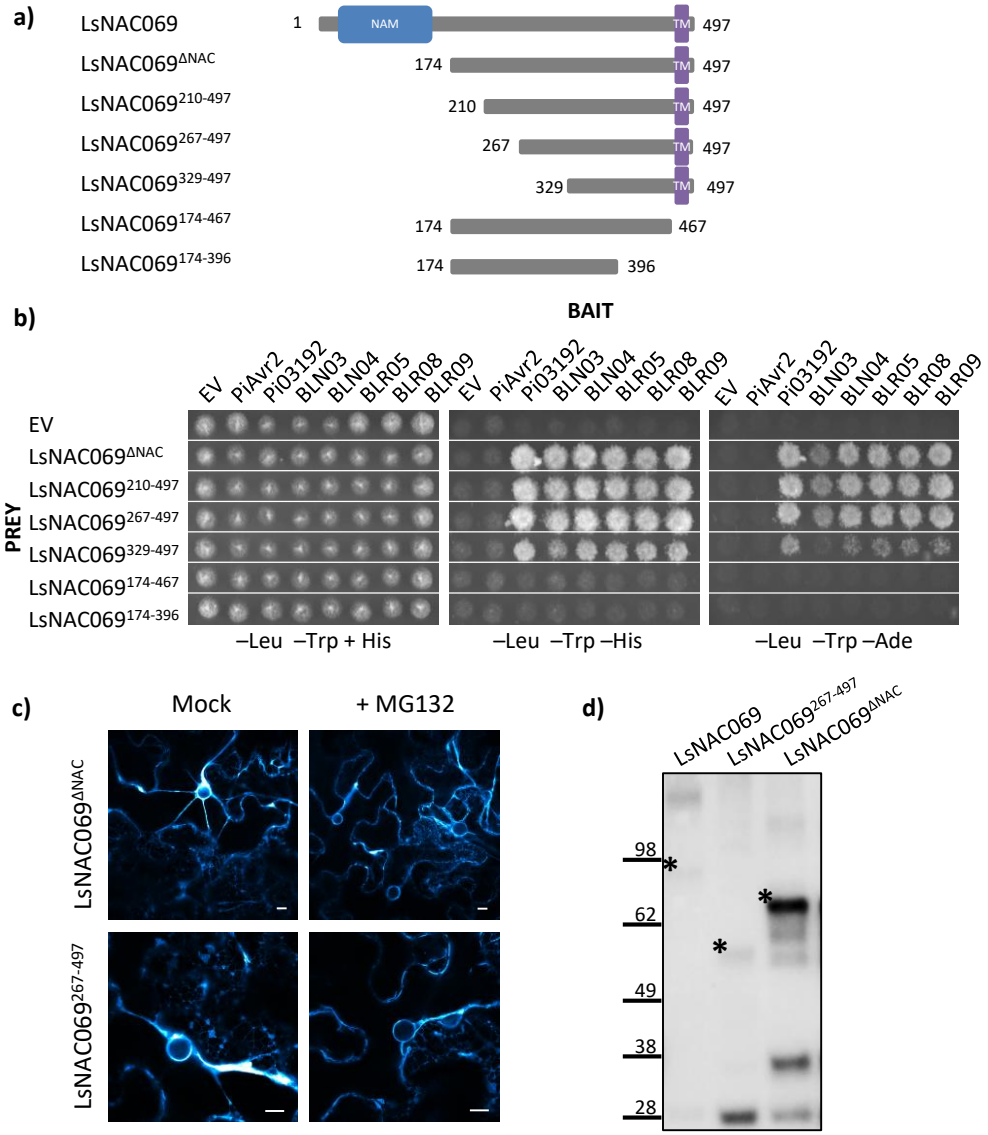

Figure 6, The transmembrane domain but not the NAC domain is required for effector interaction. a) Graphical representation of the truncated LsNAC069 constructs generated. Grey boxes represent full-length and truncated proteins. Predicted domains are shown as boxes of different colours. b) Y2H assay between *P. infestans* effectors PiAvr2, Pi03192, *B. lactucae* effectors BLN03, BLN04, BLR05, BLR08, BLR09 and truncated LsNAC069 variants. Removal of the NAC domain and parts of C-terminal region are well tolerated, but truncation of the transmembrane domain abolishes interaction. Left: permissive plate. Middle: moderately selective plate lacking histidine. Right: strongly selective plate lacking adenine. c) The N-terminally truncated proteins CFP-LsNAC069$^{\Delta NAC}$ and CFP-LsNAC069$^{267-497}$ label the ER membrane. Localization is not affected by MG132 treatment. Scale bars indicate 10 µm. d) Western blotting on lysates of CFP tagged LsNAC069 constructs in *N. benthamiana* treated with MG132 shows that CFP-LsNAC069$^{\Delta NAC}$ is more stable than CFP-LsNAC069 and CFP-LsNAC069$^{267-497}$. Asterisks (*) indicate the position of intact fusion proteins. TM = transmembrane domain, EV = empty pDEST32 (bait) or pDEST22 (prey) vector

These N-terminally truncated constructs also interacted moderately with BLN03 whereas the full-length LsNAC069 construct did not. In contrast, removal of the C-terminal transmembrane domain in LsNAC069$^{174\text{-}467}$ and LsNAC069$^{174\text{-}396}$ completely abolished interaction with all tested effectors (Figure 6b) indicating that the C-terminal region, including the transmembrane domain, is essential for effector interaction.

As truncated proteins may display altered stability and/or subcellular localization *in planta*, N-terminal CFP fusions of LsNAC069$^{\Delta NAC}$ and LsNAC069$^{267\text{-}497}$ were transiently expressed in *N. benthamiana* and analyzed by immunoblotting and confocal microscopy. CFP-LsNAC069$^{\Delta NAC}$ and CFP-LsNAC069$^{267\text{-}497}$ both labeled the ER (Figure 6c) like CFP-LsNAC069, but CFP-LsNAC069$^{\Delta NAC}$ was considerably more stable than CFP-LsNAC069 and CFP-LsNAC069$^{267\text{-}497}$ as visible on immunoblot (Figure 6d). Material for immunoblotting was incubated with the proteasome inhibitor MG132, but this did not affect the localization of CFP-LsNAC069$^{\Delta NAC}$ and CFP-LsNAC069$^{267\text{-}497}$ (Figure 6c). Due to its greater stability, further experiments were preferentially conducted with LsNAC069$^{\Delta NAC}$.

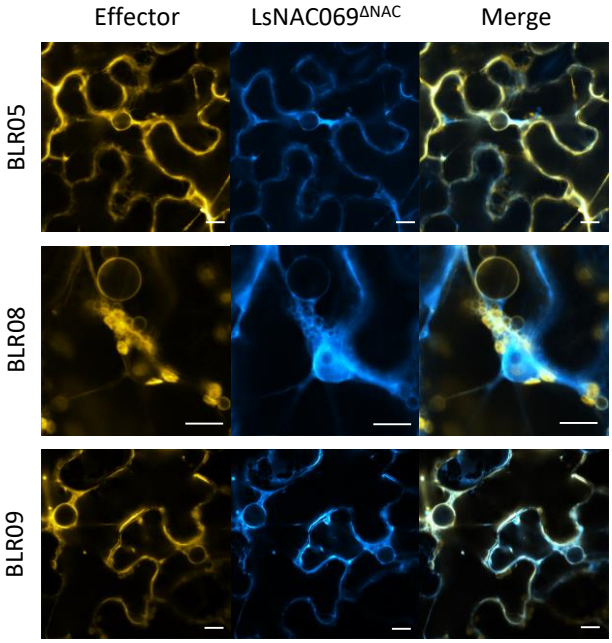

Figure 7, Co-expression of LsNAC069$^{\Delta NAC}$ with *B. lactucae* effectors in *N. benthamiana*. CFP-LsNAC069$^{\Delta NAC}$ colocalizes with YFP-BLR05 and RFP-BLR09 at the ER membrane. Co-expression of CFP-LsNAC069$^{\Delta NAC}$ with RFP-BLR08 induces a partial shift of the CFP signal from the ER membrane to the cytoplasm and nucleus. Scale bars are 10 µm.

LsNAC069$^{\Delta NAC}$ colocalizes with *B. lactucae* effectors *in planta*

The enhanced stability of CFP-LsNAC069$^{\Delta NAC}$ compared to CFP-LsNAC069 made it possible to perform co-expression experiments with *B. lactucae* effectors in *N. benthamiana*. CFP-LsNAC069$^{\Delta NAC}$ clearly colocalized with YFP-BLR05 and RFP-BLR09 at the ER (Figure 7). In the presence of RFP-BLR08, CFP-LsNAC069$^{\Delta NAC}$ was partially ER localized and partially nucleocytoplasmic localized (Figure 7). It is at this point unknown if the nucleocytoplasmic fraction represented membrane-released or degraded CFP-LsNAC069$^{\Delta NAC}$ fusion protein. CFP-LsNAC069$^{\Delta NAC}$ is found around the RFP-BLR08 ring-like structures but does not delineate the ring-like structures as sharply as RFP-BLR08. A pattern similar to LsNAC069$^{\Delta NAC}$ was observed for the RFP-tagged ER marker co-expressed with CFP-BLR08 (Figure 3a and Supplemental figure 2). The traces of CFP-LsNAC069$^{\Delta NAC}$ and ER marker around the BLR08-labeled ring-like structures suggest that the ring-like structures originate from the ER but form a separate compartment.

***LsNAC069* transcript levels are not altered during infection**

To determine if *LsNAC069* transcript levels are affected during infection, *L. sativa* cv. Olof seedlings were spray inoculated with *B. lactucae* race Bl:24 and mRNA abundance was determined at 3 hours, 1 day, 3 days, and 6 days after inoculation. At 6 days a slight increase in transcript abundance of *LsNAC069* was observed but the difference was not significant over three independent experiments (Supplemental figure 6). *B. lactucae ACTIN* mRNA abundance increased exponentially in inoculated plant samples during the time-course, which is indicative of the rapid growth of *B. lactucae* within the lettuce cotyledons. Effector transcript levels were determined relative to *B. lactucae ACTIN*. Expression of the effector genes *BLN04, BLR05, BLR08* and *BLR09* was detected at all stages of the *B. lactucae* infection. BLR08 transcripts levels peaked at 3 hours after inoculation and expression levels dropped at later stages of infection. Expression of *BLR09* peaked at day 1 and was not strongly reduced on days 3 and 6 (Supplemental figure 6). Our data do not provide evidence of transcriptional activation of *LsNAC069* during *B. lactucae* infection, yet, the activity of the protein may be controlled at the post-transcriptional level.

***B. lactucae* effectors reduce relocalization of LsNAC069 $^{\Delta NAC}$ to the nucleus**

Membrane-associated transcription factors can quickly respond to stress signals by cleavage of the transmembrane domain allowing relocalization of the active transcription factor to the nucleus. Treatment of *N. benthamiana* with *P. infestans* culture filtrate was shown to increase expression of PTI responsive genes suggesting that *Phytophthora* specific PAMPs are present in culture filtrate and are recognized by the plant (McLellan *et al.*, 2013; Yang *et al.*, 2016). To assess if culture filtrate can trigger relocalization of LsNAC069, *N. benthamiana* leaf sections were incubated with culture filtrate of the related oomycete *P. capsici* and the localization of CFP-LsNAC069 $^{\Delta NAC}$ was analyzed. All samples were treated with the inhibitor MG132 to block 26S proteasomal degradation. In the presence of culture filtrate and MG132, CFP-LsNAC069$^{\Delta NAC}$ accumulated strongly in the nucleus although signals were still detected at the ER, whereas CFP-LsNAC069$^{\Delta NAC}$ remained at the ER with MG132 treatment alone (Figure 8a).

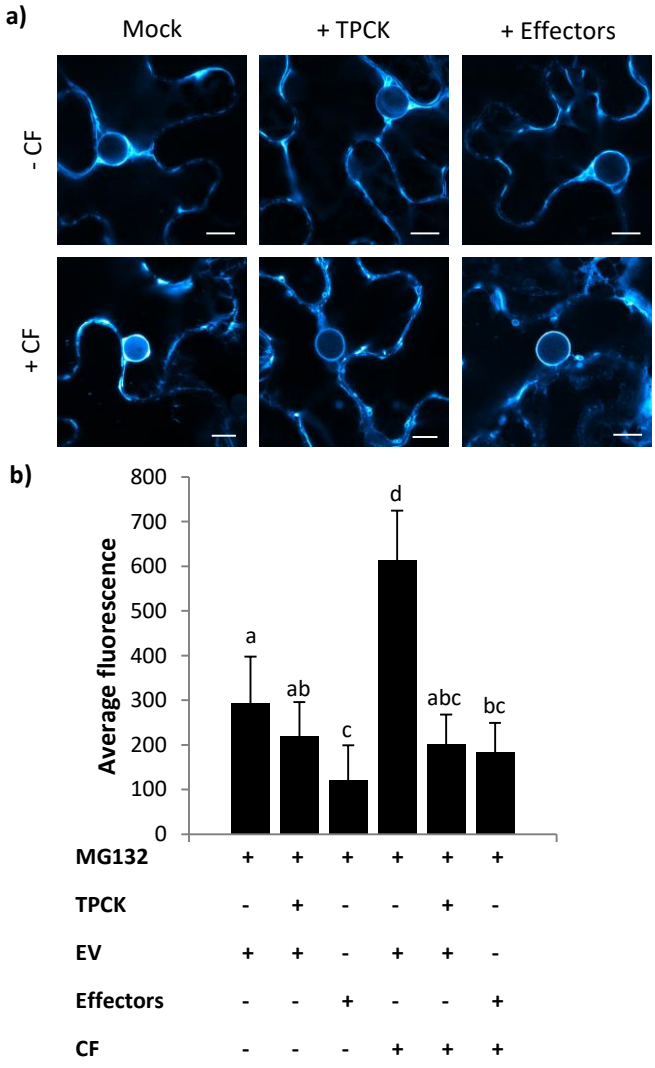

Figure 8, Culture filtrate-induced nuclear accumulation of LsNAC069$^{\Delta NAC}$ is reduced in the presence of TCPK or *B. lactucae* effectors. a) Localization of CFP-LsNAC069$^{\Delta NAC}$ in *N. benthamiana* leaf sections treated with proteasome inhibitor MG132. Leaf sections were incubated with *P. capsici* culture filtrate to induce translocation. TPCK treatment and co-expression of effectors (HA-BLR05, HA-BLR08, and HA-BLR09) both reduced translocation. Scale bars indicate 10 µm. b) Quantification of fluorescence inside the nucleus. Confocal settings were identical between samples. Bars represent the mean + SD from 13 images per treatment. Statistical differences were assessed using ANOVA with *post-hoc* Tukey testing. CF = culture filtrate, EV = empty vector

Proteolytic cleavage of ANAC017 was inhibited upon treatment with TPCK (Ng *et al.*, 2013), a serine and cysteine protease inhibitor (Bond & Butler, 1987). To determine if TPCK can prevent relocalization of CFP-LsNAC069$^{\Delta NAC}$, leaves were treated with TPCK in the presence or absence of culture filtrate. TPCK treatment effectively retained CFP-LsNAC069$^{\Delta NAC}$ at the ER membrane even with culture filtrate treatment (Figure 8a) suggesting that proteolytic cleavage is responsible for CFP-LsNAC069$^{\Delta NAC}$ relocalization.

Given that *B. lactucae* effectors BLN04, BLR05, BLR08 and BLR09 required the LsNAC069 transmembrane domain for interaction in Y2H assays, the possibility that *B. lactucae* effectors block proteolytic cleavage of LsNAC069 was explored. Three effectors, HA-tagged BLR05, BLR08 and BLR09, were together co-expressed with CFP-LsNAC069$^{\Delta NAC}$ in *N. benthamiana* leaves. Culture filtrate-induced nuclear accumulation of CFP-LsNAC069$^{\Delta NAC}$ was significantly reduced upon co-expression of the three effectors (Figure 8a). These findings were quantified by measuring the mean fluorescence intensities of the areas inside the nuclei per treatment. Statistical analysis confirmed that nuclear accumulation of CFP-LsNAC069$^{\Delta NAC}$ was induced by culture filtrate treatment in MG132 treated cells. However, culture filtrate treatment did not significantly induce relocalization of CFP-LsNAC069$^{\Delta NAC}$ in TPCK treated cells or upon co-expression of *B. lactucae* effectors (Figure 8b). These results support a model in which *P. capsici* culture filtrate components trigger proteolysis dependent relocalization of LsNAC069$^{\Delta NAC}$ from the ER to the nucleus and this process can be negatively impacted by *B. lactucae* effector expression.

Silencing of *LsNAC069* does not affect susceptibility to *B. lactucae* infection

To investigate if LsNAC069 plays a role in lettuce susceptibility to *B. lactucae* infection, two independent hairpin(hp)RNA constructs were designed (Figure 9a) to silence *LsNAC069* in transgenic *L. sativa* cv. Wendell lines. Gene silencing efficiency was assessed by quantitative RT-PCR. Both hpRNA constructs reduced LsNAC069 transcript levels by ~80% (Figure 9b). To assess the specificity of the hpRNA constructs transcript levels of *LsNAC091*, the most closely related gene with a NAC and C-terminal region, were determined. Transcript levels of *LsNAC091* were not affected in transgenic plants with either hpRNA construct (Figure 9c). Susceptibility of the silenced lines to *B. lactucae* race Bl:24 infection was determined by scoring the percentage of leaf disc surface covered in sporangiophores at 11 days after inoculation. Untransformed Wendell plants were highly susceptible to Bl:24 with infection severity ranging between 50-80%. Silenced plants displayed slightly enhanced infection in subsets of plants but these effects were not significant over the whole experiment (Figure 9d). Hence, our data indicate that silencing of a single *NAC MTF* gene, *LsNAC069*, does not significantly alter susceptibility to *B. lactucae* infection.

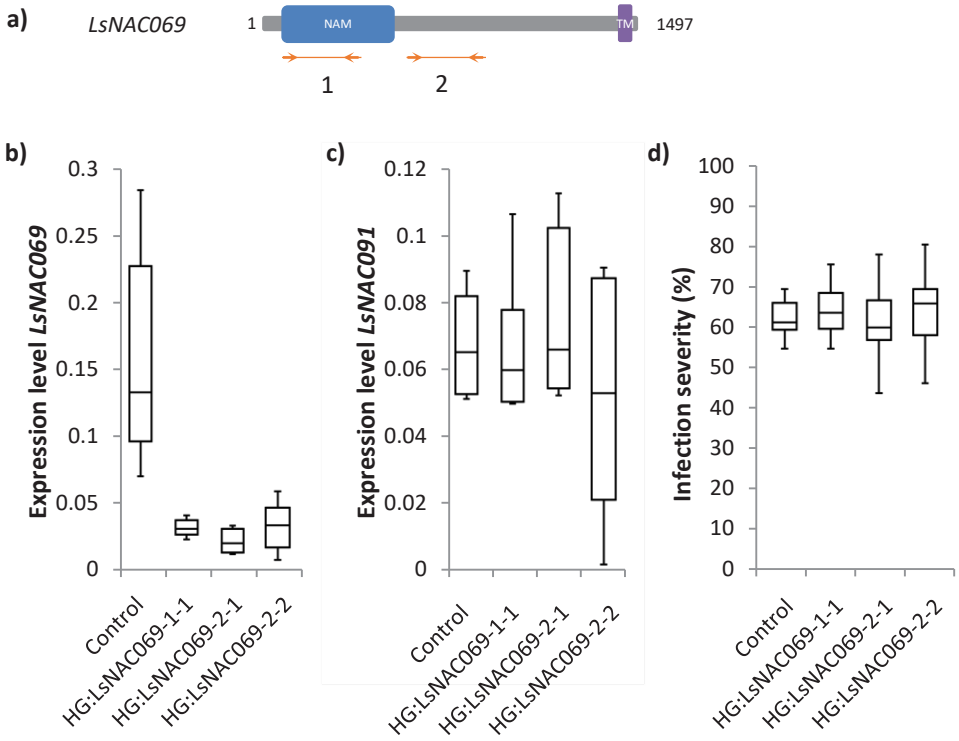

Figure 9, HpRNA constructs effectively silence *LsNAC069* but transformants do not show altered *B. lactucae* susceptibility. a) Graphical representation of the two hpRNA target sites (double orange arrows) in *LsNAC069*. Transcript levels of b) *LsNAC069* and c) *LsNAC091* in untransformed *L. sativa* Wendell plants (control) and T2 lines harbouring hpRNA construct 1 and 2. Transcript levels are relative to lettuce *ACTIN*. Each boxplot contains the values of 4-6 plants per line. d) Susceptibility to race Bl:24 infection in hpRNA T2 transformants. Leaf discs were inoculated with Bl:24 and infection levels were scored at 11 days after inoculation. Infection severity was scored as the percentage of the leaf disc surface area covered in sporangiophores. Each boxplot contains the values of 10-20 plants per line.

Discussion

Using the Y2H system, we identified a protein-protein interaction network involving five *B. lactucae* effectors and three lettuce proteins. Effectors BLR05, BLR08 and BLR09 are canonical RXLR effectors, whereas BLN03 and BLN04 lack the RXLR motif but do contain an EER motif. Although the effectors do not display overall sequence homology, strikingly, they all contain a predicted C-terminal transmembrane domain. The identified targets belong to different gene families: a NAC transcription factor (LsNAC069), a reticulon-like domain-containing protein (LsRTNLB05) and a protein of unknown function (LsPUF001). Interestingly, all targets also contain one or more predicted transmembrane domains. The C-terminus of LsNAC069, including the transmembrane domain, was also crucial for interaction with *B. lactucae* effectors whereas the NAC domain and part of the C-terminal region were dispensable. The presence of transmembrane domains raised the possibility that the effectors interact with their targets in or at one of the many membrane systems within plant cells. To test this hypothesis, initially the effectors and targets were expressed separately as N- or C-terminal fluorophore fusion proteins in *N. benthamiana* using *Agrobacterium*. Only N-terminal fluorophore fusions of BLR05, BLR08, BLR09, LsNAC069 and LsPUF001 were successfully visualized. BLR05, BLR09, LsNAC069 and LsPUF001 were predominantly localized at the ER membrane although BLR05 was also found in Golgi bodies. Co-expression of BLR05 and BLR09 with a truncated, more stable variant of LsNAC069, LsNAC069$^{\Delta NAC}$, confirmed that these proteins also colocalize at the ER membrane.

BLR08 weakly targeted the ER but was mainly found in irregularly sized ring-like structures. The smaller structures that tended to cluster together resembled enlarged multivesicular bodies (Figure 3 and Supplemental figure 2). In Arabidopsis protoplasts, formation of enlarged multivesicular bodies was induced by expression of a constitutively active Rab small GTPase (ARA7) mutant protein or by wortmannin treatment (Jia *et al.*, 2013). Multivesicular bodies or prevacuolar compartments mediate protein trafficking to the vacuole (Cui *et al.*, 2016). The larger structures (up to 10 µm in diameter) labeled by BLR08 resembled tonoplast 'bulbs' (Saito *et al.*, 2002, 2011). The large ring-like structures in *N. benthamiana* are likely artefacts of ectopic overexpression as transient expression in lettuce, which is weaker, resulted in more pronounced ER labeling along with small, homogeneously sized mobile structures. Yet, low expression levels of effector targets in lettuce forced us to continue experiments in *N. benthamiana*. Upon co-expression of CFP-LsNAC069$^{\Delta NAC}$ with RFP-BLR08 the CFP signal was detected at both the ER membrane and in the cytoplasm and nucleus. Ectopic expression of RFP-BLR08 may inadvertently induce a stress signal that triggers release of CFP-LsNAC069$^{\Delta NAC}$ from the membrane. The nucleocytoplasmic CFP signal could thus represent a form of activated CFP-LsNAC069$^{\Delta NAC}$. It is also possible that the CFP signal originates from free CFP, which is nucleocytoplasmic localized, due to RFP-BLR08-induced degradation of the fusion protein.

The effector target LsNAC069 and its interacting effectors BLN04, BLR05, BLR08 and BLR09 can be classified as tail-anchored proteins due to the presence of a transmembrane domain within 40 amino acids of the C-terminus (Borgese & Fasana, 2011; Shao & Hegde, 2011) and the absence of a signal peptide. Tail-anchored proteins are inserted post-translationally into the membrane. Proteins with a moderately hydrophobic transmembrane domain may insert via an unassisted pathway whereas those with a strongly hydrophobic transmembrane domain require assistance from chaperones (Brambillasca *et al.*, 2006; Borgese & Fasana, 2011). The chaperones shield the hydrophobic transmembrane domain

from the aqueous cytoplasmic environment to prevent aggregation of the hydrophobic domains. It is likely that chaperones interact with LsNAC069 and *B. lactucae* effectors to guide their entry into the ER membrane in plant cells. After the identification of TRC40 in mammalian cells and the Guided Entry of Tail-anchored proteins 3 (Get3) system in yeast cells (Borgese & Fasana, 2011), candidates for guiding membrane insertion of tail-anchored proteins in plants are being elucidated (Maestre-Reyna *et al.*, 2017).

The Y2H system is generally considered unsuitable for detection of protein-protein interactions between integral membrane proteins. Despite the presence of nuclear localization signals in both bait and prey vectors, membrane proteins fused to the GAL4 DBD or AD are assumed to be unable to accumulate in the yeast nucleus or expected to fold incorrectly due to their hydrophobic nature (Thaminy *et al.*, 2004). Yet, we identify multiple targets for tail-anchored *B. lactucae* effectors indicating that these effectors localize to the nucleus in yeast cells as GAL4-DBD fusion proteins. Peculiarly, the ability of Get3 to recognize the transmembrane domain of tail-anchored proteins was demonstrated using Y2H assays (Schuldiner *et al.*, 2008). A plausible mechanism to explain these observations is that (some) tail-anchored proteins can be inserted into the inner nuclear membrane (Laba *et al.*, 2014). For this particular scenario, we hypothesize that the tail-anchored GAL4 fusion proteins enter the nucleus as soluble proteins in complex with chaperones via nuclear localization signal-dependent import receptors and are inserted into the membrane using the GET system. Although the GET system is associated with the ER membrane, these membranes are continuous with the nuclear inner and outer membranes and passive diffusion could lead to low numbers of GET complexes in the inner nuclear membrane (Laba *et al.*, 2014).

Analysis of the NAC transcription factor family in lettuce identified six additional putative tail-anchored NAC transcription factors. All seven tail-anchored NAC proteins were placed in subfamily 9 of the phylogenetic tree encompassing a total of 94 lettuce NAC transcription factors. In Arabidopsis at least fourteen tail-anchored NAC transcription factors are present (Kim *et al.*, 2007a; Klein *et al.*, 2012; Yang *et al.*, 2014; Liang *et al.*, 2015; Zhao *et al.*, 2016) and ten were found in potato (Singh *et al.*, 2013). Phylogenetic analysis showed that LsNAC069 is closely related to StNTP2, ANAC016 and ANAC017. *B. lactucae* effectors also interacted strongly with these potato and Arabidopsis NAC transcription factors in Y2H assays as well as with the more distantly related proteins StNTP1 and ANAC116. Although the positive Y2H interactions indicate that these tail-anchored proteins also enter the yeast nucleus, negative results with tail-anchored proteins in Y2H assays need to be interpreted cautiously when it has not been demonstrated that these proteins enter the yeast nucleus.

Activation of MTFs including LsNAC069 is thought to be dependent on regulated intramembrane proteolysis resulting in release of the transcription factor from the membrane (Seo *et al.*, 2008). Four classes of plant proteases mediate proteolysis within membranes: rhomboid serine proteases, site-2 metalloproteases, and aspartyl proteases of the presenilin/γ-secretases and signal peptide peptidases type. We observed that relocalization of CFP-LsNAC069$^{\Delta NAC}$ was reduced in the presence of TPCK. TPCK is a broad-spectrum inhibitor of chymotrypsin-like serine proteases and some cysteine proteases (Bond & Butler, 1987). This makes the family of intramembrane rhomboid proteases a likely candidate for cleavage of LsNAC069 as it is the only intramembrane serine protease family (Weihofen & Martoglio, 2003; Adam, 2013). Two mechanisms have been proposed to govern substrate recognition by rhomboid proteases that are not mutually exclusive (Freeman, 2014). Multiple intramembrane protease substrates contain helix-destabilizing residues such as serine (S), glycine (G), and proline (P) (Cheng Li & Deber, 1994; Strisovsky

et al., 2009) in their transmembrane region. This results in an intrinsically unstable transmembrane domain that may unwind in the aqueous environment within the protease to expose the cleavage site (Moin & Urban, 2012). Indeed, LsNAC069 contains helix-destabilizing residues in its predicted transmembrane domain increasing the likelihood that it is an intramembrane protease substrate. Alternatively, rhomboid substrate specificity may be determined by recognition of a specific amino acid motif surrounding the cleavage site (Strisovsky *et al.*, 2009). So far, only the recognition motif for a bacterial rhomboid protease has been determined (Strisovsky *et al.*, 2009) and differences in rhomboid protease recognition motifs within and between species are expected to occur (Freeman, 2014). Unfortunately, this restricts extrapolation of the recognition motif to potential rhomboid protease substrates, including LsNAC069, in plants.

Normally, LsNAC069 is localized at the ER indicating that proteolysis is tightly regulated and requires a trigger. A common mechanism to prevent uncontrolled intramembrane proteolytic cleavage of protease substrates is differential subcellular compartmentalization i.e. the protease and its substrate(s) reside in different organelles and trafficking of the protease or substrate is required to initiate proteolysis (Lemberg, 2011). This was demonstrated among others for the ER-localized *Drosophila* protein Spitz that requires trafficking to the Golgi for Rhomboid-1 mediated proteolysis to occur (Tsruya *et al.*, 2002). We found that treatment of *N. benthamiana* cells with *P. capsici* culture filtrate triggers relocalization of CFP-LsNAC069$^{\Delta NAC}$ to the nucleus. *P. capsici* culture filtrate likely contains a mix of PAMPs, as was found previously for *P. infestans* culture filtrate that triggers upregulation of multiple markers of PTI (McLellan *et al.*, 2013). However, the signaling pathways that bridge the gap between perception of triggers and execution of regulated intramembrane proteolysis are still poorly understood. We observed that nuclear accumulation of CFP was strongly reduced upon co-expression of HA-BLR05, HA-BLR08 and HA-BLR09. Furthermore, the hypersensitive-like response observed in *N. benthamiana* leaves infiltrated with pUBN-CFP-LsNAC069 was partially diminished upon co-expression of pUBN-YFP-BLR05 or BLR09 (Supplemental figure 1). Considering that a nuclear CFP signal was observed when CFP-LsNAC069$^{\Delta NAC}$ was co-expressed with RFP-BLR08 or HA-BLR08 (data not shown), we infer that co-expression of HA-BLR05 and HA-BLR09 is sufficient to reduce both culture filtrate-induced relocalization of CFP-LsNAC069$^{\Delta NAC}$ and of BLR08-triggered nuclear accumulation of CFP. More research will be required to validate these assumptions and determine if BLR05 and BLR09 are functionally redundant in reducing relocalization.

Stable transformants expressing *LsNAC069* hpRNA were generated to evaluate the role of LsNAC069 in disease susceptibility. *LsNAC069* transcript levels were reduced by 90% in T2 transformants. *LsNAC069*-silenced plants did not display altered susceptibility phenotypes to *B. lactucae* infection. We would have expected that LsNAC069 contributes to disease resistance and silencing would enhance disease susceptibility based on the enhanced susceptibility phenotypes to *P. infestans* infection observed in *N. benthamiana* plants silenced for *NbNTP1* or *NbNTP2* (McLellan *et al.*, 2013). The high level of susceptibility observed in the parental line *L. sativa* cv. Wendell may have obscured an enhanced susceptibility phenotype following *LsNAC069* silencing. Furthermore, considering the vast size of the NAC family in lettuce, it may be required to silence multiple family members to overcome genetic redundancy.

Acknowledgements

We thank Hazel McLellan for providing pDONR221-Pi03192, pDONR201-PiAVR2, pDONR221-StNTP1 and pDONR221-StNTP2; Olivier van Aken for providing pDONR221-ANAC013, pDONR221-ANAC017, pDONR221-ANAC053 and pDONR221-ANAC078; Bart Edelbroek & Marcel Proveniers for supplying the Arabidopsis transcription factor Y1H library; Christopher Grefen for donating the pUBN-Dest vectors and Christine Falkner for providing the RFP-ER marker pB7WGR-KDEL.

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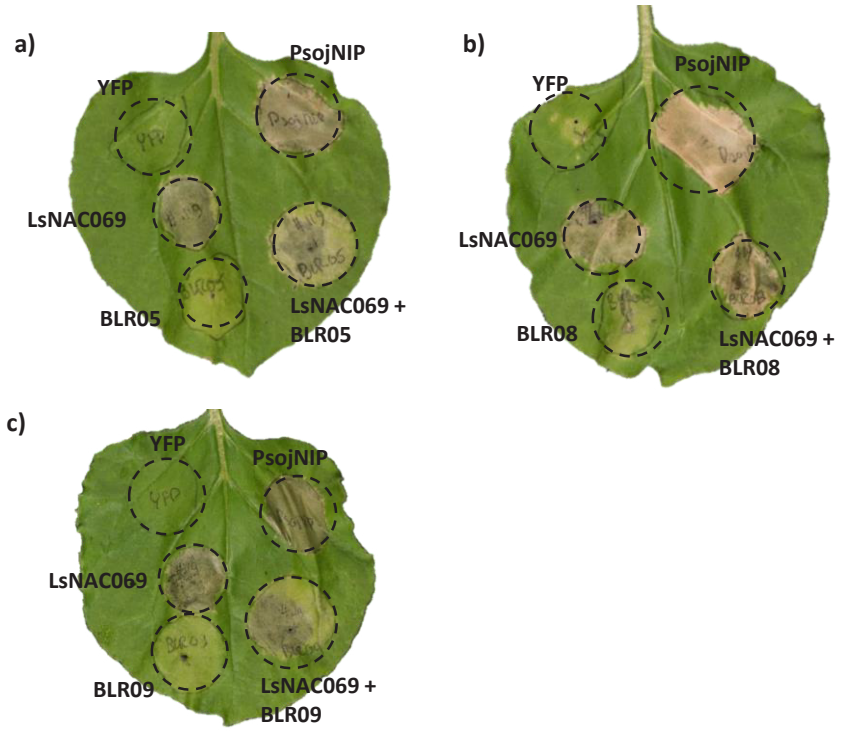
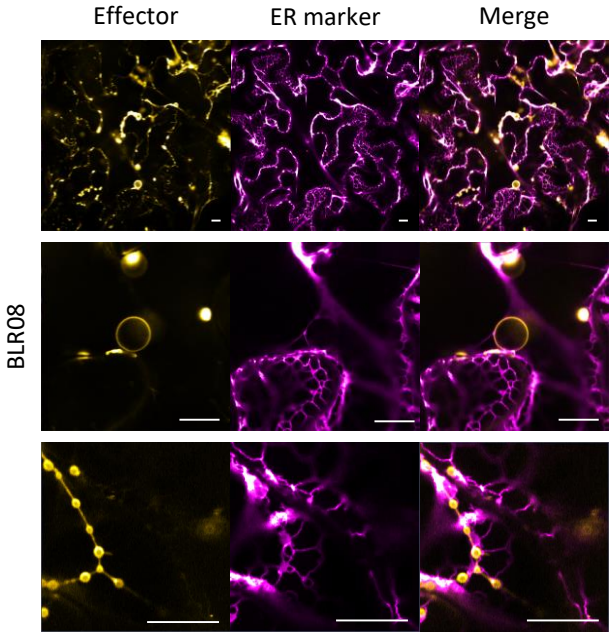
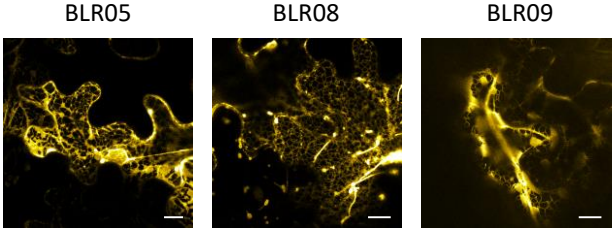
Supplemental data



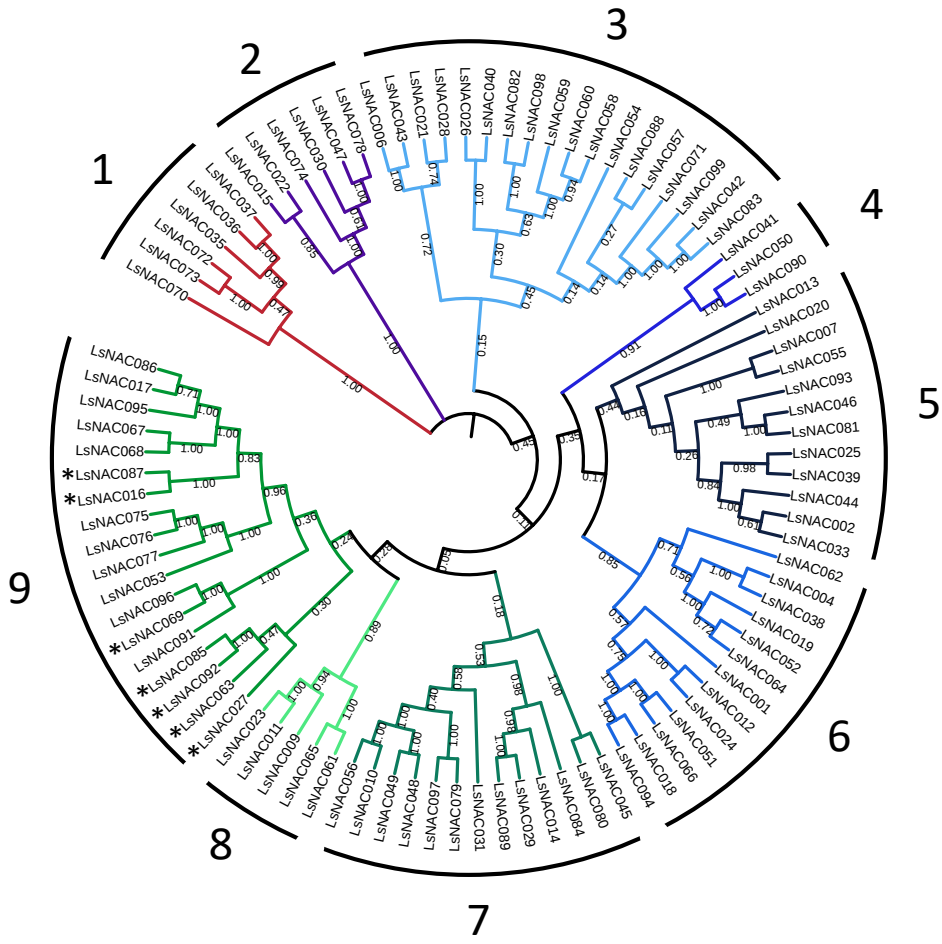
Supplemental figure 1, CFP-LsNAC069 expression using the pUBN-Dest vector results in a hypersensitive-like response in *N. benthamiana*. LsNAC069 was expressed as CFP fusion protein using the pUBN-Dest CFP vector in combination with free YFP or YFP tagged a) BLR05, b) BLR08 and c) BLR09 using the pUBN-Dest YFP vector. Co-expression of CFP-LsNAC069 with YFP-BLR05 or YFP-BLR09 had a mildly attenuating effect on development of necrosis especially at the edges of the infiltration sites whereas YFP-BLR08 did not affect the development of necrosis. Co-expression of YFP-BLR05, YFP-BLR08 or YFP-BLR09 with free YFP did not induce necrosis. *Phytophthora sojae* necrosis-inducing protein (PsojNIP) was included as a positive control for necrosis induction. Two leaves were infiltrated per effector. Pictures were takes 10 days after infiltration. Necrosis was not observed using the pB7WGC2 vector that contains the widely applied cauliflower mosaic virus 35S promoter to drive expression of fusion constructs. In contrast, the Arabidopsis ubiquitin-10 promoter drives expression in pUBN vectors.



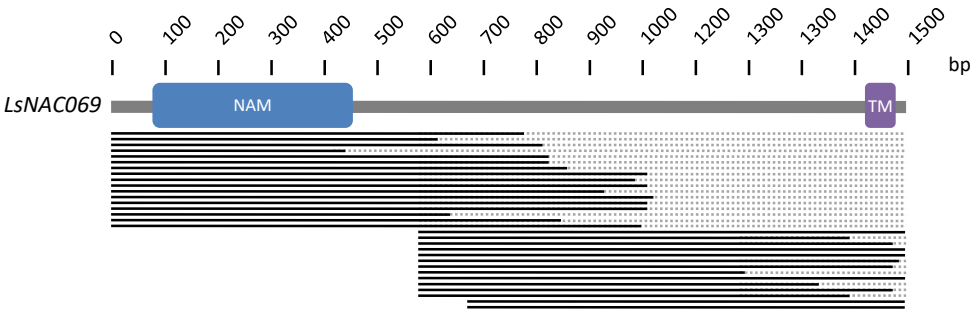
Supplemental figure 2, Subcellular localization of *B. lactucae* effector BLR08 in *N. benthamiana*. CFP-BLR08 was co-expressed with the RFP-tagged ER lumenal marker and localizes to ring-like structures of various sizes. The ring-like structures are closely associated with the ER and the ER marker also weakly outlines multiple ring-like structures. Scale bars are 10 µm.



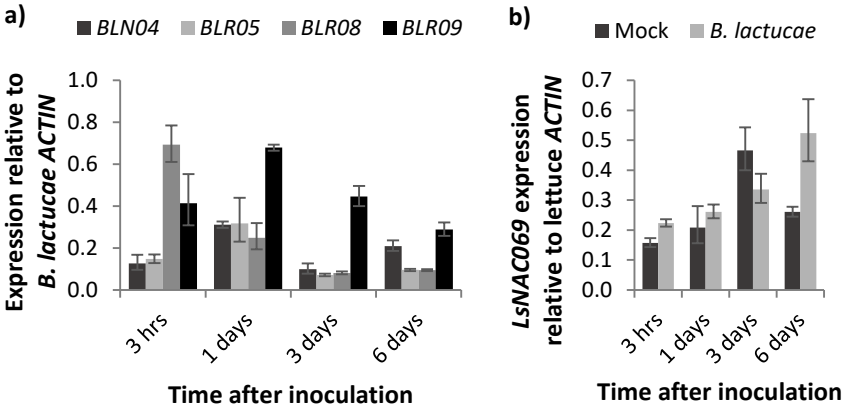
Supplemental figure 3, *B. lactucae* effectors BLR05, BLR08 and BLR09 predominantly localize to the ER membrane in lettuce. YFP-BLR05, YFP-BLR08 and RFP-BLR09 were transiently expressed in *L. sativa* cv. Olof using *Agrobacterium*. All three effectors localize to the ER membrane in lettuce and YFP-BLR08 also labels small homogenously sized mobile ring-like structures. Scale bars are 10 µm.



Supplemental figure 4, Phylogenetic relationship of NAC proteins in lettuce. Multiple alignments were generated using Clustal Omega. A Neighbor-Joining tree was constructed in MEGA 7.0 using pairwise gap deletion with 1000 bootstrap replicates. The bootstrap values are indicated at each node. Classification into subfamilies is indicated. Asterisks (*) indicate tail-anchored NAC proteins i.e. with a single C-terminal transmembrane domain.



Supplemental figure 5, Alignment of yeast isolated prey inserts to *LsNAC069*. Graphical representation of the *LsNAC069* coding sequence in grey with the position of the NAC and transmembrane domain. Prey inserts were amplified from yeast clones and Sanger sequenced with a forward primer. Reads were aligned to *LsNAC069* as indicated with black lines below the *LsNAC069* coding sequence. Prey inserts were expected to extend to the end of the *LsNAC069* sequence as is indicated with grey dotted lines.



Supplemental figure 6, Expression of *LsNAC069* and effectors *BLR05*, *BLR08*, *BLR09* and *BLN04* during *B. lactucae* infection. a) Transcript abundance of *B. lactucae* effector genes *BLN04*, *BLR05*, *BLR08* and *BLR09.* b) Transcript abundance of *LsNAC069* in mock and *B. lactucae* inoculated samples. *L. sativa* cv. Olof seedlings were harvested 3 hours, 1 day, 3 days and 6 days after infection with race Bl:24. Effector expression levels were normalized to *B. lactucae ACTIN*. *LsNAC069* expression was normalized to *L. sativa ACTIN*. Data of a single experiment with three biological replicates is depicted (mean + SE). Data are representative for three independent experiments.

Supplemental table 1, Primers used in this study.

Primers with BP cloning sites for prey gene amplification[1]

Gene	*Orientation*	*Sequence*
LsRTNLB05	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGTCGTTATGGCGCCAGC
LsRTNLB05	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTT*TATGCAAACTTCTTCGCTTTTG
LsNAC069	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGGGTTCTGACTTGATTGAAG
LsNAC069	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTT*TACCATATACACCTACCCAAT
LsPUF001	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGGCGTCTACGACCACCG
LsPUF001	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTC*TATTTCATTGCATCGACTTCA
LsNAC069 pos.523	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGAGTGGGCCCGGTCCCAAA
LsNAC069 pos.631	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGCGGAATCTGATTCCAATTCC
LsNAC069 pos.802	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGGAAAGTTGTGGCGATTTGAAA
LsNAC069 pos.988	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*ATGGGTAATGATGAATTTCAAATAAGT
LsNAC069 pos.1188	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTT*TAACCCGTTCCATTGCCATTAT
LsNAC069 pos.1401	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTTT*TAACCCCTACCCCTACTACTC

[1]Sequences in italic represent attB cloning sites

Primers with BP cloning sites for hairpinRNA construct amplification[1]

Gene	*Orientation*	*Sequence*
LsNAC069	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*GTGCTTTCCACCTGGTTTC
LsNAC069	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTT*GCCCGACCTTGGTAATAAAC
LsNAC069	Fwd	*GGGGACAAGTTTGTACAAAAAAGCAGGCTTC*GAGGAGGAATGGAGTGATGA
LsNAC069	Rev	*GGGGACCACTTTGTACAAGAAAGCTGGGTT*AATTCCGCCAAAGACCCGA

[1]Sequences in italic represent attB cloning sites

Primers for sequencing of hairpinRNA constructs in the destination vector

Name	*Orientation*	*Sequence*
pHellsgate12 promoter	Fwd	GGGATGACGCACAATCC
pHellsgate12 terminator	Rev	GAGCTACACATGCTCAGG
pHellsgate12 intron	Rev	CCGAATTCCTCGAGACCAC
pHellsgate12 intron	Fwd	AGGGTCCTAACCAAGAAAATG

Primers for qRT-PCR

Gene	*Orientation*	*Sequence*
LsNAC069	Fwd	ATAATGGCAATGGAACGGG
LsNAC069	Rev	AGAAGCTGGAGTTGTTGGT
LsNAC091	Fwd	CATCCAGTCAACAGTGCAAA
LsNAC091	Rev	GGCACCTCCTCCACACAA
Lettuce *ACTIN*	Fwd	CTATCCAGGCTGTGCTTTCC
Lettuce *ACTIN*	Rev	ACCCTTCGTAGATCGGGACT
BLN04	Fwd	AGGGGACATGCTGTATATGG
BLN04	Rev	TGCTCTCGACATGGTGGTT
BLR05	Fwd	AGCAAGAACGAGAAAAAGGAAA
BLR05	Rev	ACAGGCAAAACGGAAGACA
BLR08	Fwd	CCACACCCTATCCAACTCTC
BLR08	Rev	ACGGTAATTCGTGCTTCG
BLR09	Fwd	TGAATTGGAAGAGCGAGGAGGA
BLR09	Rev	GATGCCGTAGCAAGCAGAGA
B. lactucae ACTIN	Fwd	GCGAGAAATTGTGCGTGATA
B. lactucae ACTIN	Rev	ACTCGGCTGCAGTCTTCATT

Supplemental table 2, Overview of lettuce NAM domain containing genes.

Gene symbol	Gene model name	Genome position			Protein length	NAM domain		
		Linkage group	*Start*	*End*		*Start*	*End*	*E-value*
LsNAC001	Lsat_1_v5_gn_1_10240.1	1	12,227,757	12,230,461	309	9	136	4.20E-28
LsNAC002	Lsat_1_v5_gn_1_14220.1	1	15,786,722	15,787,976	353	19	143	9.50E-28
LsNAC003	Lsat_1_v5_gn_1_18141.1	1	21,746,417	21,747,420	279	13	116	5.50E-08
LsNAC004	Lsat_1_v5_gn_1_24600.1	1	29,136,204	29,139,463	374	9	136	7.10E-28
LsNAC005	Lsat_1_v5_gn_1_28440.1	1	32,676,601	32,678,665	263	4	126	1.10E-10
LsNAC006	Lsat_1_v5_gn_1_32381.1	1	34,666,420	34,667,623	259	31	162	8.80E-24
LsNAC007	Lsat_1_v5_gn_1_58960.1	1	70,566,384	70,571,924	332	16	143	2.60E-29
LsNAC008	Lsat_1_v5_gn_1_103201.1	1	145,597,821	145,598,768	243	4	132	1.40E-05
LsNAC009	Lsat_1_v5_gn_2_20040.1	2	44,143,440	44,145,457	231	7	135	1.90E-30
LsNAC010	Lsat_1_v5_gn_2_59341.1	2	126,887,182	126,888,710	189	4	134	2.60E-22
LsNAC011	Lsat_1_v5_gn_2_71740.1	2	145,468,699	145,473,294	646	7	133	1.70E-29
LsNAC012	Lsat_1_v5_gn_2_78720.1	2	150,703,694	150,705,830	358	12	139	6.40E-28
LsNAC013	Lsat_1_v5_gn_2_83520.1	2	160,245,039	160,247,124	304	6	130	1.40E-28
LsNAC014	Lsat_1_v5_gn_2_89180.1	2	167,230,906	167,232,854	354	6	132	1.40E-25
LsNAC015	Lsat_1_v5_gn_2_91301.1	2	170,133,486	170,135,795	378	56	196	3.40E-13
LsNAC016	Lsat_1_v5_gn_2_103401.1	2	180,824,269	180,825,975	581	34	160	3.20E-28
LsNAC017	Lsat_1_v5_gn_2_103381.1	2	180,865,873	180,868,404	419	19	145	2.40E-27
LsNAC018	Lsat_1_v5_gn_2_128940.1	2	208,403,411	208,404,763	346	8	135	1.40E-28
LsNAC019	Lsat_1_v5_gn_3_14701.1	3	18,927,812	18,929,268	379	19	146	4.40E-26
LsNAC020	Lsat_1_v5_gn_3_24581.1	3	33,861,259	33,863,722	341	13	139	4.60E-28
LsNAC021	Lsat_1_v5_gn_3_31741.1	3	41,645,489	41,646,340	223	15	138	3.00E-25
LsNAC022	Lsat_1_v5_gn_3_57981.1	3	77,434,182	77,437,780	428	59	197	3.40E-12

Supplemental table 2 continued.

Gene symbol	Gene model name	Genome position			Protein length	NAM domain		
		Linkage group	*Start*	*End*		*Start*	*End*	*E-value*
LsNAC023	Lsat_1_v5_gn_3_67400.1	3	88,629,595	88,632,365	616	7	133	6.70E-30
LsNAC024	Lsat_1_v5_gn_3_73920.1	3	100,071,231	100,073,922	370	8	135	1.00E-28
LsNAC025	Lsat_1_v5_gn_3_99141.1	3	158,082,895	158,084,338	300	17	141	2.60E-28
LsNAC026	Lsat_1_v5_gn_3_101901.1	3	164,242,582	164,243,654	285	9	132	2.90E-27
LsNAC027	Lsat_1_v5_gn_3_111800.1	3	187,662,822	187,665,497	470	67	213	1.60E-25
LsNAC028	Lsat_1_v5_gn_4_1821.1	4	1,565,681	1,566,619	248	15	140	4.80E-27
LsNAC029	Lsat_1_v5_gn_4_9861.1	4	14,770,157	14,771,784	344	18	144	3.60E-25
LsNAC030	Lsat_1_v5_gn_4_66101.1	4	101,545,430	101,547,273	286	58	197	2.60E-15
LsNAC031	Lsat_1_v5_gn_4_71141.1	4	110,153,206	110,154,897	340	11	138	3.30E-25
LsNAC032	Lsat_1_v5_gn_4_86540.1	4	137,865,442	137,866,005	163	7	138	4.10E-06
LsNAC033	Lsat_1_v5_gn_4_109600.1	4	189,520,982	189,522,167	339	17	141	2.70E-28
LsNAC034	Lsat_1_v5_gn_4_122120.1	4	216,689,480	216,691,072	343	14	119	1.00E-09
LsNAC035	Lsat_1_v5_gn_4_128500.1	4	240,707,777	240,708,961	298	8	133	2.90E-18
LsNAC036	Lsat_1_v5_gn_0_37660.1	4	240,740,779	240,742,610	508	8	133	8.50E-18
LsNAC037	Lsat_1_v5_gn_4_128561.1	4	240,806,284	240,808,154	521	20	145	1.80E-17
LsNAC038	Lsat_1_v5_gn_4_131860.1	4	252,280,221	252,281,522	366	9	136	6.50E-28
LsNAC039	Lsat_1_v5_gn_4_169300.1	4	342,539,544	342,540,683	318	14	138	1.30E-29
LsNAC040	Lsat_1_v5_gn_4_176860.1	4	366,815,870	366,817,283	287	9	132	2.00E-27
LsNAC041	Lsat_1_v5_gn_5_981.1	5	1,700,069	1,701,817	299	7	135	6.70E-25
LsNAC042	Lsat_1_v5_gn_5_7401.1	5	13,223,290	13,224,519	338	16	142	1.40E-28
LsNAC043	Lsat_1_v5_gn_5_10860.1	5	20,847,110	20,848,268	282	30	166	4.40E-23
LsNAC044	Lsat_1_v5_gn_5_20601.1	5	42,767,877	42,769,155	353	18	142	1.50E-27

Supplemental table 2 continued.

Gene symbol	Gene model name	Genome position			Protein length	NAM domain		
		Linkage group	*Start*	*End*		*Start*	*End*	*E-value*
LsNAC045	Lsat_1_v5_gn_5_106180.1	5	226,143,436	226,144,570	200	10	128	3.70E-21
LsNAC046	Lsat_1_v5_gn_5_127660.1	5	254,383,630	254,384,913	352	17	143	1.40E-28
LsNAC047	Lsat_1_v5_gn_5_146440.1	5	275,861,073	275,867,973	462	41	182	2.80E-16
LsNAC048	Lsat_1_v5_gn_5_165541.1	5	304,866,764	304,867,860	247	5	140	4.30E-23
LsNAC049	Lsat_1_v5_gn_5_165561.1	5	304,892,484	304,893,491	192	9	142	8.20E-23
LsNAC050	Lsat_1_v5_gn_5_183661.1	5	327,709,492	327,711,357	290	11	137	4.20E-28
LsNAC051	Lsat_1_v5_gn_6_2320.1	6	6,160,420	6,161,994	331	8	135	4.90E-29
LsNAC052	Lsat_1_v5_gn_6_11620.1	6	15,544,807	15,546,568	403	19	146	2.50E-27
LsNAC053	Lsat_1_v5_gn_6_9421.1	6	16,161,397	16,163,770	352	7	133	4.10E-25
LsNAC054	Lsat_1_v5_gn_6_30441.1	6	40,387,758	40,388,827	300	15	141	1.90E-26
LsNAC055	Lsat_1_v5_gn_6_38101.1	6	51,855,065	51,856,829	398	19	148	1.70E-29
LsNAC056	Lsat_1_v5_gn_6_38501.1	6	52,435,582	52,436,572	258	62	193	1.10E-20
LsNAC057	Lsat_1_v5_gn_6_46681.1	6	63,353,107	63,354,860	295	10	136	7.80E-23
LsNAC058	Lsat_1_v5_gn_6_56480.1	6	76,365,689	76,366,842	315	22	155	1.10E-26
LsNAC059	Lsat_1_v5_gn_6_56440.1	6	76,491,582	76,492,676	297	22	155	3.80E-27
LsNAC060	Lsat_1_v5_gn_6_56420.1	6	76,505,858	76,507,021	315	22	155	1.10E-27
LsNAC061	Lsat_1_v5_gn_6_64840.1	6	96,651,570	96,653,422	297	7	133	3.50E-28
LsNAC062	Lsat_1_v5_gn_6_80600.1	6	133,559,537	133,561,131	370	12	141	5.90E-28
LsNAC063	Lsat_1_v5_gn_6_80780.1	6	133,849,495	133,851,678	495	5	135	2.00E-28
LsNAC064	Lsat_1_v5_gn_6_81580.1	6	135,108,603	135,110,031	416	19	146	8.10E-27
LsNAC065	Lsat_1_v5_gn_6_87981.1	6	145,367,395	145,369,561	325	7	135	1.30E-28
LsNAC066	Lsat_1_v5_gn_6_90240.1	6	150,446,902	150,449,053	354	8	135	6.60E-29

Supplemental table 2 continued.

Gene symbol	Gene model name	Genome position			Protein length	NAM domain		
		Linkage group	*Start*	*End*		*Start*	*End*	*E-value*
LsNAC067	Lsat_1_v5_gn_6_100121.1	6	164,708,939	164,712,652	373	23	149	1.80E-25
LsNAC068	Lsat_1_v5_gn_6_99940.1	6	164,831,438	164,835,152	373	23	149	2.80E-25
LsNAC069	Lsat_1_v5_gn_6_99960.1	6	165,606,225	165,608,625	497	25	151	4.50E-27
LsNAC070	Lsat_1_v5_gn_7_19701.1	7	25,325,183	25,326,707	399	12	138	6.30E-16
LsNAC071	Lsat_1_v5_gn_7_19440.1	7	26,316,056	26,317,812	327	16	142	6.30E-28
LsNAC072	Lsat_1_v5_gn_7_18921.1	7	26,771,462	26,773,467	554	27	151	1.20E-16
LsNAC073	Lsat_1_v5_gn_7_18860.1	7	26,809,288	26,811,293	554	27	151	1.20E-16
LsNAC074	Lsat_1_v5_gn_7_22221.1	7	30,031,303	30,035,598	297	58	197	1.30E-15
LsNAC075	Lsat_1_v5_gn_7_30761.1	7	42,728,749	42,730,060	276	7	95	1.20E-12
LsNAC076	Lsat_1_v5_gn_7_30741.1	7	42,749,401	42,750,757	291	7	95	1.30E-12
LsNAC077	Lsat_1_v5_gn_7_29640.1	7	42,968,513	42,969,996	262	7	132	4.70E-22
LsNAC078	Lsat_1_v5_gn_7_38860.1	7	54,102,720	54,111,327	422	41	183	2.60E-16
LsNAC079	Lsat_1_v5_gn_7_68761.1	7	104,905,439	104,907,951	424	29	154	1.70E-24
LsNAC080	Lsat_1_v5_gn_7_80581.1	7	132,916,534	132,917,644	189	10	128	7.80E-24
LsNAC081	Lsat_1_v5_gn_7_104460.1	7	175,389,708	175,391,178	349	16	142	1.40E-28
LsNAC082	Lsat_1_v5_gn_7_105640.1	7	178,654,542	178,655,750	339	15	139	6.20E-26
LsNAC083	Lsat_1_v5_gn_7_105761.1	7	179,013,105	179,014,349	350	16	142	6.60E-28
LsNAC084	Lsat_1_v5_gn_7_112441.1	7	188,738,853	188,740,123	305	26	153	6.00E-25
LsNAC085	Lsat_1_v5_gn_8_3961.1	8	5,248,060	5,250,116	596	8	136	2.30E-24
LsNAC086	Lsat_1_v5_gn_8_17420.1	8	27,090,627	27,092,616	391	27	153	3.50E-28
LsNAC087	Lsat_1_v5_gn_8_17440.1	8	27,095,473	27,097,461	499	15	141	3.70E-28
LsNAC088	Lsat_1_v5_gn_8_50780.1	8	70,509,598	70,510,605	279	11	135	5.20E-26

Supplemental table 2 continued.

Gene symbol	Gene model name	Genome position			Protein length	NAM domain		
		Linkage group	*Start*	*End*		*Start*	*End*	*E-value*
LsNAC089	Lsat_1_v5_gn_8_51721.1	8	72,925,339	72,927,123	405	2	128	1.40E-25
LsNAC090	Lsat_1_v5_gn_9_10341.1	9	12,510,052	12,513,020	300	11	137	1.60E-27
LsNAC091	Lsat_1_v5_gn_9_42720.1	9	46,238,296	46,245,937	617	24	150	3.10E-26
LsNAC092	Lsat_1_v5_gn_0_9760.1	9	55,317,702	55,319,739	506	10	138	1.60E-25
LsNAC093	Lsat_1_v5_gn_9_48560.1	9	57,064,130	57,067,246	383	30	157	1.30E-26
LsNAC094	Lsat_1_v5_gn_9_52241.1	9	59,095,500	59,096,870	374	8	135	1.00E-28
LsNAC095	Lsat_1_v5_gn_9_71421.1	9	93,944,388	93,947,127	373	24	150	1.30E-28
LsNAC096	Lsat_1_v5_gn_9_79840.1	9	113,783,762	113,785,562	91	11	89	2.10E-10
LsNAC097	Lsat_1_v5_gn_9_82461.1	9	126,472,364	126,474,183	376	33	158	8.90E-25
LsNAC098	Lsat_1_v5_gn_9_95120.1	9	149,888,638	149,889,438	242	2	76	1.10E-14
LsNAC099	Lsat_1_v5_gn_9_96041.1	9	151,834,170	151,835,700	366	16	142	2.60E-28

Supplemental table 3, Overview of lettuce reticulon-like domain containing genes.

Gene symbol	Gene model name	Genome position			Protein length	Reticulon domain		
		Linkage group	*Start*	*End*		*Start*	*End*	*E-value*
LsRTNLB01	Lsat_1_v5_gn_1_4680.1	1	6,644,280	6,647,428	564	381	539	6.90E-28
LsRTNLB02	Lsat_1_v5_gn_2_38560.1	2	85,598,848	85,602,363	584	378	545	9.20E-28
LsRTNLB03	Lsat_1_v5_gn_2_124901.1	2	204,934,241	204,935,492	230	44	200	1.20E-44
LsRTNLB04	Lsat_1_v5_gn_3_9921.1	3	12,639,478	12,641,267	251	63	219	4.10E-55
LsRTNLB05	Lsat_1_v5_gn_3_35581.1	3	45,357,575	45,359,500	296	118	268	1.30E-43

Supplemental table 3 continued.

Gene symbol	Gene model name	Genome position			Protein length	Reticulon domain		
		Linkage group	*Start*	*End*		*Start*	*End*	*E-value*
LsRTNLB06	Lsat_1_v5_gn_3_38820.1	3	47,766,761	47,768,395	404	176	330	6.60E-28
LsRTNLB07	Lsat_1_v5_gn_3_109960.1	3	183,059,625	183,066,155	575	386	544	1.20E-30
LsRTNLB08	Lsat_1_v5_gn_3_138621.1	3	250,644,139	250,645,616	269	83	239	4.10E-59
LsRTNLB09	Lsat_1_v5_gn_4_14841.1	4	22,593,621	22,594,748	220	38	190	1.80E-40
LsRTNLB10	Lsat_1_v5_gn_4_168300.1	4	341,964,829	341,965,572	196	1	157	2.10E-35
LsRTNLB11	Lsat_1_v5_gn_5_114901.1	5	235,522,903	235,524,111	203	18	174	1.40E-43
LsRTNLB12	Lsat_1_v5_gn_5_137201.1	5	266,596,872	266,598,173	201	43	176	6.60E-16
LsRTNLB13	Lsat_1_v5_gn_5_155541.1	5	291,299,234	291,300,673	210	24	180	2.30E-50
LsRTNLB14	Lsat_1_v5_gn_6_84741.1	6	142,114,021	142,115,683	249	64	220	2.50E-54
LsRTNLB15	Lsat_1_v5_gn_7_86441.1	7	144,576,744	144,577,965	203	18	174	3.30E-41
LsRTNLB16	Lsat_1_v5_gn_8_6361.1	8	8,165,945	8,167,426	263	78	233	6.30E-49
LsRTNLB17	Lsat_1_v5_gn_8_41400.1	8	56,703,898	56,704,710	188	6	158	1.10E-37
LsRTNLB18	Lsat_1_v5_gn_8_61721.1	8	89,271,221	89,274,182	588	338	497	1.40E-23
LsRTNLB19	Lsat_1_v5_gn_8_86660.1	8	125,173,282	125,173,770	139	19	135	3.70E-23
LsRTNLB20	Lsat_1_v5_gn_8_98740.1	8	145,533,241	145,534,560	232	61	210	3.50E-39
LsRTNLB21	Lsat_1_v5_gn_8_99581.1	8	146,842,784	146,845,673	179	88	179	2.90E-21
LsRTNLB22	Lsat_1_v5_gn_8_141581.1	8	235,547,630	235,549,734	219	36	189	4.40E-37
LsRTNLB23	Lsat_1_v5_gn_9_3321.1	9	4,457,341	4,457,744	110	3	106	6.00E-17
LsRTNLB24	Lsat_1_v5_gn_9_42821.1	9	46,319,343	46,322,443	564	381	539	4.30E-34
LsRTNLB25	Lsat_1_v5_gn_9_57040.1	9	67,584,026	67,585,588	259	74	230	2.30E-55

Chapter 5

General discussion

Plant pathogenic downy mildews and *Phytophthora* spp. belonging to the order Peronosporales cause diseases on many agriculturally important crops (Kamoun *et al.*, 2015). These filamentous pathogens secrete effector molecules during host colonization that act extracellularly (apoplastic effectors) or inside the plant cells (host-translocated effectors) to suppress the plant immune system and rewire host processes to accommodate the invaders. Host-translocated effectors can be recognized by specialized intracellular receptors and trigger activation of the plant immune system leading to resistance. Successful pathogens may stay one step ahead by deploying effectors that inhibit immune responses following effector recognition, or through mutation or deletion of the recognized effector. And so, plant and pathogen go through continuous cycles of adaptation and counter-adaptation, which is also referred to as a co-evolutionary molecular arms race. In modern agriculture, crops with single newly-introduced resistance genes are deployed in monocultures resulting in extreme selection pressures on pathogens to break resistance. As a consequence, the duration of counter-adaptation cycles is strongly reduced.

In my thesis, I have focused on effectors of the plant pathogenic oomycete *Bremia lactucae* that causes downy mildew disease on lettuce. Most lettuce cultivars are susceptible to infection and it is proposed that *B. lactucae* effectors contribute to disease susceptibility. Following the sequencing of the *B. lactucae* transcriptome, multiple effectors were identified, predominantly of the RXLR(-like) type. To date, recognition of four *B. lactucae* effectors in specific lettuce accessions has been described (Stassen *et al.*, 2012, 2013; Giesbers *et al.*, 2017). The work described here expands our knowledge of *B. lactucae* effectors by on the one hand describing the response of >150 lettuce accessions to fourteen previously uncharacterized effectors, and on the other hand identifying effector targets in lettuce to explore the molecular mechanisms underlying disease susceptibility. Here, I recapitulate some of the main findings, discuss the observations in a broader context, point out technical limitations, and provide ideas for future work on effectors and their targets.

From gene-for-gene resistance to networks

Disease resistance is often mediated by dominant resistant genes that operate in a gene-for-gene manner, i.e. for each host gene mediating resistance, there is a complementary pathogen effector gene that is responsible for recognition. Consequently, resistance genes can be identified by probing germplasm with candidate effector genes. An advantage of this strategy is that resistance to a pathogen can be dissected into effector–resistance (*R*) gene pairs. Subsequently, the effectiveness of specific *R* genes against multiple pathogen races can be determined and *R* genes can be strategically deployed. Effector-assisted *R* gene discovery has been successfully applied in multiple host-pathogen systems (Vleeshouwers *et al.*, 2008; Wroblewski *et al.*, 2009; Stassen *et al.*, 2013; Vleeshouwers & Oliver, 2014; Giesbers *et al.*, 2017). In **Chapter 2**, a lettuce germplasm set was screened using *Agrobacterium*-mediated transient expression for induction of hypersensitive responses (HR) to fourteen *B. lactucae* effectors. Effector BLR40 was specifically recognized in *Lactuca sativa* cv. Design. Furthermore, effectors BLN06 and BLR38 were recognized in *L. serriola* LS102 and BLN06 was also recognized in *L. sativa* NunDm17 and RYZ2164.

To determine the genetic basis of BLR38 recognition in *L. serriola* LS102, quantitative trait locus (QTL) analysis was performed on a segregating F2 population. This resulted in the identification of two independent loci on chromosomes 4 and 8 that together are required for BLR38 recognition. Based on the current knowledge of effector recognition, it is likely that the loci encode nucleotide-binding domain and leucine-rich repeat-containing (NLR)

proteins. A recent review paper describing a meta-analysis on the molecular mechanisms involved in resistance gene functioning, identifies NLRs as the only class of receptors mediating intracellular recognition of effectors (with the exception of executor genes that are activated by *Xanthomonas* effectors) (Kourelis & van der Hoorn, 2018). In lettuce, NLRs predominantly reside in one of the five major resistance clusters (MRCs) located on chr 1, 2, 3, 4 and 8. Indeed, positioning of the markers flanking the loci conferring BLR38 recognition on the *L. sativa* cv. Salinas reference genome, indicated that the locus on chr 4 partially overlaps with MRC4. The locus on chr 8 is located downstream of MRC8A in a region that also contains NLRs suggesting that the responsible genes underlying the loci may be NLRs. There are two main types of NLRs: those with an N-terminal TIR domain (TNLs) and those with an N-terminal coiled-coil (CC) domain (CNLs). *L. sativa* cv. Salinas encodes 216 TNL (-type) genes and 167 CNL (-type) genes. Most NLRs belong to multigene *Resistance Gene Candidate* (*RGC*) families that tend to colocalize on the same chromosome. MRC4 in *L. sativa* cv. Salinas contains 18 TNL(-type) genes that all belong to *RGC12*, and three CNL(-type) genes of different families. MRC8A harbors four TNL(-type) genes (*RGC4*) and two CNL(-type) genes (*RGC27*) (Christopoulou *et al.*, 2015a,b).

So far, two scenarios have been reported in which two NLRs were required to mediate effector recognition. To begin with, multiple, genetically tightly linked NLR pairs were identified in Arabidopsis and rice. The gene pairs are positioned in head-to-head orientation in the genome with a shared bidirectional promoter to ensure transcriptional coregulation (Birker *et al.*, 2009; Narusaka *et al.*, 2009; Césari *et al.*, 2014; Sukarta *et al.*, 2016). Tight genetic linkage prevents aberrant activation of immune signaling in these NLR pairs that operate by a negative feedback mechanism, such as the rice resistance gene pair *RGA4/RGA5*. RGA4 constitutively activates immune signaling even in the absence of an effector and requires *RGA5* expression to suppress this aberrant immune activation. Suppression is relieved upon effector perception by RGA5 and apt immune signaling ensues (Césari *et al.*, 2014). We have no indications that BLR38 recognition is dependent on a negative feedback mechanism: *L. serriola* LS102 x *L. sativa* GreenTowers F3 families that lack the *L. serriola* LS102 locus on chr 4 or chr 8 do not show signs of auto-immune responses nor did we observe distorted segregation patterns in the F2 population.

Secondly, an immune signaling network was identified in multiple species belonging to the asterids clade (Wu *et al.*, 2017) to which lettuce also belongs. Similar to RGA4/RGA5, pairs formed within the network are comprised of one NLR acting as 'sensor' to identify the effector and the other NLR acting as 'helper' to activate immune signaling. Phylogenetic analyses showed that the 'helper' NLRs form a family that, together with the 'sensor' NLR clade, constitutes a superclade within the CNL phylogenetic tree (Wu *et al.*, 2017). In contrast to RGA4/RGA5, the network 'sensor' and 'helper' NLRs appear to operate by a positive feedback mechanism, are dispersed over the genome in tomato and form a genetically unlinked network (Wu *et al.*, 2017).

There are multiple parallels between the NLR network scenario and BLR38 recognition: 1) two gene products are required to mediate recognition 2) loss of one component abrogates recognition but does not induce auto-immune responses 3) the loci are genetically unlinked and located on different chromosomes. Unfortunately, we currently have no evidence that the loci conferring BLR38 recognition are part of a network.

The 'helper' NLRs within the *Nicotiana benthamiana* NLR network display a considerable amount of functional redundancy that contributes to robustness in the network (Wu *et al.*, 2016, 2017). In contrast, we have no indications that there are functionally

redundant loci in the QTL regions on chr 4 and chr 8 in the *L. serriola* LS102 and *L. sativa* cv. GreenTowers genomes as we have not identified a single plant that was BLR38 responsive in the absence of either the *L. serriola* LS102 locus on chr 4 or chr 8.

Considering all characteristics described above regarding the loci conferring BLR38 recognition, we propose three distinct models that are compatible with our observations.

1. *The loci encode NLRs but are not part of a larger network.*

The evolutionary advantage of using two unlinked loci that are both required and specific for a single effector is unclear, as recombination events would easily result in loss of one of the two loci.

2. *The loci encode NLRs and are part of a larger network.*

In this scenario, the 'sensor' NLR requires a specific 'helper' NLR as we did not observe functionally redundant loci. The 'helper' NLR may also be required for immune signaling in combination with other 'sensor' NLRs.

3. *One locus encodes an NLR and the other locus encodes a guardee or decoy*.

The Guard Model and the Decoy Model postulate that resistance proteins can also recognize effectors indirectly by monitoring effector-mediated alterations to host proteins; these are either guardees or decoys. Guardees, by definition, function in plant immunity and their interaction with a pathogen effector has a beneficial effect on pathogen fitness in the absence of the resistance protein but triggers an immune response in the presence of the guarding resistance protein. On the other hand, interaction of decoys with effectors does not benefit the pathogen since decoys have no function other than activating the corresponding resistance protein (Hoorn & Kamoun, 2008). If the Guard Model is applied, then BLR38 can only interact with the guardee in one (*L. serriola* LS102) or possibly a few lettuce lines and the guardee is absent or mutated in all other lettuce lines. Yet, BLR38 is conserved in all six *B. lactucae* races that we tested and allelic variants are still recognized in *L. serriola* LS102. So, either BLR38 has more targets or neither the locus on chr 4 nor on chr 8 in *L. serriola* LS102 encodes a guardee. If the Decoy Model is applied, then the target of BLR38 for which a decoy has evolved in *L. serriola* LS102, can be a conserved protein in lettuce. This fits better with a relatively conserved effector than the Guard Model.

Alternative sources of resistance genes

Introgression of resistance genes from wild species into cultivated lettuce (*L. sativa*) is the major method by which new resistant varieties are generated in breeding programs. *L. serriola*, which is relatively closely related to cultivated lettuce, is the most common source, but more distantly related species, e.g. *L. saligna*, are also used (Michelmore & Truco, 2017). *B. lactucae* resistance has also been observed in *L. aculeata* and *L. virosa* (Maisonneuve, 2003; Jemelková *et al.*, 2015), although sterility of F1 *L. sativa x L. virosa* hybrids complicates breeding efforts (Maisonneuve, 2003). Thus, even though *B. lactucae* evolves rapidly, a broad range of resistance sources is still available.

However, introgression of resistance genes by conventional breeding methods from wild species may not be an option in all crops due to reproductive barriers or lack of adequate resistance genes in the wild germplasm. Furthermore, rapid breakdown of newly deployed resistance genes is frequently observed. Also, genetic resources in many crops are slowly being depleted and alternative sources of *R* genes may be required for these species in the (near) future. The development of transgene technology has allowed the engineering of disease resistance in crops where conventional methods failed. Heterologous expression of NLRs from related species was effective in providing resistance against Asian soybean

rust (ASR) caused by the fungal pathogen *Phakopsora pachyrhizi* and bacterial spot disease in tomato caused by *Xanthomonas campestris* pv. *vesicatoria (Xcv)*. Commercial soybean cultivars are susceptible to ASR as deployment of individual soybean resistance genes has led to rapid breakdown of resistance and depleted the soybean *R* gene reservoir. Taking advantage of the broad host range of *P. pachyrhizi* on legumes (Bonde *et al.*, 2008; Slaminko *et al.*, 2008), resistant accessions of related legumes were explored as alternative source of resistance genes resulting in the introduction of a pigeonpea NLR in soybean (Kawashima *et al.*, 2016). Similarly, the *Bs2* gene that confers recognition of effector avrBs2 and induces resistance against bacterial spot disease in pepper, was introduced in tomato (Tai *et al.*, 1999). Multi-year field trials showed that *Bs2* conferred effective field resistance and increased product yield (Horvath *et al.*, 2012). These are just a few of the examples in which implementation of transgene technology resulted in enhanced disease resistance in crops [for more (Chen *et al.*, 2007; Dangl *et al.*, 2013)].

Currently, commercialization of genetically modified (GM) crops is hindered by two major hurdles: legal authorization and consumer acceptance. In the European Union approval for commercialization of GM crops takes between four to six years and costs up to 15 million euros (Hartung & Schiemann, 2014) and, after approval, individual EU member states can still prohibit cultivation of authorized GM crops (Mühlböck & Tosun, 2017). Understandingly, breeding companies may be reluctant to financially support the de-regulation process of GM crops when it is unsure if individual countries will allow GM crop cultivation and if the public will buy GM produce. A 2010 survey in 27 European countries found that only 22% of respondents thought GM food was safe for their health while 59% disagreed with this statement (Special Eurobarometer 341: Biotechnology, 2010; Gaskell *et al.*, 2011). These results illustrate the need to win over the public if GM crops are to become mainstream food in Europe.

Durable resistance breeding by combining qualitative and quantitative resistance genes

It is widely recognized that single newly-introduced resistance genes do not provide durable resistance but need to be part of a larger resistance management strategy to avoid boom-and-bust cycles. The boom phase is characterized by rapid adoption of cultivars harboring the new resistance gene by farmers. Consequently, strong selection pressure is exerted on the pest or pathogen. After the initial boom comes the bust as breakdown of resistance occurs and a new epidemic erupts.

Boom-and-bust cycles are generally associated with qualitative resistance genes. These tend to be monogenic, dominant NLRs that confer race-specific complete or near-complete resistance. In contrast, quantitative resistance is often associated with (multiple) genes of small effect, that confer partial, race-nonspecific resistance. Quantitative resistance loci (QRLs) can have additive effects when combined. A promising strategy to obtain durable resistance using host-dependent resistance (i.e. excluding chemical pathogen control methods) is to combine multiple qualitative and/ or quantitative resistances in one genome. This process is also referred to as gene 'pyramiding' or 'stacking' (Nelson *et al.*, 2018).

Pyramiding of qualitative resistance genes can be used to generate broad-spectrum disease resistance. Broad-spectrum resistance in potato differentials Ma*R8* and Ma*R9* was attributed to the presence of four and seven stacked resistance genes respectively (Kim *et al.*, 2012). Similarly, dissection of *Phytophthora infestans* disease resistance in potato cultivar Sarpo Mira revealed that resistance was mediated by four qualitative resistance genes and a quantitative field resistance gene (Rietman *et al.*, 2012).

Furthermore, the durability of qualitative resistance genes can be enhanced by combining them with quantitative resistance traits. This was elegantly demonstrated in a study on oilseed rape (*Brassica napus*) by Brun and coworkers. Resistance to the fungal pathogen *Leptosphaeria maculans,* mediated by qualitative resistance gene *Rlm6,* was broken by *L. maculans* after three years of recurrent selection. In contrast, the *L. maculans* population selected on oilseed rape harboring *Rlm6* in combination with multiple QRLs had not broken *Rlm6*-mediated resistance at the end of the five-year experiment (Brun *et al.*, 2010).

We observed that recognition of *B. lactucae* effector BLR38 in *L. serriola* LS102 x *L. sativa* cv. GreenTowers F3 families and *L. serriola* LS102 x *L. sativa* cv. CobhamGreen families conferred (near-complete) resistance to multiple *B. lactucae* races (**Chapter 2**). Though recognition of BLR38 does not confer resistance to Bl:24 and possibly other races, considering the plethora of known *B. lactucae* resistance genes and QRLs (Parra *et al.*, 2016), we would expect that stacking of the loci conferring BLR38 recognition with other qualitative and quantitative resistances could contribute to the development of lettuce cultivars with durable broad-spectrum resistance.

The yeast-two-hybrid system for the identification of effector targets

The genomes of downy mildews and *Phytophthora* spp. encode vast RXLR effector repertoires, yet the plethora of intracellular effector-host protein interactions that collectively result in suppression of immunity and establishment of symbiosis are only slowly being elucidated (Zheng *et al.*, 2014; Whisson *et al.*, 2016). In **Chapter 3**, protein-protein interactions between *B. lactucae* effectors and lettuce proteins were mapped using the yeast-two-hybrid (Y2H) system to build a foundation from which specific effector-target interactions could be explored (described in **Chapter 4**). The Y2H system was previously applied to construct a plant-pathogen interactome map using effectors from the fungal pathogen *Golovinomyces orontii*, bacterial pathogen *Pseudomonas syringae* and oomycete pathogen *Hyaloperonospora arabidopsidis* against an *Arabidopsis thaliana* library of ~8000 full-length proteins (Mukhtar *et al.*, 2011; Weßling *et al.*, 2014). The Y2H system is widely applied because it allows for high-throughput detection of protein-protein interactions *in vivo* and is independent of endogenous protein expression levels. Only direct interactions are identified which can be considered both an advantage and disadvantage, when proteins are expected to operate in complexes. Since both bait and prey are directed to the nucleus in yeast, interactions between spatially separated proteins under physiological conditions, may be identified that are biologically irrelevant (Mering *et al.*, 2002). Also, the identification of interactors for transcriptional activators is often complicated due to the auto-activating nature of these proteins in bait vectors.

Nevertheless, Y2H experiments have been successfully used to identify the targets of multiple *P. infestans* RXLR effectors. Effector AVR3a interacted with potato E3 ubiquitin ligase CMPG1 in Y2H assays and stabilized CMPG1 resulting in the suppression of infestin 1-triggered cell death (Bos *et al.*, 2010). Effector PexRD2 was shown to interact with MAPKKKε leading to suppression of MAPKKKε-dependent cell death signaling pathways (King *et al.*, 2014). Whereas effectors AVR3a and PexRD2 targeted host proteins that contribute to host immune responses, effectors Pi02860, Pi04314 and Pi04089 interacted with susceptibility factors (Wang *et al.*, 2015; Boevink *et al.*, 2016b; Yang *et al.*, 2016). Genes that facilitate and support pathogen infection can be considered susceptibility (*S*) factors (Lapin & Van den Ackerveken, 2013; Schie & Takken, 2014). For example, Pi04314 is proposed to form a complex with potato phosphatase type 1c (PP1c) possibly to redirect PP1c phosphatase

activity to the benefit of the pathogen (Boevink *et al.*, 2016b). Consequently, silencing of susceptibility factors may attenuate pathogen infection or their overexpression may enhance infection (Boevink *et al.*, 2016a).

Our library screens identified 46 unique lettuce targets that interacted with 21 (46% of tested) *B. lactucae* effectors (**Chapter 3**). This interactome map is likely far from complete. This is in part due to inherent limitations of the Y2H system. For example, only direct interactions between two proteins that do not require other proteins for complex formation can be detected. Also, the GAL4 domains fused to the N-terminus of bait and prey proteins may sterically block interaction sites. Finally, misfolding of proteins may prevent interaction.

Apart from inherent limitations, differences in experimental set-up and selection strategy can have profound effects. This was demonstrated by two research groups that had independently screened ~6000 full-length *Saccharomyces cerevisiae* proteins in all possible combinations in Y2H screens resulting in 691 and 841 interactions. Surprisingly, only 141 interactions overlapped between the two datasets (Uetz *et al.*, 2000; Ito *et al.*, 2001). An important difference in experimental set-up was the use of low-copy Y2H vectors with a single reporter gene by one group versus multicopy Y2H vectors with three reporter genes by the other group. The use of low-copy vectors such as vectors pDEST22/ pDEST32 in our study, was shown to reduce the number of false-positive interactions compared to high-copy vectors but also negatively affects the fraction of true-positives recovered (Braun *et al.*, 2009). Bait-prey pairs that activate multiple reporter genes with unique promoter regions generally constitute more reliable interactors than interaction pairs that activate a single reporter as the latter is more likely to occur by chance. We assessed the activation of reporter genes *HIS3* and *ADE2* and found that 34 bait-prey pairs (56% of the total) activated both reporters. Interestingly, nine of the top ten effector targets ranked by total number of yeast colonies identified, activated both reporter genes.

Furthermore, prey library construction and screening depth affect the number of recovered protein-protein interactions. Gene fragment libraries typically identify more interactions than full-length coding sequence libraries (Boxem *et al.*, 2008; Koorman *et al.*, 2016). Our prey cDNA library contains both full-length coding sequences and gene fragments. Amplified prey sequences from positive Y2H clones were compared with *L. sativa* cv. Salinas predicted gene models to determine the region of interaction. For 20 of the 46 Y2H identified lettuce genes, at least one amplified prey insert contained the start codon of the predicted lettuce gene model. The coding sequences of these 20 lettuce genes were smaller than 1.62 kb (Figure 1). The group of 26 lettuce genes for which only gene fragments were identified included 12 genes that ranged in coding sequence size from 1.65 kb to 2.9 kb (Figure 1). On the one hand, this illustrates the value of gene fragments in prey libraries as some proteins will not interact as full-length sequences. On the other hand, this likely also reflects technical limitations as we did not identify prey inserts >2-3kb in yeast. Before transformation of yeast with the prey library, prey inserts were amplified from 96 random *E. coli* colonies to determine the average insert size, which was estimated at 1.1kb. The largest prey inserts were ~2kb indicating that larger prey inserts may be poorly represented in the library. Finally, our cDNA library was not normalized implying that low transcript levels in the harvested lettuce material likely resulted in underrepresentation in the library. It is therefore difficult to reach saturation in screening. As an example, we found additional interactions between PRA1 proteins and *B. lactucae* effectors when assessed by direct cotransformation that had not been detected in the library screens (**Chapter 3**).

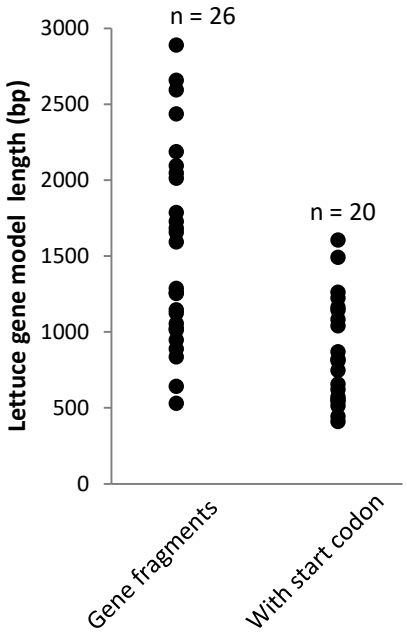


Figure 1, Distribution of coding sequence length for lettuce genes identified in yeast-two-hybrid screens. Each dot represents an effector target and the length of the corresponding coding sequence according to the gene models predicted for *L. sativa* cv. Salinas is plotted. Yeast amplified prey inserts of 20 effector targets contained the lettuce gene start codon and prey inserts of 26 lettuce genes did not.

Future perspectives for Y2H-identified effector targets

To assess the biological relevance of the effector targets identified in the Y2H screens, hairpin(hp)RNA constructs of four effector targets were transformed in *L. sativa* cv. CobhamGreen and Wendell. Although transcript levels of three out of four targets decreased by ≥ 80% in successful transformants, subsequent disease assays did not reveal consistent alterations in *B. lactucae* susceptibility. Assuming the identified lettuce proteins represent true effector targets, there may be several explanations for the lack of altered susceptibility phenotypes. First, the Wendell and CobhamGreen parental lines sporulated profusely upon infection with *B. lactucae* race Bl:24 at moderate inoculation densities (40-60 spores/µl), which may have obscured enhanced susceptibility phenotypes. Another possibility is that silencing of the host gene mimicked the result of effector-target interactions during infection of wildtype plants. For example, in **Chapter 4**, we demonstrate that a NAC transcription factor relocalizes to the nucleus in *P. capsici* culture filtrate-treated *N. benthamiana* cells and relocalization is strongly reduced in the presence of three *B. lactucae* effectors. Thus, silencing of the NAC transcription factor in lettuce and effector delivery by *B. lactucae* in plant cells would have comparable outcomes: no active NAC transcription factor in the nucleus. In this case, overexpression of the effector target may be required to demonstrate its relevance. Thirdly, effector targets that are members of larger gene families, may be functionally

redundant and silencing of multiple genes may be required for disease susceptibility to be affected. BLR26 interacted with two membrane steroid-binding proteins that belong to a larger gene family of steroid-binding domain-containing proteins but shared insufficient sequence identity to be silenced by the same construct. Finally, individual effectors and their targets may not have large effects. For example, the *B. lactucae* effectors BLG01 and BLG03 were shown to be recognized in specific lettuce lines but did not affect susceptibility to race Bl:24 infection when transiently overexpressed in leaf discs (Stassen, 2012).

Even though silencing of selected effector targets did not result in altered susceptibility phenotypes, these and other Y2H identified interactions are worth exploring further. Here, I focus on BLR38 target Lsa007018.1 to illustrate the research possibilities. Interestingly, co-expression of effector and target fluorophore fusions in *N. benthamiana* revealed four interaction pairs in which the effector or target induced relocalization of the interaction partner to the nucleus (**Chapter 3**). Effector BLR38 induced a relocalization of target Lsa007018.1 from the cytoplasm to the nucleus. Lsa007018.1 is homologous to Arabidopsis protein FLX-like 2 (FLOWERING LOCUS C EXPRESSOR-like 2/ FLL2). *FLX-like 2* is a member of a gene family comprised of five genes in Arabidopsis. Alignment of Lsa007018.1 with the Arabidopsis FLX(-like) gene family reveals multiple conserved leucine residues (Figure 2). Lsa007018.1 also contains a leucine repeat featuring leucine residues separated by six other amino acids (Figure 2), which is typically associated with leucine zippers that mediate protein-protein interactions. The Lsa007018.1 sequence was analyzed for the presence of a leucine zipper using the 2ZIP software (Bornberg-Bauer *et al.*, 1998), yet, Lsa007018.1 is not predicted to form a leucine zipper due to the absence of a coiled-coil. Mutation of three conserved leucine residues in Arabidopsis FLX-like 4 abrogated interaction with FRIGIDA, a flowering time determinant (Ding *et al.*, 2013). FLX and FLX-like 4 were both shown to be involved in flowering time regulation (Choi *et al.*, 2011; Ding *et al.*, 2013; Lee & Amasino, 2013). The function of the other three family members is unknown. The identification of Lsa007018.1 in a screen for *B. lactucae* effectors may hint towards a role of FLX-like family members in the integration of environmental signals in flowering time regulation. Bacterial, fungal and downy mildew infection induce early flowering of Arabidopsis plants (Korves & Bergelson, 2003; Lyons *et al.*, 2015) although the molecular mechanism by which biotic stress is integrated in flowering time regulation remains poorly understood. Thus, it would be interesting to explore if *B. lactucae* infection affects regulation of flowering time in lettuce and if Lsa007018.1 is involved in this or other processes.

A thorough understanding of the biological function of Y2H-identified effector targets in healthy and infected lettuce plants can help determine their value in controlling disease caused by *B. lactucae*. In various pathosystems, *S* genes have been described and proposed to provide a more durable form of resistance. Yet, applicability is in many cases hindered due to pleiotropic effects, e.g. developmental defects, and trade-offs in resistance to other pathogens. Controlled mutation of *S* genes using CRISPR/Cas9 to prevent effector interaction while maintaining the biological function of the *S* gene could provide opportunities for deployment of *S* genes (Gawehns *et al.*, 2013; Dangl *et al.*, 2013).

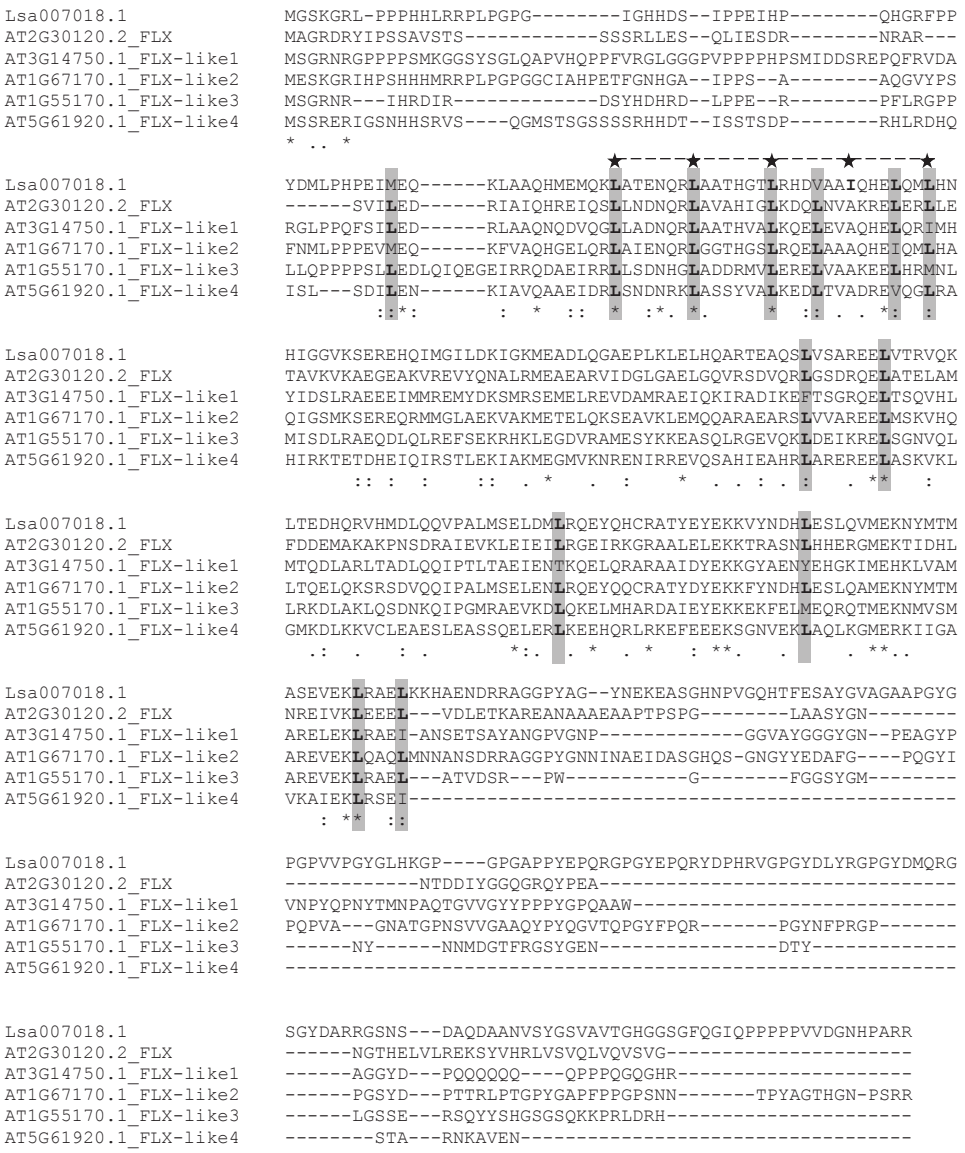


Figure 2, Alignment of the lettuce *Lsa007018.1*-encoded protein with members of the Arabidopsis Flowering Locus C Expressor (FLX) protein family. Leucine residues that are conserved in at least four of the six proteins are indicated in grey when there is also a leucine or similar (methionine (M), isoleucine (I) or valine (V)) residue present in Lsa007018.1. A potential leucine repeat comprised of leucine residues separated by six other amino acids, is indicated with stars over the repeat residues.

Secretion of transmembrane domain-containing effectors

In sharp contrast to the majority of effectors that interacted specifically with their targets in the Y2H system, we identified five effectors - BLN03, BLN04, BLR05, BLR08 and BLR09 - that converged on three lettuce proteins. In **Chapter 4**, we demonstrate that one of these effector targets, LsNAC069, is a membrane-associated NAC transcription factor that resides at the endoplasmic reticulum. LsNAC069 interacting effectors BLR05, BLR08 and BLR09 also localized to the secretory pathway and both BLR05 and BLR09 colocalized with LsNAC069 at the ER in *N. benthamiana*. Furthermore, *P. capsici* culture filtrate- induced accumulation of LsNAC069 in the nucleus was reduced upon co-expression of BLR05, BLR08 and BLR09. Similar results were reported for potato NAC transcription factors, StNTP1 and StNTP2, that localized and interacted at the ER with *P. infestans* effector Pi03912 (McLellan *et al.*, 2013). Strikingly, *B. lactucae* effectors BLN03, BLN04, BLR05, BLR08 and BLR09 as well as *P. infestans* effector Pi03912 contain a C-terminal transmembrane domain. Here I explore the implications of a C-terminal transmembrane domain in effectors with regard to *in planta* expression using *Agrobacterium* and expression in oomycetes during infection.

Tail-anchored proteins are proteins with a C-terminal transmembrane domain that do not contain a signal peptide. A signal peptide is required for cotranslational ER targeting of transmembrane proteins by the signal recognition particle (Kim & Hwang, 2013). Instead membrane integration of tail-anchored proteins occurs posttranslationally and is dependent on the C-terminal transmembrane domain that functions as a targeting signal. Some proteins with a moderately hydrophobic transmembrane domain insert unassisted in the ER, others with a highly hydrophobic transmembrane domain require assistance. In yeast, the transmembrane domain of tail-anchored proteins is recognized by components of the

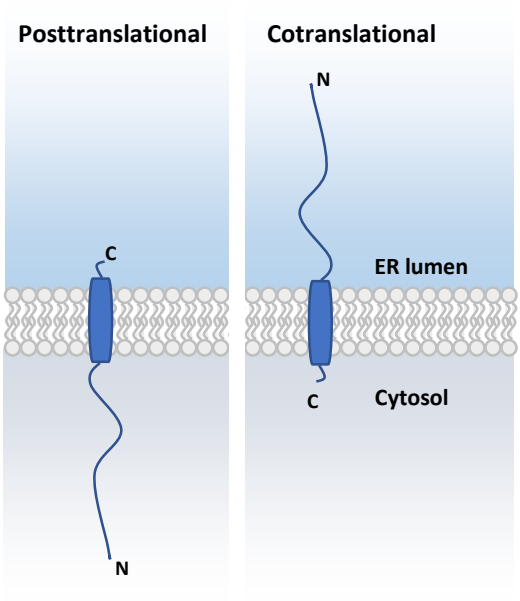


Figure 3, Effector topology resulting from cotranslational (protein with a signal peptide) and posttranslational (protein without a signal peptide) insertion into the ER membrane.

GET system including Get3 (TRC40 in mammals), delivered to the ER-resident Get1/Get2 receptor complex and inserted into the membrane (Schuldiner *et al.*, 2008; Shao & Hegde, 2011). LsNAC069 and multiple other membrane-associated transcription factors are plant-encoded tail-anchored proteins. Yet, for *in planta* expression using *Agrobacterium,* the *B. lactucae* effectors were cloned without their signal peptide, thereby creating artificial tail-anchored proteins whose observed ER targeting could not have occurred cotranslationally. This strongly suggests that *in planta* effectors without signal peptides can behave as tail-anchored proteins.

In tail-anchored proteins the long N-terminus (70-143 amino acids for *B. lactucae* effectors BLN03, BLN04, BLR05, BLR08 and BLR09) resides in the cytosol whereas the short C-terminus (2-10 amino acids for the *B. lactucae* effectors) after the transmembrane domain is committed to the ER lumen. However, during plant infection *B. lactucae* expresses BLR05, BLR08 and BLR09 with an N-terminal signal peptide. The signal peptide would direct the effectors to the pathogen ER and cotranslational membrane insertion would ensue. The signal peptide directs translocation of the N-terminus through the Sec61 channel before the transmembrane domain has been translated (Shao & Hegde, 2011). This favors a reversed topology for the effectors in *B. lactucae*, in which the N-terminal domain is lumenal and the C-terminus cytosolic, compared to posttranslational effector insertion *in planta* (Figure 3).

Lumenal N-terminal domains are accessible to ER resident enzymes for co- and posttranslational modifications. In a recent study, cleavage of *P. infestans* effector AVR3a directly after the RXLR motif was observed (Wawra *et al.*, 2017). The oomycete RXLR motif resembles the *Plasmodium* export element (PEXEL) motif (RxLxE/Q/D) found in effectors of *Plasmodium falciparum*. *Plasmodium* effectors containing the PEXEL motif are cleaved by the aspartyl protease Plasmepsin V after the RxL in the PEXEL motif. Effector processing by Plasmepsin V occurs cotranslationally in the ER (Sleebs *et al.*, 2014). It is unclear if cleavage of AVR3a was also mediated by an aspartyl protease since none of the cloned *P. infestans* aspartyl proteases were able to cleave AVR3a *in vitro* (Wawra *et al.*, 2017).

In *Plasmodium*, the PEXEL motif guides the export of effectors via secretory vesicles (Koning-Ward *et al.*, 2016). It is conceivable that the oomycete RXLR motif plays a similar role although the exact mechanism likely differs. *Plasmodium* effectors bud off from the ER in COPII vesicles and pass through the Golgi (Coffey *et al.*, 2016). Secretion of the apoplastic *P. infestans* effector EPIC1 is also dependent on Golgi-mediated secretion as it was sensitive to Brefeldin A treatment (Wang *et al.*, 2017). Brefeldin A induces fusion of Golgi membranes with the ER and a collapse of the Golgi complex, thereby effectively prohibiting conventional ER-to-Golgi transport (Ritzenthaler *et al.*, 2002; Nebenführ *et al.*, 2002). In contrast, secretion of the host-translocated *P. infestans* effector Pi04314 was Brefeldin A insensitive indicating that secretion occurred via an unconventional, COPII-independent pathway (Boevink, 2017; Wang *et al.*, 2017). Mutation of the RXLR motif to alanine residues in effector AVR3a abrogated translocation into plant cells but did not prevent secretion from haustoria (Whisson *et al.*, 2007). These data fit with a model where the RXLR motif functions in directing effectors to the unconventional pathway (Van den Ackerveken, 2017).

Interestingly, *Plasmodium* also secretes PEXEL-negative proteins (PNEPs) that contain a transmembrane domain. PNEPs are inserted into the ER membrane and appear to follow a similar secretory pathway to the plasma membrane as PEXEL effectors (Coffey *et al.*, 2016; Koning-Ward *et al.*, 2016). So far, secretion and host-translocation of downy mildew RXLR effectors with a transmembrane domain has not been demonstrated. However, one might speculate that, in an extension of the analogy between RXLR and PEXEL effectors, host

translocated RXLR effectors with or without a transmembrane domain could both follow an unconventional secretory pathway. Indeed, signal peptide- and transmembrane domain-containing proteins can reach the plasma membrane via an unconventional pathway that bypasses the Golgi in multiple eukaryotic organisms (Rabouille, 2017) although this pathway has not been described for oomycetes yet.

Similar to *P. infestans* effectors, host-translocated and apoplastic effectors of the rice blast fungus *Magnaporthe oryzae* use unconventional and Golgi-dependent secretory pathways respectively. The secretion of host-translocated effectors was found to be dependent on Exo70 and Sec5, two components of the exocyst complex that mediates vesicle fusion (Giraldo *et al.*, 2013). The Exo70 subunit has multiple paralogs in Arabidopsis and of particular interest is Arabidopsis protein Exo70E2 that locates to exocyst-positive organelles (EXPO). Secretion of EXPO organelles is Brefeldin A insensitive and, due to their double membrane structure, results in release of single membrane structures outside the plant cell (Wang *et al.*, 2010). Membrane-bound vesicles were also observed in the extrahaustorial matrix during infection of Arabidopsis by the powdery mildew pathogen *G. orontii* although it could not be determined if these originated from the plant or pathogen (Micali *et al.*, 2011). Vesicular transport of soluble and membrane-associated effectors could provide a mechanism for delivery of effectors to the extrahaustorial membrane. Upon fusion, soluble effectors would be released into the host cytoplasm whereas membrane-associated effectors would be inserted in the extrahaustorial membrane. Note that due to the cotranslational membrane insertion of effectors with a C-terminal transmembrane domain in the pathogen ER, it would be expected that the N-terminus is lumenal in both the pathogen ER and subsequent intra- and/or extracellular vesicles until vesicle fusion with the extrahaustorial membrane. The effector N-terminus would then be exposed to the host cytoplasm.

Endocytosis is used in plant cells to target plasma membrane localized proteins for degradation or recycling e.g. of cargo receptors (Reyes *et al.*, 2011). The composition of the extrahaustorial membrane is likely also regulated by endocytosis and could provide a mechanism for extrahaustorial membrane localized effectors to reach different subcellular compartments.

The sequence of events described above could provide a model for the secretion of effectors (Figure 4) like BLR05 and Pi03192, but remains for the near future, pure conjecture. Just over a decade ago, the RXLR motif in effectors was proposed as host translocation signal (Whisson *et al.*, 2007), since then a plethora of theories has emerged describing RXLR-dependent effector translocation (Petre & Kamoun, 2014). However, a new wind is already blowing claiming a role for the RXLR motif in secretion (Wawra *et al.*, 2017). Thus, it will be fascinating to see how our current thoughts and understandings are rocked in the years to come.

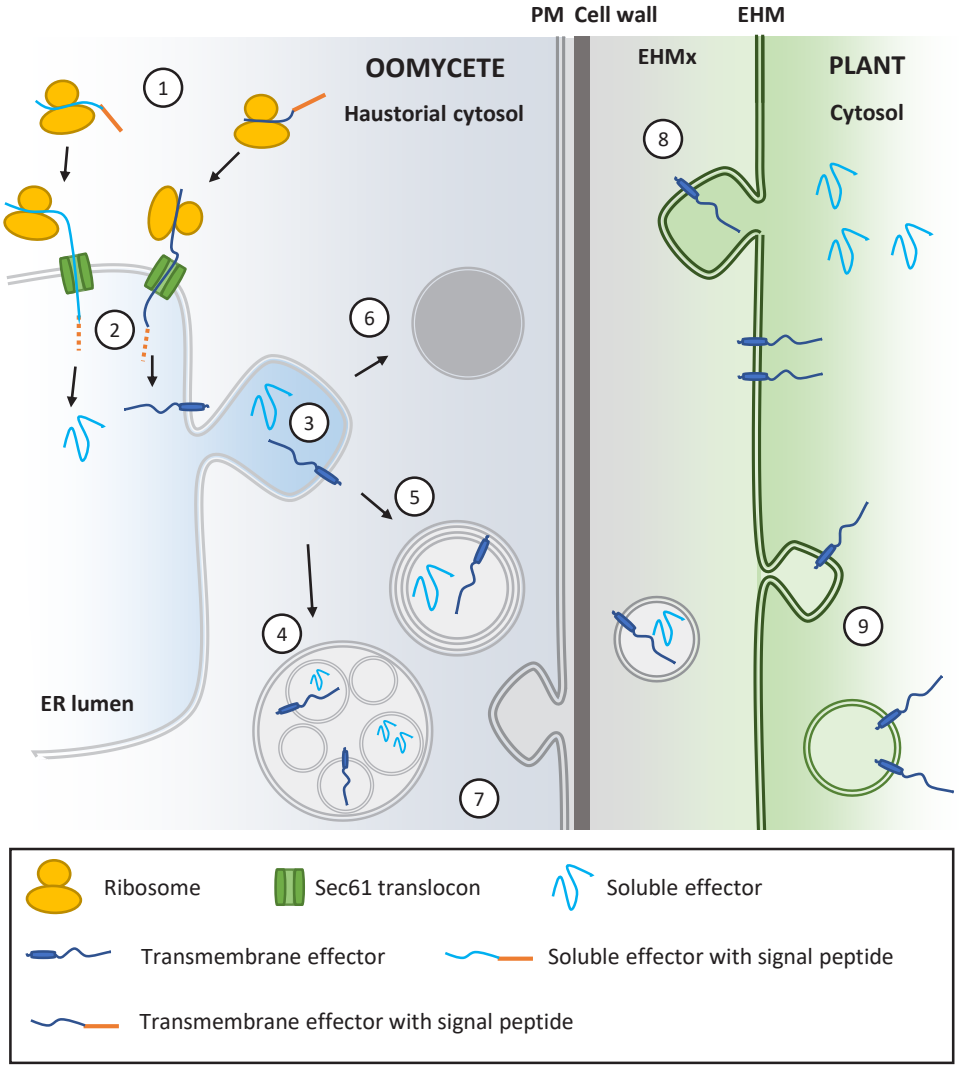


Figure 4, Hypothetical model for secretion and translocation of RXLR effectors at the haustorial interface. (1) During translation, both soluble and membrane proteins are recognized via their signal peptide by the signal recognition particle. The ribosome is trafficked to the ER (2) where the signal peptide directs translocation of soluble proteins through the Sec61 translocon into the ER lumen and directs cotranslational membrane insertion of proteins with a transmembrane domain. The signal peptide is cleaved off in the process. The RXLR-motif of effectors may be recognized during or after translocation and (3) is required for sorting of hosttranslocated proteins to a Golgi-independent secretory pathway. The carriers loaded with soluble and membrane-associated effectors may be similar to (4) multivesicular bodies, (5) EXPO structures or (6) present an unknown type of vesicle. (7) Upon fusion of the carriers with the oomycete plasma membrane (PM), the internal vesicle(s) are released and pass the oomycete cell wall through an unknown mechanism before reaching the extrahaustorial matrix (EHMx). (8) Fusion of effector-loaded vesicles with the plant-derived extrahaustorial membrane (EHM) releases soluble cargo into the plant cytosol and inserts membrane-associated proteins into the EHM. (9) Membrane-associated effectors may follow an endocytic pathway to reach their final destination.

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Appendix

Summary

In modern agriculture, cultivated lettuce (*Lactuca sativa*) is typically grown in large monocultures with multiple crops per year. This makes lettuce, like many intensively produced crops, susceptible to disease outbreaks. The plant pathogenic oomycete *Bremia lactucae* that causes downy mildew disease on lettuce, is considered the most important disease affecting the production of cultivated lettuce worldwide. Lettuce is susceptible to infection at all developmental stages. Infected leaves may develop yellowish lesions and white sporangiophores on the lower leaf surface. Severely infected leaves turn brown, dry out or rot due to secondary infections. The application of pesticides helps to control *B. lactucae* but is associated with high costs and the development of pesticide-resistant species. Furthermore, European regulations concerning pesticide use have become stricter in recent years to reduce the negative impact of pesticides on the environment. Consequently, the introgression of dominant resistance *(R)* genes that provide resistance to specific *B. lactucae* races, is a major aspect of breeding programs. Deployment of *R* genes exerts strong selection pressure on the pathogen resulting in the rapid breakdown of resistance. Achieving durable resistance against *B. lactucae* is therefore still a major challenge. *R* genes frequently encode specialized intracellular receptors with a nucleotide-binding domain and leucine-rich repeats, called NLRs. Activation of NLRs is dependent on the recognition of classes of proteins secreted by pathogens, termed effectors. Effectors of plant pathogenic bacteria, fungi and oomycetes manipulate host cell processes using a wide variety of mechanisms to facilitate the infection process. An overview of the strategies utilized by effectors is described in **Chapter 1**.

Following the recognition of effectors or effector-modified host proteins, an effector-triggered immune response ensues, which is frequently associated with a hypersensitive response resulting in localized cell death (necrosis). In **Chapter 2**, we exploited this phenomenon to discover novel *R* genes. Fourteen *B. lactucae* effectors were transiently expressed in >150 lettuce lines and accessions. Expression of three effectors - BLN06, BLR38 and BLR40 – resulted in yellowing (chlorosis) or necrosis of the infiltrated leaf sections in specific lettuce lines indicating the presence of lettuce host proteins that recognize these effectors. We focused on BLR38 that is recognized in the prickly lettuce (*Lactuca serriola*) accession LS102. Plants that recognized BLR38 were resistant to multiple *B. lactucae* races but not against race Bl:24 from which *BLR38* was originally cloned. Possibly, race Bl:24 contains one or more effectors that are absent in other *B. lactucae* races and effectively interfere with BLR38-triggered immune responses in lettuce. Interestingly, recognition of BLR38 required two loci on distinct chromosomes and was gene dosage dependent. Resistances to downy mildew in lettuce, identified so far, are mostly monogenic. Interestingly, in multiple plant species it was recently found that effector recognition or disease resistance can also be mediated by two genetically closely linked genes or, similar to BLR38 recognition, by two unlinked genes.

Deployment of *R* genes exerts strong selection pressure on the pathogen resulting in the rapid breakdown of resistance. Theoretically, susceptibility (*S*) genes in the host could provide a form of durable resistance that *R* genes fail to confer. The term *S* gene is used to describe host genes that facilitate infection, for example, those encoding negative regulators of immunity. The pathogen thus requires these *S* genes for successful infection, and loss or mutation of *S* genes would be difficult to compensate for, whereas loss or mutation of effectors recognized by R proteins is sufficient to subvert R protein mediated immune

responses. An improved understanding of effector targets in lettuce can help to elucidate the underlying molecular mechanisms leading to disease susceptibility and, potentially, lead to the identification of *S* genes. To identify effector targets in lettuce, we performed an effector- lettuce protein interaction study using the yeast-two-hybrid (Y2H) system (**Chapter 3**). Both novel and previously described *B. lactucae* effectors were assayed and 61 interactions were identified between 21 effectors and 46 lettuce proteins. To determine if the Y2H-identified interaction partners were likely to interact *in planta,* the subcellular localization of selected effectors and their plant targets was investigated. Effectors and lettuce proteins tagged with distinct fluorescent proteins were transiently expressed in tobacco plants (*Nicotiana benthamiana*) and visualized using confocal microscopy. Indeed, in nine out of twelve tested combinations the effector and plant target (partly) localized to the same subcellular compartment. Furthermore, we observed in four cases that the effector or the plant target relocalized to the nucleus upon co-expression of their Y2H-identified interaction partner that already resided in the nucleus. These findings strongly suggest that our Y2H screens resulted in the identification of multiple relevant effector targets. Finally, we studied whether the individual silencing of three effector target genes would alter susceptibility to *B. lactucae* infection. Although transcript levels of two out of three targets were reduced by >90%, these plants were not affected in their susceptibility to *B. lactucae* infection.

The Y2H screens also uncovered a small protein-protein interaction network comprised of five *B. lactucae* effectors and three lettuce proteins. In **Chapter 4**, we explored this network further with a focus on the lettuce membrane-associated NAC transcription factor LsNAC069. Membrane-associated transcription factors allow plants to respond rapidly to sudden changes in the environment. Following perception of environmental cues, the membrane-associated transcription factors are activated by controlled proteolytic cleavage. Upon release from the membrane, the activated transcription factors relocalize to the nucleus to regulate transcription. We performed transient expression assays of LsNAC069 and its interacting *B. lactucae* effectors tagged with distinct fluorescent proteins to visualize their intracellular localization pattern in lettuce and *N. benthamiana* plants. Due to poor stability of full-length LsNAC069 *in planta*, a truncated form of LsNAC069 that lacks the NAC domain, LsNAC069$^{\Delta NAC}$, was mainly used. Using the Y2H system, we confirmed that both LsNAC069 and LsNAC069$^{\Delta NAC}$ interact with the network *B. lactucae* effectors: interaction of the effectors with LsNAC069 was dependent on the presence of the C-terminal region, including the transmembrane domain in LsNAC069, but did not require the N-terminal NAC domain. Furthermore, we confirmed that in their dormant state, LsNAC069 and LsNAC069$^{\Delta NAC}$ localized to the membrane of the endoplasmic reticulum in *N. benthamiana*. LsNAC069$^{\Delta NAC}$ also colocalized with *B. lactucae* effectors BLR05 and BLR09 at the endoplasmic reticulum membrane. Relocalization of LsNAC069$^{\Delta NAC}$ to the nucleus could be triggered by incubating *N. benthamiana* leaf sections with culture filtrate of the plant-pathogenic oomycete *Phytophthora capsici*. Culture filtrate contains pathogen-associated molecular patterns that are recognized by pattern-recognition receptors on the plant membrane and activate plant immune responses. Relocalization of LsNAC069$^{\Delta NAC}$ was strongly reduced in the presence of the serine protease inhibitor TPCK (N-p-Tosyl-L-phenylalanine chloromethyl ketone) indicating that proteolytic cleavage is required for membrane release of LsNAC069$^{\Delta NAC}$. Furthermore, relocalization of LsNAC069$^{\Delta NAC}$ was reduced upon co-expression of *B. lactucae* effectors BLR05, BLR08 and BLR09. To evaluate the role of LsNAC069 in disease susceptibility, stably silenced lettuce lines were generated. These did not display altered susceptibility to

B. lactucae infection, possibly due to genetic redundancy. Thus, the role of LsNAC069 and its interacting *B. lactucae* effectors during infection of lettuce remains elusive.

In **Chapter 5**, the main findings described in this thesis are summarized and discussed in a broader perspective. Furthermore, the challenges that lie ahead to obtain durable resistance in lettuce are outlined and propositions for further research are done that may allow application of our findings in the future.

Samenvatting

In de moderne landbouw wordt sla (*Lactuca sativa*) verbouwd in monoculturen en wordt vaak meerdere keren per jaar dezelfde slasoort geplant op hetzelfde stuk land. Dit maakt dat sla, net zoals andere intensief verbouwde gewassen, gevoelig is voor ziekte epidemieën. De belangrijkste ziekteverwekker die de productie van sla bedreigt, is de oömyceet *Bremia lactucae*, de veroorzaker van valse meeldauw op sla. De ziekte heeft een verwoestend karakter en verspreidt zich in hoog tempo over de velden, waardoor hele oogsten verloren kunnen gaan. Het bestrijden van *B. lactucae* met behulp van pesticiden wordt door de Europese Unie steeds verder aan banden gelegd om de impact die pesticiden op het milieu hebben, te verminderen. Boeren zijn daardoor sterk afhankelijk van de beschikbaarheid van slarassen die resistent zijn. Veredelaars zijn daarom continu op zoek naar nieuwe resistentiebronnen die gebruikt kunnen worden in veredelingsprogramma's. Het inkruisen van dominante resistentiegenen (*R*-genen) levert een sterke bescherming op tegen specifieke fysiologische varianten (fysio's) van *B. lactucae*. Het toepassen van resistentiegenen heeft echter als nadeel dat deze sterke selectiedruk uitoefenen op de ziekteverwekker, waardoor de evolutie van resistentie-doorbrekende ziekteverwekkers vaak niet lang op zich laat wachten. Hierdoor blijft het een enorme uitdaging om slarassen met duurzame resistentie te ontwikkelen. Resistentie-eiwitten herkennen bepaalde, door ziekteverwekkers uitgescheiden, eiwitten; effectoren genoemd. Ziekteverwekkers produceren effectoren om infectie van de plant mogelijk te maken en succesvolle infecties in stand te houden. Effectoren grijpen daarvoor aan op verschillende processen in plantencellen; de werkwijze van verschillende effectoren geproduceerd door bacteriën, schimmels en oömyceten is beschreven in **Hoofdstuk 1**.

De directe of indirecte herkenning van effectoren door resistentie-eiwitten leidt tot een immuunreactie van de plant en deze gaat vaak gepaard met een overgevoeligheidsreactie waarbij lokaal de plantencellen afsterven (necrose). In **Hoofdstuk 2** maken we gebruik van dit zichtbare fenomeen om nieuwe resistentiegenen te ontdekken. Veertien effectoren van *B. lactucae* werden tot expressie gebracht in ruim 150 slalijnen. De expressie van drie effectoren - BLN06, BLR38 en BLR40 – resulteerde in een geelverkleuring (chlorose) of necrose van de geïnfiltreerde stukjes blad bij specifieke slalijnen. Dit geeft aan dat deze slalijnen eiwitten bevatten die de effectoren herkennen. In **Hoofdstuk 2** is de herkenning van effector BLR38 in de wilde sla (*Lactuca serriola*) soort LS102 beschreven. Slaplanten die effector BLR38 herkenden, waren sterk verminderd vatbaar voor verschillende *B. lactucae* fysio's, maar waren nog steeds vatbaar voor *B. lactucae* fysio Bl:24 waaruit het *BLR38* gen gekloneerd was. Een mogelijke verklaring hiervoor is dat Bl:24, één of meerdere effectoren bevat die de immuunreactie van de gastheer na herkenning van BLR38, onderdrukken. Bij de herkenning van effector BLR38 zijn twee onafhankelijke gebieden met genen (genetisch niet-gekoppelde gebieden) betrokken die partiële dominantie vertonen. Dat laatste betekent dat planten die twee kopieën van de betrokken gebieden uit *L. serriola* LS102 bevatten, sterker reageren dan planten met slechts één kopie. Deze resultaten waren verassend omdat in sla resistentie meestal gekoppeld is aan een enkel gen (monogeen). Daarentegen, in meerdere planten is recentelijk aangetoond dat bij effector herkenning of ziekteresistentie twee genetisch gekoppelde of genetisch niet-gekoppelde genen, vergelijkbaar met BLR38 herkenning, betrokken kunnen zijn.

Naast het gebruik van resistentiegenen, kunnen in de toekomst mogelijk vatbaarheidsgenen (Engelse term: susceptibility (*S*) genes) toegepast worden om de duurzame resistentie te creëren die resistentiegenen tot nu toe niet hebben geboden. De term vatbaarheidsgen wordt gebruikt voor plantengenen die het infectieproces bevorderen, zoals genen die coderen voor negatieve regulatoren van het immuunsysteem van de plant. De ziekteverwekker heeft de vatbaarheidsgenen van de plant nodig om te infecteren. Verlies of mutatie van deze genen in de plant geeft resistentie die lastig te compenseren is door de ziekteverwekker. Een eerste stap naar de identificatie van vatbaarheidsgenen is het bepalen van de eiwitten die gemanipuleerd worden door effectoren, zodat er een beter begrip van de moleculaire mechanismen die leiden tot vatbaarheid ontstaat. Om de doelwitten van *B. lactucae* effectoren in sla te identificeren, hebben we een interactiestudie uitgevoerd tussen *B. lactucae* effectoren en sla eiwitten met behulp van het 'yeast-two-hybrid system'. Door gebruik te maken van dit gistsysteem konden we op een efficiënte manier eiwit-eiwit interacties in kaart brengen (**Hoofdstuk 3**). In totaal hebben we 61 interacties geïdentificeerd, waarbij 21 *B. lactucae* effectoren en 46 sla eiwitten betrokken zijn. Om te bepalen of het aannemelijk is dat deze interacties zich ook in plantencellen afspelen, is bekeken waar effectoren en hun doelwitten zich bevinden in de plantencel. Fluorescente eiwitten werden gekoppeld aan zowel effectoren als hun doelwitten en deze werden tot expressie gebracht in tabaksplanten (*Nicotiana benthamiana*). Daarna werd met behulp van confocale microscopie op het niveau van individuele plantencellen naar de lokalisatie van de fusie eiwitten gekeken. In negen van de twaalf onderzochte combinaties bevonden de effectoren zich geheel of gedeeltelijk in hetzelfde compartiment als de doelwitten. Daarnaast zagen we in vier gevallen dat ofwel de effector dan wel het doelwit zich naar de celkern verplaatste, zodra hun interactiepartner mede tot expressie werd gebracht. Deze bevindingen laten zien dat het gistsysteem meerdere relevante interacties heeft voortgebracht. Tot slot hebben we onderzocht of het onderdrukken van de transcriptie van drie doelwitgenen in sla gevolgen had voor de vatbaarheid van sla. Alhoewel de mRNA niveaus van twee van de drie doelwitten met >90% verminderd waren, had dit geen gevolgen voor de vatbaarheid van sla voor *B. lactucae* infectie.

De gistexperimenten onthulden ook een klein interactienetwerk, bestaande uit vijf *B. lactucae* effectoren en drie sla eiwitten. In **Hoofdstuk 4** is dit netwerk uitgebreider onderzocht, waarbij de nadruk lag op de sla membraangebonden NAC-transcriptie factor LsNAC069. Membraangebonden transcriptiefactoren maken het voor planten mogelijk om snel te reageren op signalen uit de omgeving. Nadat de plant signalen heeft waargenomen volgt activatie van membraangebonden transcriptiefactoren door ze los te knippen (proteolyse). Hierbij komt de transcriptiefactor los van het membraan en verplaatst zich naar de celkern om transcriptie te reguleren. Wij brachten LsNAC069 en de interacterende *B. lactucae* effectoren, allen gekoppeld aan fluorescente eiwitten, tot expressie in sla en *N. benthamiana* om de intracellulaire lokalisatie te bestuderen. Dit werd bemoeilijkt door de instabiliteit van LsNAC069 in planten. Daarom werd in de meeste experimenten gebruik gemaakt van een verkorte vorm van LsNAC069 waarbij het NAC-domein ontbreekt, genaamd LsNAC069$^{\Delta NAC}$. Om aan te tonen dat voor onze doeleinden LsNAC069$^{\Delta NAC}$ een geschikte vervanger is voor LsNAC069 hebben we een aantal eigenschappen vergeleken. Zowel LsNAC069 als LsNAC069$^{\Delta NAC}$ interacteerden met de netwerk effectoren in gist. Hierbij was de interactie tussen de *B. lactucae* effectoren en LsNAC069 afhankelijk van de C-terminale regio, waaronder het transmembraan domein in LsNAC069. Het N-terminaal gelokaliseerde NAC-domein droeg niet bij aan de interactie. Daarnaast bleken LsNAC069

en LsNAC069$^{\Delta NAC}$ gebonden te zijn aan het membraan van het endoplasmatisch reticulum in *N. benthamiana*. De lokalisatie van LsNAC069$^{\Delta NAC}$ overlapte ook met de lokalisatie van de interacterende *B. lactucae* effectoren BLR05 en BLR09 in *N. benthamiana*. Aangezien *N. benthamiana* niet als gastheer kan dienen voor *B. lactucae*, maakten we gebruik van de *N. benthamiana* infecterende oömyceet *Phytophthora capsici* om de verplaatsing van LsNAC069$^{\Delta NAC}$ naar de celkern te stimuleren. De verplaatsing van LsNAC069$^{\Delta NAC}$ was sterk verminderd als een remmer voor proteases (eiwitten die proteolytische reacties uitvoeren) genaamd TPCK (N-p-Tosyl-L-phenylalanine chloromethyl ketone) werd toegediend. Deze bevinding bevestigt de theorie dat gecontroleerde protease activiteit noodzakelijk is om LsNAC069$^{\Delta NAC}$ los te maken van het membraan. Daarnaast was verplaatsing verminderd als de interacterende *B. lactucae* effectoren BLR05, BLR08 en BLR09 mede tot expressie werden gebracht. Deze bevinding suggereert dat *B. lactucae* er baat bij heeft als LsNAC069 niet geactiveerd wordt. Om de rol van LsNAC069 in vatbaarheid van sla voor *B. lactucae* te onderzoeken, werden stabiele slalijnen ontwikkeld met sterk onderdrukte *LsNAC069* transcriptie. De lage *LsNAC069* mRNA niveaus leidden echter niet tot een verandering in de vatbaarheid. Dit komt mogelijk door genetische redundantie: meerdere genen uit dezelfde genfamilie voeren gedeeltelijk dezelfde taken uit, waardoor het verlies van één of enkele genen opgevangen kan worden. Hierdoor blijft de rol van LsNAC069 en de interacterende *B. lactucae* effectoren tijdens infectie onbekend.

In het laatste hoofdstuk (**Hoofdstuk 5**) worden de belangrijkste resultaten van mijn thesis samengevat en in een bredere context geplaatst. De uitdagingen die voor ons liggen om in de toekomst duurzame resistentie in sla te ontwikkelen worden besproken en suggesties worden gegeven hoe verder gewerkt kan worden aan opgestarte onderzoekslijnen, zodat de bevindingen hieruit mogelijk in de toekomst een toepassing zullen vinden.

Dankwoord – Acknowledgements

Mijn jaren als PhD zitten erop. In die periode hebben zoveel mensen bijgedragen aan mijn ontwikkeling tot de persoon en onderzoeker die ik nu ben, dat ik hier een moment wil nemen om jullie, allemaal, te bedanken.

Als eerste wil ik mijn dagelijkse begeleider en promotor Guido bedanken dat ik een tweede kans heb gekregen om aan een PhD te beginnen. Je durfde het aan om me veel vrijheid te geven. Bedankt voor al je adviezen en nuchtere blik. Ik ben trots op deze thesis en de wetenschap die we daarvoor samen hebben verricht. Daarnaast wil ik ook Corné bedanken voor het stroomlijnen van de feedback tijdens de halfjaarlijkse besprekingen.

Furthermore, I would like to acknowledge the breeding companies that invested both time and resources in this project. Daarbij kan ik Anne en Marieke als Wageningse partners in dit STW project niet overslaan, bedankt voor de samenwerking. Ook Thijs en Mike van Ontwikkelingsbiologie ben ik dankbaar voor alle hulp met de Y2H screens.

Claudia, Danke sehr (and that's as far as my German goes) for joining me on this project. I hoped for an awesome biochemist, who would perform all the protein interaction assays in plants, but also got a kind colleague that was just as passionate about research as I was. Thank you for all the discussions on science and your contributions to this thesis. Ook een bedankje voor Joyce, voor al het werk dat je in de beginfase van het project hebt gedaan en een bedankje naar mijn studenten Lex en Jelle voor jullie bijdrage. Miek, dank voor je zorgzaamheid voor iedereen binnen de afdeling. En verder, lieve Kim(mie), ik vond het zo leuk om je in mijn laatste jaar als kamergenootje te hebben. Natuurlijk ook Manon, het zonnestraaltje van de afdeling, bedankt voor de gezelligheid en je positiviteit. Mijn paranimfen, Joël en Tijmen, bedankt voor alle slechte grappen. Mijn PhD zou een stuk saaier zijn geweest zonder jullie. And of course I also need to thank all my other (former) colleagues at PMI for answering questions, helping out and making the breaks more fun: Anja, Ainhoa, Aster, Christos, Colette, Dongping, Eline, Erqin, Giannis, Gilles, Hao, Hans, Irene, Ivan, Ke, Lotte, Marcel, Marciel, Marco, Merel, Niels, Nicole, Nora, Paul, Pauline, Peter, Pim G, Pim V, Richard, Roeland, Ronnie, Sarah, Saskia, Silvia C, Silvia P, Tom, Yang and Yeling.

Marco en Bert, dank voor de kans om aan de VU te mogen beginnen met mijn eerste PhD. Het is wellicht niet zo gelopen als we destijds hadden gehoopt, maar het was een leerzame ervaring waar ik geen spijt van heb. Ik heb dan ook veel goede herinneringen aan mijn tijd in Amsterdam en wil bij deze alle oud-collega's bedanken: Anne, Anitha, Azra, Daniel, Danny, David, Folkert, Henry, Herman, Jan-Paul, Jody, Marija, Ovi, Raymond, Reggie, Reka, Sabrina en Saskia.

I would also like to acknowledge those people that taught me about science long before I started my PhDs. In particular, Rumyana Karlova, Jan-Willem Borst and Sacco de Vries during my BSc thesis, Ruud Wilbers and Arjen Schots during my MSc thesis and Jurgen Denecke during my internship in Leeds.

Tim en Ivon (en natuurlijk Nena, Martyn en de kids), bedankt voor jullie steun en de gezellige etentjes in de afgelopen jaren. Ik wil ook mijn schoonfamilie bedanken dat jullie me vanaf het begin welkom hebben laten voelen. Mama, papa en Pascalle (en Cyril), bedankt voor alle steun en hulp in de afgelopen jaren en jullie vertrouwen in een goede afloop. Martien, bij jou zijn, is thuiskomen. Je was er de afgelopen zes jaar voor me op goede en slechte dagen met onvoorwaardelijke steun, humor en liefde. Dank je.

Bussum, mei 2018

Curriculum vitae

Alexandra Pelgrom was born in Eindhoven, the Netherlands, on July 27th 1988. She attended the Carolus Borromeus College in Helmond and graduated in 2006. The same year she started her BSc education in Biotechnology at Wageningen University. She wrote her BSc thesis on “Phosphorylation dependent localization and interaction studies of AGAMOUS-LIKE 15 in *Arabidopsis thaliana*” at the department of Biochemistry. Subsequently, she chose the MSc specialization Medical Biotechnology and wrote her MSc thesis on “Interleukin-10 signal transduction” at the Department of Nematology. For her internship, she studied “ERD2 and ERP1 in the plant secretory pathway” at the Centre for Plant Sciences at Leeds University in the United Kingdom. In the fall of 2011, she obtained her MSc degree and started a PhD on “The role of 14-3-3 proteins in GPCR mediated signaling in mammalian cells” at the department of Medicinal Chemistry at the Vrije Universiteit in Amsterdam in 2012. She moved back to the field of plant science in November 2013, when she started a PhD on the subject of effectors from the plant pathogenic oomycete *Bremia lactucae* as described in this thesis, at the department of Plant-Microbe Interactions at Utrecht University.

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