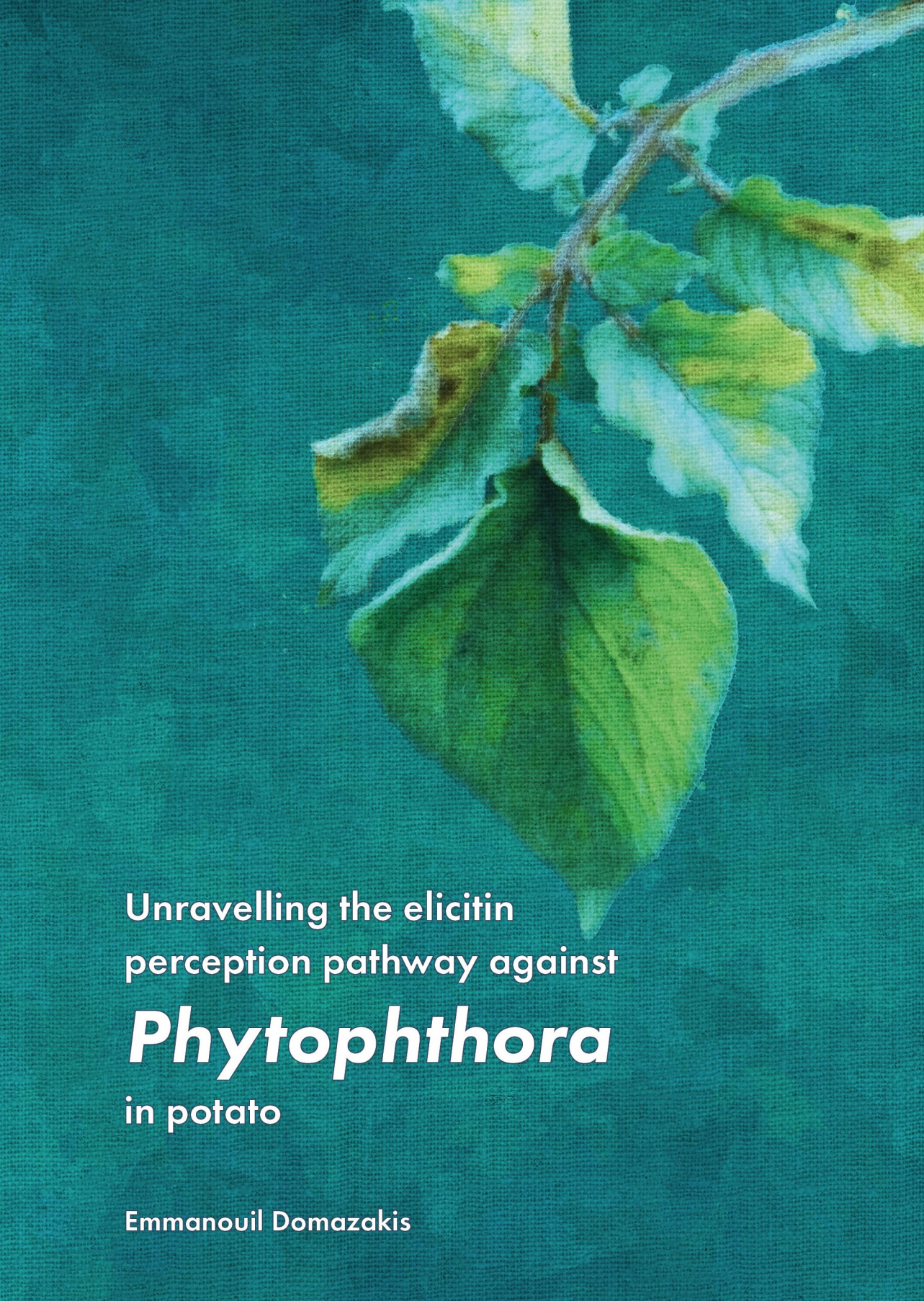

Unravelling the elicitin perception pathway against *Phytophthora* in potato

Emmanouil Domazakis

Propositions

1. The identification of immune receptors can be accelerated by using proteomics.
   (this thesis)

2. Binding of an effector to a receptor does not necessarily mean downstream response activation.
   (this thesis)

3. Genome editing will be a requirement for reverse genetic studies in the future.

4. Results from human nutrition studies are only reliable when the test population is large enough.

5. A course "designing experiments" should be an integral part of MSc and PhD training programs.

6. Innovation is driven by either real problems or plain laziness.

7. To achieve real sustainability, every crop should be planted in areas where minimal input is required.

Propositions belonging to the thesis entitled
"Unravelling the elicitin perception pathway against *Phytophthora* in potato"
Emmanouil Domazakis
Wageningen, 1 June 2018

Unravelling the elicitin perception pathway against *Phytophthora* in potato

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This research was conducted under the auspices of the Graduate School EPS (Experimental Plant Sciences).

Unravelling the elicitin perception pathway against *Phytophthora* in potato

Emmanouil Domazakis

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Chapter 1

General introduction

A never-ending war between plants and pathogens

Plants have to cope with harsh biotic and abiotic stresses in their environment in order to thrive. To do so, they employ physical barriers (trichomes, cuticle, epidermis and cell wall) and induced immunity triggered by innate immune receptors (Agrios, 2005; Freeman, 2008; Dodds and Rathjen, 2010; Wirthmueller et al., 2013). Induced plant immunity is based on two factors; the capacity of individual cells to recognize invading organisms and the rapid activation of defense mechanisms. It is important to note that in natural systems, disease is the exception rather than the rule and most plants are able to resist infection from potential pathogens, while at the same time, they can establish symbiotic interactions with other organisms. Unfortunately, this is not the case for most agricultural systems; that is, due to the limited genetic variation in the plant material used and intensive agricultural practices, pathogens may cause serious crop losses with immense effects for the farmers and the society in general (Dangl et al., 2013). Therefore it is of major importance to continuously breed for disease resistant crops by exploiting all genetic variation available or even precisely engineer plant genomes to outcompete the co-evolving pathogens.

Plant immunity: conceptual models and a contemporary overview

A first explanation of plant immunity came from Flor (1942) who, by studying the flax-flax rust interaction, proposed that inheritance of plant immunity to pathogens as well as the ability of the latter to cause disease is controlled by corresponding gene pairs. In this 'gene for gene' model, for a specific plant-pathogen interaction, the plant has a resistance (*R*) gene that matches with the corresponding avirulence (*Avr*) gene of the pathogen strain. Upon recognition of the corresponding gene products, defense responses are mounted, which can lead to resistance (Flor, 1942; Keen, 1990). However, later studies showed that some R proteins monitor (guard) the state of host components that are targeted by pathogen molecules, leading to the formulation of the guard hypothesis (van Der Biezen and Jones, 1998). From this time, it became evident that certain pathogen molecules are promoting pathogen virulence by modulating host components (virulence targets). But in turn, this manipulation can be sensed by the R proteins, and subsequently lead to resistance. A later view on plant immune system is described in the zig-zag model, proposed by Jones and Dangl (2006). This model describes that plants sense invading pathogens through pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), which are typically well-conserved molecules that pathogens need for survival. Recognition of PAMPs

can activate host defense responses leading to PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). Since PAMPs can be found in non-pathogenic microbes as well, the terms microbe-associated molecular patterns (MAMPs) and MAMP-triggered immunity (MTI) became more relevant. In addition, plants recognize the self-damage caused by biotic or abiotic factors through damage-associated molecular patterns (DAMPs) (Dangl et al., 2013) (Fig. 1).

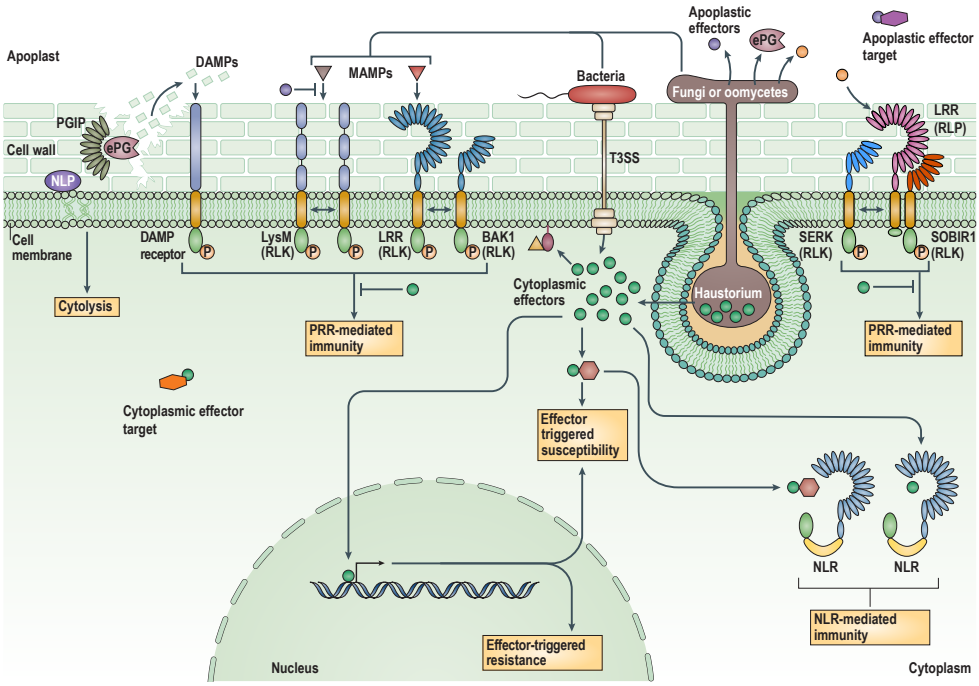

FIGURE 1 | A simplified view of the molecular players in induced plant immunity (adapted from Wirthmueller et al. (2013)). Plants are continuously threatened by several enemies such as fungi, oomycetes and bacteria. Pathogens secrete a wide range of apoplastic and cytoplasmic effectors during infection. For example, they secrete enzymes (e.g. endo-polygalacturonases-ePGs), toxins (e.g. ethylene-induced Nep1-like proteins-NLPs), nutrient uptake proteins (e.g. oomycete elicitins) or effectors that target specific host components (apoplastic/cytoplasmic effector targets) for the benefit of the pathogen. To defend themselves, plants evolved protease inhibitors (e.g. PG-inhibiting proteins-PGIPs), cell surface pattern recognition receptors (PRRs) and cytoplasmic receptors (nucleotide-binding leucine-rich repeat proteins-NLRs). Via these proteins, the plants either block effector activity or trigger defense responses by binding microbe-associated molecular patterns (MAMPs) or effectors, thereby sensing the pathogen. Indirectly, plants may sense self-derived components called damage-associated molecular patterns (DAMPs) (e.g. cell wall fragments) or the manipulation of host proteins. PRRs are often functioning in complex with co-regulatory RLKs of the somatic-embryogenesis receptor kinase (SERK) family (e.g. BRI1-associated kinase-BAK1) or the suppressor of BIR1-1 (SOBIR1) while other PRRs function as homodimers (e.g. lysin motif-LysM receptors). Other abbreviations: phosphate (P), leucine-rich repeat (LRR), lysin motif (LysM), type III secretion system (T3SS).

During evolution, plant pathogens have developed effector proteins to circumvent MTI and establish disease, a phenomenon called effector triggered susceptibility (ETS). According to Hogenhout et al. (2009) and Win et al. (2012), effectors are "microbial and pest secreted molecules that alter host-cell processes or structures generally promoting the microbe lifestyle. Effector functions are as diverse as suppressing immune responses or enhancing access to nutrients". These molecules are important components in the interaction between microbes and host plants, as they form the molecular keys to establish a parasitic or symbiotic interaction. Initially it was considered that only bacteria and filamentous pathogens secrete effectors, but increasing evidence supports the widespread use of such molecules from a plethora of organisms including nematodes, insects or even parasitic plants (Spallek et al., 2017). In turn, plants have evolved cytoplasmic resistance proteins that are able to recognize cytoplasmic pathogen effectors and activate defense responses leading to effector triggered immunity (ETI) (Jones and Dangl, 2006).

Following the zig-zag model, immunity against pathogens is quantitative with the strongest responses leading to hypersensitive response (HR) and weakest responses being ineffective for defense. As more knowledge was obtained during the past decade, it became clear that pathogen molecules that were initially considered as avirulence factors, are in fact virulence factors, and also the distinction between MAMPs and effectors cannot be strictly maintained (van Der Biezen and Jones, 1998; Thomma et al., 2011). In some cases, conserved so-called 'core effectors' could be considered as MAMPs, since they are conserved among strains of the pathogen or even among distant kingdoms of life (Bohm et al., 2014; Oome et al., 2014). An example of effectors with virulence contribution and MAMP features is the Nep1-like protein (NLP) (Gijzen and Nürnberger, 2006; Bohm et al., 2014). From this protein, a small 20-amino acid epitope (nlp20) is recognized by some plants (Albert et al., 2015). Recently, an alternative model called the invasion model has been proposed to deal with this dilemma, (Cook et al., 2015). This model highlights that cell surface PRRs are also co-evolving with their corresponding ligands, similar to cytoplasmic R-genes, and may target less conserved patterns or effectors. Overall, the plant immune system is highly dynamic and complex and needs to be tightly regulated (Couto and Zipfel, 2016; Huang et al.; Wu et al., 2017).

The first layer of defense; recognition of MAMPs and apoplastic effectors by plant PRRs

The MAMP definition entails molecules that are innate components found widespread in whole classes of microbes and are important for the fitness of the invading organism (Newman et al., 2013). MAMPs act as signature for recognition by the host and they are found exposed in the plant apoplast where they can interact with the host. Some well-known MAMPs include elongation factor thermo unstable (EF-Tu) (elf18/26), flagellin (flg22, 28), chitin, lipopolysaccharides, activator of XA21 (Ax21) and ethylene-inducing xylanase (EIX) (Newman et al., 2013). MTI in plants has been most extensively studied using the peptides elf18 and flg22 derived from EF-Tu and flagellin, respectively (Felix et al., 1999; Zipfel et al., 2006).

PRRs are germline-encoded sensors, typically localizing at the cell surface (the plasma membrane). They act as the first line of recognition of self- or nonself-derived molecules found in the apoplast. This recognition triggers a defense response that in many cases results in quantitative resistance against pathogens (Jones and Dangl, 2006). In contrast to animal genomes, plants encode a vast number of PRRs and in the past 20 years, several of them have been cloned and characterized (Fig. 2). PRRs consist of an apoplastic, ligand binding domain, a single pass transmembrane domain and a cytoplasmic domain. The apoplastic ligand binding (Boutrot and Zipfel, 2017) domain varies depending on the ligand; LRR type PRRs typically recognize proteinaceous MAMPs or effectors, LysM type PRRs recognize chitin oligosaccharides, EGF-like and lectin type PRRs recognize DAMPs, proteins or other carbohydrates (Zipfel, 2014; Boutrot and Zipfel, 2017). All cloned PRRs are divided in two classes, receptor-like kinases (RLKs) and receptor-like proteins (RLPs). The main difference between those two classes is that RLKs contain a cytoplasmic kinase domain, while RLPs have only a short cytosolic tail without any obvious signaling domain (Newman et al., 2013; Couto and Zipfel, 2016). Among the most well studied PRRs are the ones recognizing EF-Tu, flagellin, chitin and nlp-20, namely EFR, FLS2 and CEBiP/CERK1/LYK4,5 and RLP23, respectively (Fig. 2) (Gómez-Gómez and Boller; Zipfel et al., 2006; Miya et al., 2007; Wan et al., 2008; Cao et al., 2014; Albert et al., 2015). However, the function and ligands of the majority of RLPs and RLKs remains unknown.

It is becoming increasingly evident that RLPs and RLKs require additional proteins for triggering downstream signaling. Members of the somatic-embryogenesis receptor kinase (SERK) family of RLKs have been found to associate with PRRs. Examples include the RLKs FLS2, EFR and RLPs such as RLP23 associate with SERK3 upon MAMP perception (Zipfel, 2014; Couto and Zipfel, 2016). So, it became evident

that SERK proteins, especially SERK3 (BAK1 in *Arabidopsis*) act as a general co-regulatory RLK from RLKs acting as PRRs. However in the case of RLPs, which lack a downstream signaling domain, another co-regulatory RLK, SOBIR1 has been found to be required for function, often in addition to SERK3 (Liebrand et al., 2014). For example, Cf-4- and Cf-9-mediated resistance to *Cladosprorium fulvum* was found to require SOBIR1 and SERK3 (Liebrand et al., 2013; Postma et al., 2016). Several other RLPs involved in immunity, such as RLP23, RBPG1, Ve1 require the same co-regulatory RLKs (Bi et al., 2014; Gust and Felix, 2014; Liebrand et al., 2014b; Albert et al., 2015; Bi et al., 2016; Postma et al., 2016). Since both SOBIR1 and SERK3 are required for PRR functioning in immunity, it is anticipated that they are involved in resistance to pathogens. Experimental evidence supports this as either *SOBIR1*- or *SERK3*-silenced or mutant plants were found to be more susceptible in a wide array of pathogens (examples include *Phytophthora parasitica, Sclerotinia sclerotiorum, Botrytis cinerea, Verticillium dahliae, C. fulvum, Magnaporthe oryzae* in tomato and Arabidopsis) (Chaparro-Garcia et al., 2011; Liebrand et al., 2013; Zhang et al., 2013; Liebrand et al., 2014; Zhang et al., 2014; Takahashi et al., 2016). Therefore, co-regulatory RLKs, also known as co-receptors, are necessary components for the induction of defense responses and resistance to pathogens conferred by PRRs (Chinchilla et al., 2009; Liebrand et al., 2014) (Figs. 1, 2).

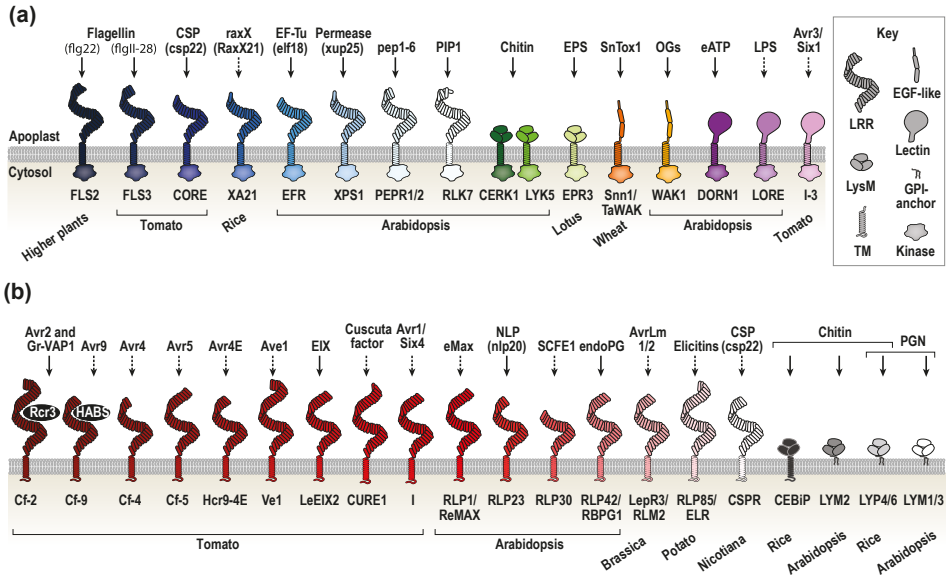

FIGURE 2 | An overview of PRR receptors cloned and characterized to date and their (potential) ligands (adapted from Boutrot and Zipfel (2017)). (a) Receptor-like kinases (RLKs) and (b) receptor-like proteins (RLPs) have been cloned from several plant species. Normal arrows indicate biochemical evidence of binding while dashed arrows indicate that the PRR-ligand interaction has not been biochemically confirmed.

The second layer of defense; recognition of cytoplasmic effectors by NLRs

Cytoplasmic effectors are secreted by multiple pathogens and aim into manipulating the host cells by serving structural roles in the formation of infection structures, promoting nutrient leakage or suppressing defense responses (Jones and Dangl, 2006). When those effectors are introduced into the plant cells, a second layer of plant immunity may be triggered (ETI), leading to a robust HR cell death. ETI is triggered by cytoplasmic NLR receptors, often called products of *R*-genes (Fig. 1). Most resistance proteins cloned to date are NLRs (Zhu et al., 2012). Those modular proteins, typically comprise of a N' domain which is either Toll-Interleukin receptor 1 (TIR) or coiled coil (CC) domain, a nucleotide-binding site (NB) and an LRR domain. The NB (NB-ARC in Pfam) domain is conserved and is responsible for binding and hydrolyzing ATP (Tameling et al., 2002), while the LRR domain is believed to mediate the protein-protein interaction with cytoplasmic effectors. However, there are also reports of NLRs that lack LRRs, yet these are still are able to recognize effectors (Duxbury et al., 2016; Sarris et al., 2016). In more recent studies, NLRs are described to be among the most rapidly evolving proteins in plants (Mondragón-Palomino et al., 2002; Ballvora et al., 2007). Several NLR proteins are recognizing cytoplasmic effectors through direct binding with the LRR domain or through interaction with an integrated decoy domain. The list of NLRs which directly bind cytoplasmic effectors has been growing over the last years. NLRs can also recognize pathogen effectors indirectly, by guarding another NLR carrying a decoy domain (Kroj et al., 2016). In this case, the actual NLR receptor is monitoring the state of another NLR, which is called 'guardee' if it is involved in signaling, or 'decoy' if it is just mimicking the actual effector host target (Sarris et al., 2016). Any modification of the integrated guardee/decoy NLRs is sensed by the NLR receptors leading to immunity. Recently, NLRs were found that originate from the fusion of NLRs with additional proteins. Such domains are serving as 'baits' as they are targeted by pathogen effectors, with three examples identified so far, carrying WRKY or HMA integrated domains (Sarris et al., 2016).

The potato crop: importance and challenges

The potato (*Solanum tuberosum* L.) is among the most nutritious crops in the world and a basic component for the diet of billions of people (King and Slavin, 2013). Potato is ranked fourth among the most consumed crops, following rice, wheat and maize (Haverkort et al., 2009; Haverkort et al., 2016). Besides growing potatoes for food, potatoes are used for industrial and dietary production of fibers

(potato skin), starch and protein (mainly patatin) (Burlingame et al., 2009; Zaheer and Akhtar, 2016). The largest areas of potato cultivation are found in Europe and Asia, together accounting for 80% of the world's production. Total production of potato tubers exceeded 381 Megatons in 2014 (FAO http://www.fao.org/faostat/en/#data/QC).

The cultivated potato originates from the central Andes mountain region and was imported in Europe by the conquistadors in the 16th century (Hawkes, 1990; Gavrilenko et al., 2013). Wild tuber bearing *Solanum* spp. belong to section Petota (~200 species) and are ranging from diploid (2n = 2x = 24) to hexaploid (2n = 6x = 72) species and are distributed over a large geographical area ranging from the south-western United States to central Argentina (Spooner and Hijmans, 2001).

Most diploid potato species are self-incompatible while the tetra- and hexaploid species are mostly self-compatible allopolyploids (Hawkes, 1990). Cultivated potato suffers from severe inbreeding depression and most cultivated species are allotetraploid. This makes breeding new cultivars carrying new traits a difficult and long process. With the sequencing of the potato genome in 2011, insights were obtained into the high heterozygosity, inbreeding depression, the evolution of tuberization and repertoire of disease resistance genes (PGSC, 2011).

Potato is susceptible to several diseases, such as stem canker and black scurf caused by *Rhizoctonia solani*, early blight caused by *Alternaria solani* and late blight caused by *Phytophthora infestans*. From the sequenced potato genome, a plethora of putative resistance genes were identified (PGSC, 2011). However, to obtain resistance against these pathogens, the wild potato relatives form a major resource (Vleeshouwers et al., 2011a; Machida-Hirano, 2015).

Phytophthora infestans, the potato late blight pathogen

Potato plants are susceptible to several pests and pathogens; however the most devastating disease in most countries is late blight (Fry et al., 2015; Kamoun et al., 2015). Late blight is caused by the oomycete *Phytophthora infestans* Mont. de Bary, which is considered the most notorious oomycete pathogen (Kamoun et al., 2015). The name of the genus *Phytophthora* comes from the Greek words phyto (φυτόν-phyto = plant) and phthora (φθορά-phthora = damage). Late blight alone is accounted for more than 16% of the total potato yield losses globally (Haverkort et al., 2009; Haverkort et al., 2016). Typically farmers in the Netherlands spray at least 10 times during the growing season in order to control this disease. This accounts for 50% of the total fungicides used in the country (Haverkort et al., 2016).

From a historic perspective, heavy late blight infection of potato fields in Ireland is renowned for triggering the Irish potato famine. Due to this devastation in the main food crop of Irish people, around 1 million people died and another million migrated outside of the island in the mid-19th century (Yoshida et al., 2013). To date, whenever left uncontrolled, *P. infestans* can destroy a potato field in a matter of days. Oomycetes in general, contain some highly adaptable species in terms of overcoming introduced plant resistances or even chemical pesticides. *P. infestans* is renowned for its ability to overcome any introduced singular resistance gene and has developed resistance to some of the chemicals used for its control (Fry, 2008; Vleeshouwers et al., 2011; Cooke et al., 2012; Randall et al., 2014).

P. infestans is able to infect all parts of the plants causing whole plant collapse or post-harvest damage on the tubers (Fig. 3). Its life cycle begins from a sporangium landing on a leaf. Then depending on the temperature and humidity, these spores of *P. infestans* can germinate directly (at >18°C) or form zoospores (at <18°C), motile spores that encyst on the host surface. In both cases, sporangia and cysts form a germination hypha, an appressorium and a penetration peg with which they invade the host (Fig. 3). Initially *P. infestans* behaves as a biotroph and does not kill the infected cells (Judelson and Blanco, 2005). Hypha grow around the mesophyll cells where later on, haustoria are formed. These are structures involved in effector secretion into the host and presumably nutrient uptake. In the final stage, the pathogen starts to form sporangiophores and sporangia which are protruding from the host stomata. At this stage and depending on the host susceptibility, the plant cells are killed by the pathogen as it enters the necrotrophic stage. In contrast to other *Phytophthora* spp., *P. infestans* is a heterothalic oomycete, with two known mating types, A1 and A2 (Fig. 3) (Judelson and Blanco, 2005; Schoina and Govers, 2015). When both mating types co-occur, specialized hyphal structures called oogonium and antheridium, are formed. Those fuse together to create an oospore. Oospores are reinforced sexual spores which can rest in the soil debris and cause infection. This mode of reproduction is assumed to contribute to the high evolutionary potential of the pathogen.

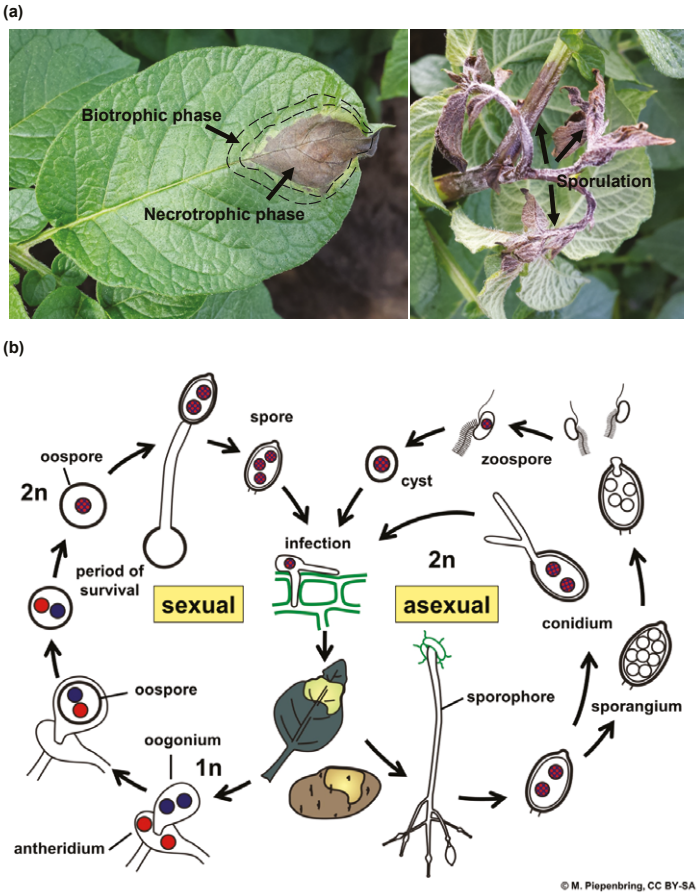

FIGURE 3 | Late blight symptoms in potato caused by *Phytophthora infestans* infection and the pathogen life cycle. (a) As a hemi-biotroph, *P. infestans* initially infects cells without causing cell death (biotrophic phase) while at a later point it switches to necrotrophy where tissue collapses (necrotrophic phase). Following colonization of potato, sporulation may occur under humid conditions. (a) Life cycle of *P. infestans* (adapted from M. Piepenbring (https://creativecommons.org/licenses/by-sa/3.0), via Wikimedia Commons).

Phytophthora infestans effectors: structures and functions

Oomycete plant pathogen genomes are known to encode for hundreds of apoplastic and cytoplasmic effectors (Kamoun, 2006; Morgan and Kamoun, 2007; Raffaele et al., 2010a). Both kinds of effectors are modular (Schornack et al., 2009). Apoplastic effectors typically consist of a signal peptide and a cysteine-rich mature protein part which serves their biological function. Apoplastic effectors often include proteases, protease inhibitors, proteins involved in nutrient uptake or in necrosis induction. Cytoplasmic effectors consist of a signal peptide, a host

translocation motif (RXLR or LXLFLAK) and a C-terminal domain which possesses the biochemical activity of the protein (Whisson et al., 2007; Schornack et al., 2009).

Phytophthora pathogens secrete a wide array of cysteine-rich effectors in the apoplast. Among those effectors, elicitins were identified as major secreted proteins in culture filtrates. Elicitins were among the first oomycete genes to ever be cloned (Ricci et al., 1989; Panabieres et al., 1995; Fawke et al., 2015).

Elicitins form a diverse family of secreted proteins, specific to *Phytophthora* and *Pythium*. They are found in complex multigene families and form diverse subclasses (Jiang et al., 2006). Elicitins sharing a highly conserved 98-amino acid domain with six cysteine residues spaced in a typical pattern are classified as ELIs while all other elicitins, with variable lengths of elicitin domains are named elicitin-likes (ELLs). So far, four ELI and 13 ELL clades have been identified (Jiang et al., 2006). Within each clade, elicitins show similar expression patterns and are under purifying selection. The crystal structure of elicitin β-CRY (aka CRY-B, class ELI-1) revealed a highly conserved region, the ω-loop, critical for sterol binding (Boissy et al.). Β-CRY and INF1 (class ELI-1) elicitins are among the most well studied (Derevnina et al., 2016). Clades besides ELI-1 possess C-terminal extensions of a variable length which are believed to serve as anchors to the cell wall (Jiang et al., 2006). Elicitins carry many MAMP attributes; they are structurally conserved, show difference to plant proteins, and fulfill an important biological function for the pathogen by taking up nutrients (Derevnina et al., 2016). Namely, elicitins bind sterols and other lipids with varying affinities (Mikes et al., 1998; Osman et al., 2001). Sterols are important for oomycete growth and sporulation (Stong et al., 2013). *Phytophthora* and *Pythium* are sterol auxotrophs and as such, elicitins are required for scavenging sterols from the host (Stong et al., 2013; Derevnina et al., 2016).

Protease inhibitors represent another class of well-characterized *P. infestans* effectors. Those effectors are known to block plant secreted serine (EPI1 and EPI10) and cysteine (EPIC1 and EPIC2B) proteases. EPI1 and EPI10 effectors contain two or three Kazal-like protein domains, respectively, and were found to interact with and inhibit the pathogenesis-related, subtilisin-like serine protease P69B of tomato (Tian et al., 2004; Tian et al., 2005). EPIC1 and EPIC2B are directly interacting and blocking the potato and tomato papain cysteine protease C14, which leads to increased pathogen fitness on potato (Kaschani et al., 2010). Both EPIC1 and EPIC2B target the tomato protease PIP1 and RCR3, but with lower affinity than C14 and only at apoplastic pH (Kaschani et al., 2010). Therefore, those effectors have similar function as Avr2 from the tomato fungal pathogen *C. fulvum* (van Esse et al., 2008), indicating that such protease inhibitor activity is

necessary for successful colonization of plants by filamentous fungi and oomycete pathogens. This hypothesis is further supported by the fact that PIP1, RCR3 and C14 are under diversifying selection in *Solanum*, probably due to being in an arms race with pathogens that manipulate them in order to colonize the host (Kaschani et al., 2010).

Nep1-like proteins (NLPs) and other small cysteine-rich (SCR) proteins form another class of apoplastic effectors of *P. infestans*. Necrosis-inducing NLPs PiNPP1.1, PiNPP1.2 and PiNPP1.3 of *P. infestans* are known to be secreted at later stages of infection, some of which are acting as toxins, promoting cell death (Kanneganti et al., 2006; Zuluaga et al., 2016). Several SCR effector proteins have been found, which show similarity to the PcF effector from a strain of *Phytophthora cactorum* thas was isolated from infected strawberry (Orsomando et al., 2003; Nicastro et al., 2009; Orsomando et al., 2011). Those effectors in *P. infestans* are named with the acronym SCR followed by the number of amino acid residues and include SCR74 and SCR91, among others (Bos et al., 2003; Liu et al., 2005). SCR74 is highly upregulated during early stages of tomato and potato colonization. It belongs to a highly polymorphic family with at least 21 members identified (Liu et al., 2005). All *P. infestans* strains tested so far, contain at least two and up to six SCR74 variants, while no strain has been identified without this effector. The polymorphism found in SCR74 is due to gene duplication and recombination and is likely the result of diversifying selection forces between wild *Solanum* and *P. infestans* (Liu et al., 2005; Kamoun, 2006). A study of Orsomando et al. (2011) showed that SCR74 and SCR91 are inducing apical withering when applied at high (10-18 μM) concentrations in tomato seedlings, suggesting a function similar to PcF. However, SCR74 was less potent in inducing those symptoms compared to PcF and SCR91 (Orsomando et al., 2011). The function of these fast evolving effectors in *P. infestans* remains to be elucidated.

During infection, *P. infestans* secretes a plethora of cytoplasmic effectors into the host cells. Such proteins are believed to be secreted via specialized hyphal structures called haustoria that are common infection structures found in biotrophic and hemi-biotrophic pathogens. Haustoria are enveloped in a modified plant plasma membrane and are not completely disrupting it. Two major cytoplasmic effector classes are found in *P. infestans*, RXLR effectors and crinkling and necrosis (CRN) inducing effectors (Kamoun, 2006; Schornack et al., 2009). RXLR effectors contain the homonymous arginine-X-leucine-arginine motif (X stands for any amino acid) in the sequence following the secretion signal peptide. Effector prediction pipelines identified hundreds (>500) of candidate RXLR effectors in the *P. infestans* genome and several have been studied using high throughput *in planta* screens and biochemical studies (Bos et al., 2003; Torto et al., 2003; Haas et al., 2009;

Vleeshouwers et al., 2011). RXLR effectors were found to target a variety of host proteins and processes. AVR3a was shown to target and stabilize the ubiquitin ligase protein CYS, MET, PRO, and GLY PROTEIN 1 (CMPG1), thereby suppressing immunity (Bos et al., 2010; Gilroy et al., 2011). Another function for AVR3a was found to be suppression of receptor endocytosis by associating with the dynamin-related protein 2 (DRP2) (Chaparro-Garcia et al., 2015). AVR2 was found to target the phosphatase BSL1 to suppress immunity (Saunders et al., 2012b). Another effector, PexRD54, was found to dampen host defenses by binding ATG8 and antagonizing a host autophagy cargo receptor Joka2 (Dagdas et al., 2016). AVRblb2 was shown to suppress C14 protease secretion, an enzyme which is also targeted with the apoplastic effectors EPIC1 and EPIC2B mentioned above (Bozkurt et al., 2011). CRN are present in all sequenced oomycete species sequenced so far (Stam et al., 2013). With only some exceptions in *Hyaloperonospora arabidopsidis* (Baxter et al., 2010), CRN effectors do not have an RXLR motif, but carry a LXLFLAK motif for translocation. In *P. infestans*, CRNs form a complex family of effectors, among which CRN1 and CRN2 were identified as necrosis-inducing proteins in *Nicotiana* and *Solanum* sp. (Torto et al., 2003). In general, there are a plethora of studies on cytoplasmic effectors providing insights on a diversity of functions.

Identified resistances against potato late blight

As mentioned above, the *P. infestans* genome contains a plethora of effectors, some of which are translocated into the host cells and some are effectors confined in the apoplast. In the center of origin of *P. infestans*, wild *Solanum* plants are a source for resistance genes as the pathogen co-evolves with the host (Vleeshouwers et al., 2011). To enable effector functional tests as well as identification of resistance genes, scientists have employed high throughput *in planta* expression of effectors in a wide late blight-resistant *Solanum* germplasm (Vleeshouwers et al., 2008; Oh et al., 2009; Vleeshouwers et al., 2011). This combination of effector prediction and wide germplasm screening was termed 'effectoromics'. The same strategy also yielded the first PRR against oomycetes which specifically recognizes elicitins (Du et al., 2015).

Response to elicitins in *Solanum* is mediated by ELR

Elicitin signaling has been found important for the basal resistance of *N. benthamiana* to *P. infestans,* as pathogen strains deficient in INF1 elicitin production showed enhanced virulence (Kamoun et al., 1998). The first cell-surface receptor RLP that specifically responds to elicitins has been recently cloned from a wild

potato species, *Solanum microdontum* (Du et al., 2015). The elicitin-response receptor (ELR) is a RLP lacking a cytoplasmic kinase domain that recognizes several elicitins ranging from 45-65% amino acid similarity (Du et al., 2015). This recognition spectrum includes *Phytophthora* elicitins belonging to class ELI-1, ELI-2 and ELI-4 elicitins (Jiang et al., 2006; Du et al., 2015). Elicitins that are recognized by ELR do not seem to share a conserved stretch of amino acids, as it is found in bacterial flagellin, EF-Tu or NLPs (Felix et al., 1999; Kunze et al., 2004; Bohm et al., 2014). Rather, the responsiveness of ELR to several elicitins likely depends on the structural similarity of this class of effectors (Derevnina et al., 2016).

Genome-editing technologies to advance plant breeding

With the advancement of plant breeding, it is always desirable to introduce novel variation in cultivars without introducing too much unwanted traits. Around the year 2000, the dominant tool for genome editing was zinc finger nucleases (ZFNs) (Kim et al., 1996; Carroll, 2011). This was later followed with the discovery of transcription activator-like effector nucleases (TALENs) (Christian et al., 2010). Both ZFNs and TALENs are artificial fusion proteins comprised of a DNA-binding domain and a *Fok*I restriction endonuclease and have been used successfully in many organisms including plants (Bortesi and Fischer, 2015). They both function as dimers, which requires the design of a pair of nucleases in order to achieve the intended mutation. Despite their success, these methods required difficult design of reagents and did not find wide applicability. Recently, the discovery of clustered regularly interspaced short palindromic repeats (CRISPR), which is part of an adaptive bacterial immune system, has emerged as an alternative genome editing technology (Jinek et al., 2012). CRISPR/CRISPR-associated 9 (Cas9) is utilizing single guide RNA (sgRNA) to target the complementary sequence in the genome in conjunction with the unspecific Cas9 endonuclease. CRISPR/Cas9-mediated genome editing is easier to design as it requires only the design and cloning of a short sgRNA sequence and is functional in a plethora of organisms. This made it the most widely used system for genome editing in plants (Belhaj et al., 2013).

Some cultivated potato genotypes have a relatively high transformation efficiency and thus, genome editing assays are applicable. Indeed, several recent studies have described the use of CRISPR/Cas9 for studying gene function in potato (Butler et al., 2015; Wang et al., 2015; Andersson et al., 2017). Therefore, assuming that several wild potato relatives are also amenable to transformation, CRISPR/Cas9 could revolutionize targeted genetic improvement for this crop and facilitate reverse genetic studies.

Objectives of the thesis

Potato late blight is the most threatening disease of potato cultivation worldwide. So far, plant breeding for late blight resistance was focused on the introduction of mostly singular NLR resistance genes (*R*-genes). However, despite their effectiveness, those introduced resistances have been defeated by the pathogen rather quickly. In my thesis, I studied how surface-triggered immunity functions against *P. infestans* in potato. I investigate how the perception of elicitins is activated by a potato PRR. Moreover, I studied how biochemical and genome editing approaches could accelerate novel immune receptor identification and gene functional analysis in this important crop.

Identifying surface receptors in crops like potato is a difficult task and effectoromics have proved a major tool for doing this (Vleeshouwers et al., 2011; Vleeshouwers and Oliver, 2014). In the case of potato late blight, effectors are predicted and screened in a wide collection of wild potato germplasm using different assays such as agroinfiltration, PVX agroinfection or protein infiltration. In **Chapter 2**, we describe the latter three methods in high detail as step by step protocols. We emphasize on the production of effector proteins in yeast with details for small scale, efficient protein production of six *P. infestans* apoplastic effectors.

PRRs are predominantly found to function in receptor complexes. Increasing experimental evidence supports the association of LRR type RLPs with co-regulatory RLKs. In **Chapter 3**, we study whether ELR requires such partner proteins for its functioning using a multidisciplinary approach combining gene silencing, genetic complementation and biochemistry. PRRs are expected to physically associate with their respective ligands in order to activate defense signaling. For ELR, such information was missing since its identification. In **Chapter 4**, we investigate whether ELR is a true PRR by physically binding elicitins and study the mechanistics of elicitin perception in *Solanaceae*.

Reverse genetics is commonly used to characterize gene function. In wild potato species, such assays were not possible, causing researchers to shift to model species. However, recent advances in technologies such as CRISPR/Cas9-mediated mutagenesis have provided opportunities for genome editing in crop plants like potato. Previously, the effect of ELR on disease resistance was studied by overexpression in cv. Désireé, but not in its own native genetic background. In **Chapter 5**, we attempt to perform functional characterization of ELR homologs in several wild *Solanum* species using CRISPR/Cas9 genome editing. We report on the efficacy of the method for genome editing by generating *ELR* mutants and discuss on its potential application for characterizing newly discovered PRRs.

In **Chapter 6**, the outcomes of this thesis research are summarized and placed in the broader research context regarding cell surface-mediated immunity. By looking at ELR as a model we gained a lot of information which is useful for the identification of novel PRRs. Being able to apply this knowledge on wild potato species has the potential to provide us with genetic tools to combat the most devastating potato pathogen; *P. infestans*.

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Chapter 2

Effectoromics-based identification of cell surface receptors in potato

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abstract

In modern resistance breeding, effectors have emerged as tools for accelerating and improving the identification of immune receptors. Effector-assisted breeding was pioneered for identifying resistance genes (*R* genes) against *Phytophthora infestans* in potato (*Solanum tuberosum*). Here we show that effectoromics approaches are also well suitable for identifying pathogen recognition receptors (PRR) that recognize apoplastic effectors. To detect genotypes that recognize apoplastic proteins of *P. infestans*, routine agroinfiltration and potato virus X (PVX) agroinfection methods can be applied. In addition, protein infiltrations are feasible for assessing responses to apoplastic effectors and aid in confirming results obtained from the aforementioned methods. Protocols for the effectoromics pipeline are provided, starting from phenotyping for effector responses, up to genotyping and PRR gene identification.

Keywords: Pattern recognition receptors (PRR), apoplastic effector, genetic mapping, agroinfiltration, PVX agroinfection, protein infiltration, effectoromics, yeast protein production, *Solanum*.

Introduction

Breeding for broad-spectrum and durable disease resistance is one of the most important prerequisites for achieving a stable food production. Potato (*Solanum tuberosum* L.) is the third staple crop world-wide, and suffers from dramatic yield losses due to the devastating late blight pathogen *Phytophthora infestans* Mont. de Bary. To understand and improve resistance to late blight in potato, effectors of *P. infestans* have been successfully exploited in breeding (Vleeshouwers et al., 2008; Vleeshouwers et al., 2011). In this 'effectoromics' strategy, effectors are functionally tested for responses in potato germplasm. After detecting responding genotypes, the receptors are being genetically mapped on progeny populations derived from crosses between responding (R) and non-responding (NR) genotypes, in which phenotyping is done by high throughput effector assays (Takken et al., 2000; Vleeshouwers et al., 2006).

Effectoromics was originally focused on detecting resistance (*R*) genes of the NBS-LRR class by assaying cytoplasmic effectors, and protocols were established for a.o. *Solanum* and *Nicotiana* species (Kapila et al., 1997; Kanneganti et al., 2007; Du and Vleeshouwers, 2014). Recently, the strategy was expanded to identifying pattern recognition receptors (PRR), or surface receptors, by functionally screening for responses to apoplastic effectors in potato (Du et al., 2015).

With the ever increasing amount of pathogen genome data, effectoromics is now being implemented for various other crops and pathosystems. Not only biotrophic pathogens, but also pathogens with other life styles can be the target of effectoromics, and e.g. toxins can be identified for necrotrophic pathogens (Oliver et al., 2012; Vleeshouwers and Oliver, 2014). The main requirements for effectoromics, is availability of 1) resistant plant material for sourcing the immune receptors, 2) candidate effectors predicted from pathogen genomes (Box 1), and 3) reliable and efficient functional assays (Box 2/Fig. 1). *Agrobacterium*-based expression systems, such as for agroinfiltration and potato virus X (PVX)-agroinfection, are well established in *Solanaceae* (Takken et al., 2000; Torto et al., 2003; Du et al., 2014) but are less applicable in e.g. monocots. An advantage of targeting apoplastic effectors is that these are typically more stable than cytoplasmic effectors and can be assayed by protein infiltrations (Vleeshouwers et al., 2006). Protein infiltration may provide a reliable way to assay responses obtained by other systems (Vleeshouwers et al., 2006), since it is not dependent on transformation efficiency and cannot be hindered by extreme resistance responses.

Box 1: Prediction of candidate apoplastic effectors

Effector candidates can be identified by applying selection criteria on a database of proteins from the pathogen of interest. Such data might already be available, or could be generated by a genome sequencing and/or RNAseq experiment.

A number of criteria used in various publications (Tan et al., 2010; Liu et al., 2012; Saunders et al., 2012a; Mirzadi Gohari et al., 2015) are generally established for the selection of apoplastic effectors:

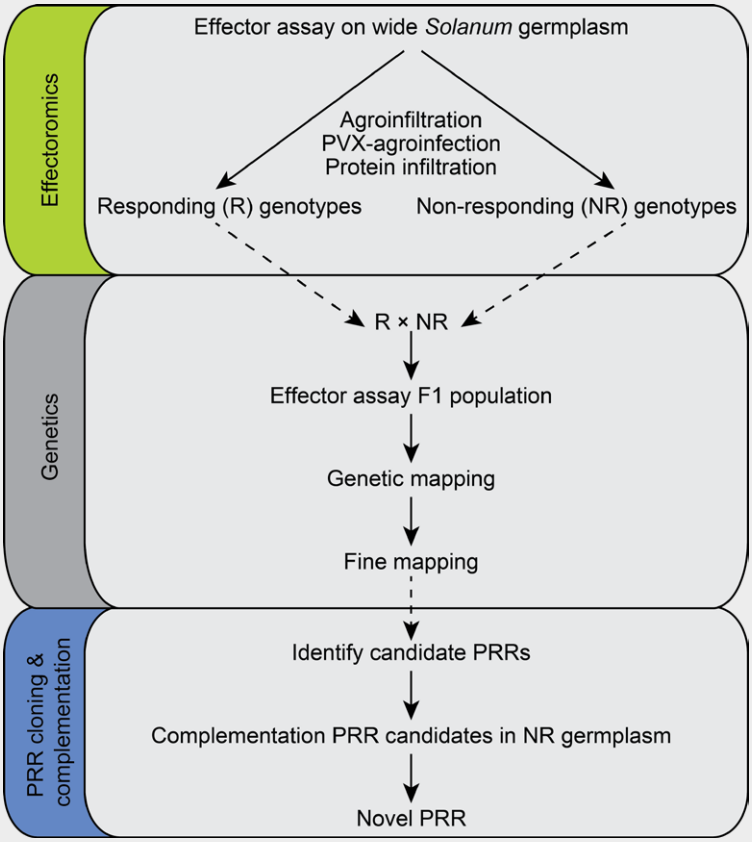
1. Presence of a secretion signal. In order for effectors to be secreted in the apoplast, they need to contain a signal peptide for secretion. Signal peptides can be predicted with software such as SignalP (Petersen et al., 2011).
2. Size. Effectors are often small proteins (e.g. <30 kDa).
3. Cysteine content. Apoplastic effectors are typically cysteine rich proteins; the formation of disulphide bonds is believed to help stabilize the proteins in the apoplastic environment
4. Expression profile. If such data are available. Functional effectors need to be expressed during the infection process.

Depending on the pathogen, additional criteria may be useful to further prioritize the list of effector candidates, for example: location in the genome (in *Phytophthora* spp., most effectors reside in repeat-rich regions (Haas et al., 2009) and in *Fusarium* spp. effectors are found close to transposable elements on so-called 'pathogenicity' chromosomes (Ma et al., 2010; Schmidt et al., 2013), presence or absence of certain domains and motifs (for example including proteins with a predicted LysM domain to find effectors that bind chitin (Vleeshouwers et al., 2006; Bolton et al., 2008), but excluding proteins with motifs like RxLR, which are required for cell entry (Vleeshouwers et al., 2006; Whisson et al., 2007; Manning et al., 2008), to filter out cytoplasmic effectors), similarity to known effectors, or information about presence/absence of homologs in virulent or avirulent strains of the pathogen.

Being able to produce high quality apoplastic effector proteins using heterologous systems is of great importance. *Pichia pastoris* has emerged as an appropriate host for secreted protein production (Ahmad et al., 2014). As an eukaryote, it is able to post-translationally modify target effector proteins and secrete them into the growth medium (Brondyk et al., 2009). This is in a way similar to eukaryotic pathogens secreting effectors in the host apoplast. In addition, compared to *Escherichia coli*-based expression systems, it is able to produce higher quality protein with appropriate folding and does not form aggregates (Brondyk et al., 2009).

Recent advances have enabled integration of novel methods, such as proteomics approaches for detecting protein activity and resistance gene enrichment sequencing (RenSeq) for accelerated genetic mapping (Jupe et al., 2013). In this chapter, we provide protocols for an effectoromics pipeline in potato (Box 2/Fig. 1). These include routine *Agrobacterium*-based methods that are applicable for all types of immune receptors known so far, and with emphasis on apoplastic effector screening methods that are useful for identifying new surface receptors.

Box 2: Effectoromics workflow to identify novel surface receptors



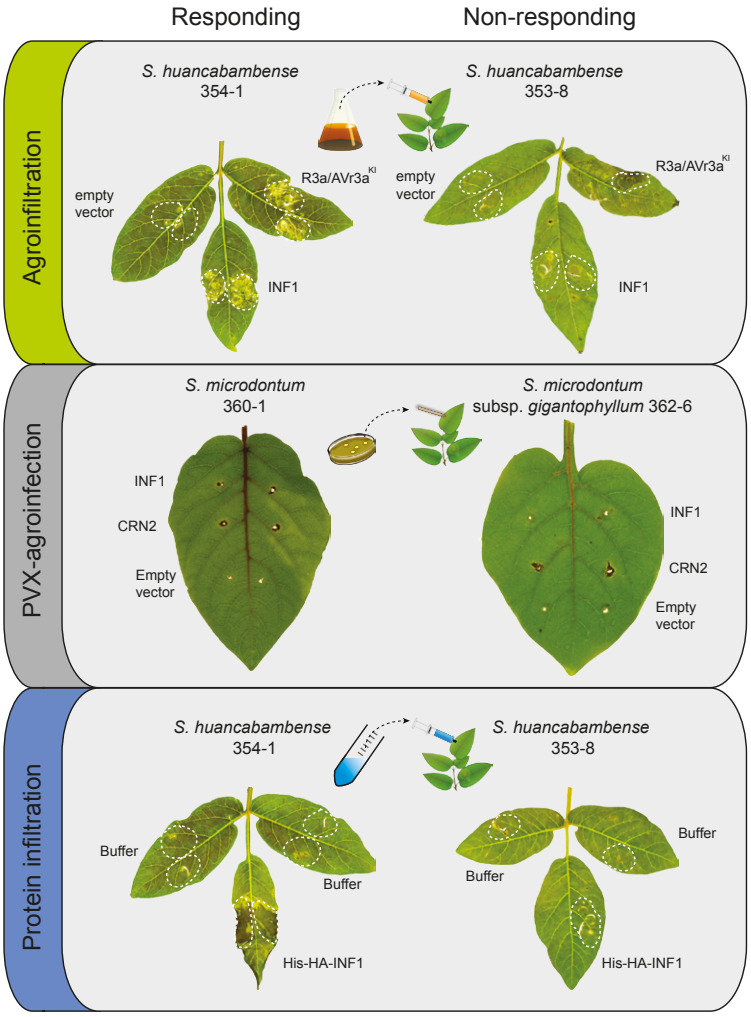


FIGURE 1 | Effectoromics screening for responses to apoplastic effectors in *Solanum* species using agroinfiltration, PVX-agroinfection or protein infiltration. Leaves of responding (R) and non-responding (NR) *Solanum* genotypes are agroinfiltrated, PVX-agroinfected or protein-infiltrated with INF1 effector expressing constructs or with His-HA-INF1 protein purified from *P. pastoris*. As negative control, in the case of agroinfiltration and PVX agroinfection, empty vector carrying Agrobacterium strains are used. In the case of protein infiltration, buffer infiltration is used. In case of agroinfiltration and PVX-agroinfection, a positive control is included (R3a/AVr3a^{KI} (Armstrong et al., 2005; Huang et al., 2005) or CRN2 (Torto et al., 2003), respectively). No cell death is observed for the negative controls and INF1 is only causing cell death in the responding genotypes as confirmed by three methods (Vleeshouwers et al., 2006).

Materials

Potato *in vitro* propagation and maintenance

MS20 medium (per l: 4.4 g Murashige and Skoog basal salt mixture without vitamins, 20 g sucrose in 700 ml of demineralized water (ddH_2O). Adjust pH to 5.8 with NaOH and HCl and bring to a final volume of 1 l. Add 8 g of micro agar and autoclave) (Du et al., 2014).

Agroinfiltration and PVX agroinfection

1. *Agrobacterium tumefaciens* strains AGL1 (Lazo et al., 1991) or GV3101 (Koncz and Schell, 1986)
2. LB (lysogeny broth) medium/LBA (LB + agar) (per l: 10 g tryptone, 5 g yeast extract and 10 g NaCl. For LBA add 10 g/l bacteriological agar. Autoclave the media).
3. (Agroinfiltration only) 200 mM acetosyringone (4-Hydroxy-3,5-dimethoxyacetophenone) stock (39,3 mg of acetosyringone in 1 ml of DMSO).
4. (Agroinfiltration only) 1 M MES (2-(N-morpholino)-ethane sulfonic acid) buffer (per 50 ml: 10.66 g of MES monohydrate, adjust pH to 5.6 with NaOH and filter sterilize).
5. (Agroinfiltration only) YEB (yeast extract broth) *Agrobacterium* Growth Medium (per l: 5 g beef extract, 5 g bacteriological peptone, 5 g sucrose, 1 g of yeast extract, 0,492 g of $MgSO_4 \bullet 7H_2O$, autoclave) (van der Hoorn et al., 2000).
6. (Agroinfiltration only) MMA agroinfiltration buffer (per l: 20 g of sucrose, 5 g of MS salts without vitamins, 1.95 g MES, 2 ml 1 M NaOH, 1 ml of 200 mM acetosyringone. Confirm that pH is 5.6. If necessary, adjust with HCL/NaOH).
7. Antibiotics: prepare stock concentration of the appropriate antibiotic. Common antibiotic 1000 × stock concentrations: kanamycin 50 mg/ml, spectinomycin 50 mg/ml, ampicillin/carbenicillin 100 mg/ml (in water) rifampicin 25 mg/ml (in DMSO), chloramphenicol 34 mg/ml, tetracycline 5 mg/ml (in ethanol).
8. (Agroinfiltration only) pK7WG2 vector (Gateway) (Karimi et al., 2002) or other CaMV 35s driven plant expression vector.
9. (PVX-agroinfection only) pGR106 vector (Lu et al., 2003) or other (CaMV 35s) PVX encoding vector.

Protein production in *Pichia pastoris*

1. pPinkα-HC vector (PichiaPink™, Invitrogen, Carlsbad, USA)
2. Restriction endonucleases and buffers
3. T4 ligase and buffer
4. DH5α, TOP10 or similar competent *Escherichia coli* cells

5. LB medium containing ampicillin or carbenicillin (100 µg/ml)
6. LBA plates containing ampicillin or carbenicillin (100 µg/ml)
7. AOX1 sequencing primers (AOX: 5′-GACTGGTTCCAATTGACAAGC-3′, AOX: 3′-GCAAATGGCATTCTGACATCC-5′)
8. Target effector gene primers
9. Plasmid DNA purification kit
10. *Pichia pastoris* strain PichiaPink™ 1, 2, 3 or 4 (*ADE2* mutants, strains 2, 3, 4 are also protease deficient strains-see PichiaPink™ manual
11. Working glycerol stock of PichiaPink™ strains (see Invitrogen manual)
12. 20% w/v dextrose solution (per l: 200 g dextrose, autoclave)
13. 1 M sorbitol (per l: 187.17 g sorbitol)
14. 1 M potassium phosphate buffer, pH 6.0 (per l: 132 ml of 1 M K_2HPO_4, 868 ml of 1 M KH_2PO_4 and confirm that the pH is 6.0 ± 0.1, use phosphoric acid or KOH to adjust pH)
15. YNB (per l: 134 g Yeast Nitrogen Base with ammonium sulfate, without amino acids. Filter sterilize)
16. YPD medium (per l: 10 g Yeast extract, 20 g Peptone. Autoclave and add 100 ml 20% Dextrose) and YPD agar plates (YPD medium containing 8 g/l agar. Autoclave and add 100 ml 20% Dextrose)
17. YPDS medium (YPD containing 1 M sorbitol)
18. PAD selection plates (adenine drop-out media) (see Invitrogen manual)
19. Sterile Milli-Q water (Merck Millipore, Darmstadt, Germany)
20. Electroporation device with yeast transformation setup (e.g. for BioRad gene pulser and 0.1 cm gap cuvette: 400 Ω resistance, 25 µF capacitance and 1.4 kV voltage)
21. 125 ml and 1 l baffled culture flasks, sterile
22. 500 ml centrifuge bottles, sterile
23. 10% glycerol (per l: 100 ml glycerol, autoclaved)
24. 10% methanol (per l: 100 ml absolute methanol, filter sterilized)
25. 0.02% biotin (per 100 ml: 20 mg biotin, filter sterile)
26. BMGY (per l: 10 g yeast extract, 20 g peptone, 700 ml Milli-Q water, autoclave. Then add 100 ml of 1 M potassium phosphate pH 6.0, 100 ml YNB, 2 ml of 0.02% biotin and 100 ml 10% glycerol)
27. BMMY (per l: dissolve 10 g yeast extract and 20 g peptone in 700 ml Milli-Q water, autoclave. Then add 100 ml of 1 M potassium phosphate pH 6.0, 100 ml YNB, 2 ml 0.02% biotin and 100 ml 10% methanol)
28. Baffled or normal flasks
29. 96-deep well plates (2 ml capacity)
30. Amicon Ultra 400 ml stirred cell and appropriate filtration membranes (Merck

Millipore, Darmstadt, Germany)
31. 4× laemli SDS-PAGE loading buffer (per 10 ml: 2 ml 1M Tris-HCl pH 6.8, 0.8 g SDS, 4 ml 100% glycerol, 0.4 ml 14.7 M β-mercaptoethanol, 1 ml 0.5 M EDTA, 8 mg bromophenol blue)
32. 96-well PCR plates
33. Antibodies raised against HA (hemagglutinin) or His (histidine) epitopes.

Genetic mapping

1. SSR markers (Milbourne et al., 1998)
2. 4300 DNA Analyzer (LI-COR, Lincoln, USA)
3. LightScanner® System (Bio Fire, Utah, USA)
4. Phire Green Hot Start II DNA Polymerase (Thermo-Fisher Scientific)
5. *S. tuberosum* Group Phureja DM1-3 genome v4.03 (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml (Hardigan et al., 2016))

Methods

Potato plant maintenance and propagation

Maintain potato plants *in vitro* in sterile jars containing MS20 medium. Incubate in climate chambers at 24 °C under long day conditions (16 h light/ 8 h dark) for two weeks (Du et al., 2014). Then transfer them into pots of sterilized soil in climate regulated greenhouse compartments within the temperature range of 18-22 °C and under 16 h/ 8 h day/night regime (Du et al., 2014).

Agroinfiltration

1. Inoculate the *Agrobacterium* strains containing the gene of interest in 15 ml of YEB containing 1.5 µl of 200 mM acetosyringone solution, 150 µl of 1 M MES buffer and the appropriate antibiotics.
2. Incubate on a shaking incubator for 24-48 h at 28°C at 200 rpm until the culture has grown to an OD_{600} of approximately 1.
3. Harvest the cells by centrifugation at 3,000 x g for 10 min. Decant supernatant and resuspend the pellet in freshly made MMA buffer to an OD_{600} of 0.4. Gently vortex the cells. For co-infiltration of two strains, mix the cultures in a 1:1 ratio.
4. Incubate the bacterial strains in MMA for 1-6 h at room temperature before infiltrations.
5. Use a 1 ml needleless syringe to infiltrate the lower side of the potato leaf. Use around 4-5 weeks old plants grown from *in vitro* rooted plantlets (see note 1 and 3). A successful infiltration becomes visible by a change in colour of the infiltrated area from light green to dark green.

6. Use three plants for agroinfiltration with each strain and three leaves per plant (see note 3). Be sure to include a negative control (e.g. a strain carrying empty vector) and a positive control (e.g. a co-infiltration of matching avirulence and resistance gene) (see note 4).
7. Score for cell death after 3 days of infiltration on a scale from 0% (no symptoms) to 100% (confluent cell death) (Du et al., 2014) (Fig. 1) (see note 4, 5 and 6).

PVX agroinfection

1. Inoculate the *Agrobacterium* strains containing the gene of interest in 3 ml of YEB. Incubate the cultures at 28°C, shaking at 200 rpm for 24-48 h.
2. Pipette 100 µl of each *Agrobacterium* strain and spread them on LB agar plates containing the appropriate antibiotics. Incubate at 28°C for 24-48 h.
3. Use a bacterial spreader to thoroughly collect the *Agrobacterium* culture at the centre of the plate. Dip the tip of a toothpick in the *Agrobacterium* cells.
4. Inoculate each strain on the leaf by piercing the leaf with the toothpick. Use around 2-3 weeks-old plants from *in vitro* (see note 2). Inoculate at least three plants for each strain and three leaves per plant. Toothpick-inoculate each *Agrobacterium* strain in duplicate, on each side of the mid vein. Be sure to include a positive (e.g. CRN2) and a negative control (empty pGR106 vector) in every treated leaf (Torto et al., 2003).
5. Score for cell death around the inoculation wound at 14 days post inoculation (Fig. 1) (see note 4 and 5). Record the qualitative/quantitative cell death response (score as 0 for no response, 1 for intermediate and 2 for strong response) for each spot and compare with the negative control. (Du et al., 2014) (see note 4, 5 and 6).

Resistance gene mapping in *Solanum*

1. Identify diploid *Solanum* genotypes that respond to the target effector by agroinfiltration and PVX agroinfection with clear phenotypes (an overview of the map-based cloning strategy is presented in Fig. 2, see note 4 and 5).
2. Cross the genotypes with clear responses (R) with genotypes that do not respond (NR) (Box 2, Fig. 2). Harvest the F1 seeds.
3. Sow 50 F1 seeds in greenhouse and phenotype the response for the target effector. If the F1 population shows a 1:1 segregating ratio, it indicates that the target gene is heterozygous in the responding parents.
4. If the F1 population does not show segregation (indicating that the receptor is homozygous in the response parent), select 2-3 F1 response progenies with good phenotypes for a backcrossing with the non-responding genotypes. The resulting BC1 population should show a segregation for the effector response.

5. Run about 80 SSR markers that are equally distributed over the potato chromosomes (Milbourne et al., 1998) to determine on which chromosome the resistance gene is located.
6. Test markers spanning the target chromosome (identify known markers from literature or develop new markers based on the published potato genome (PGSC, 2011) (DMv4.03).
7. Sow more seeds to screen a higher number of progeny plants from the same cross with the flanking markers. Maintain the recombinants *in vitro* for further phenotyping and marker testing.

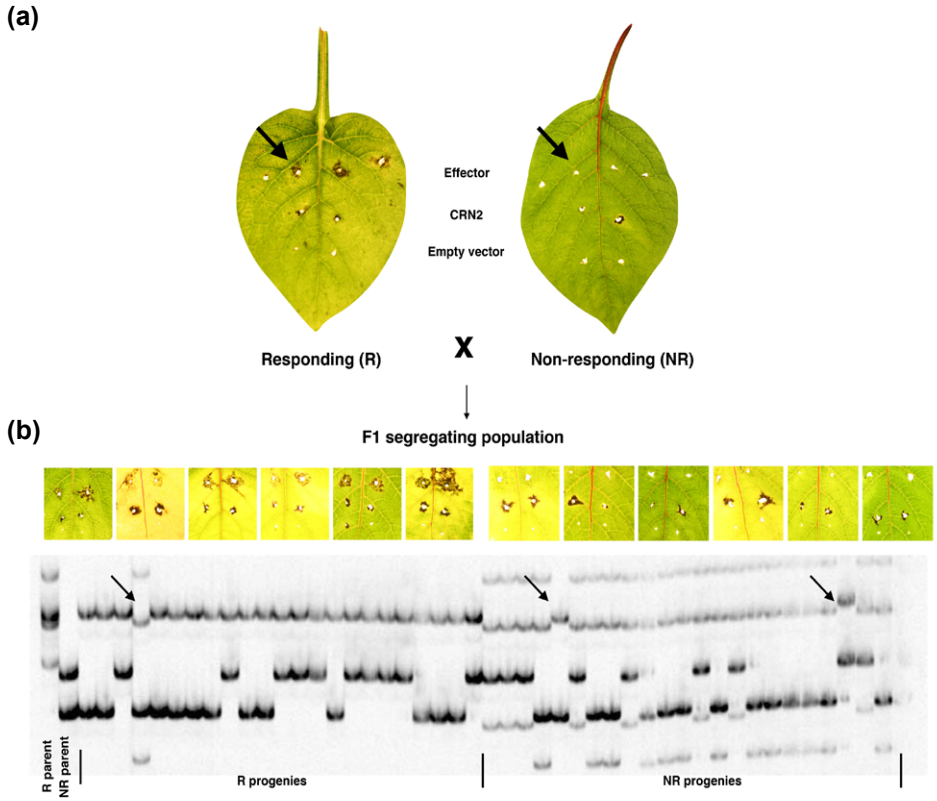


FIGURE 2 | Map-based cloning of surface immune receptors following the effectoromics screening in diploid *Solanum* species. a) The selected responding (R) *Solanum* genotype is crossed with a non-responding (NR) genotype. The F1 progenies are screened for effector responses as described in this chapter. b) The F1 population shows 1:1 segregation ratio. Eighty SSR markers (Milbourne et al., 1998) are used to determine the chromosome where the target gene is located. The depicted SSR marker is linked with the phenotype and three recombinants are indicated (arrows).

Marker development

1. Develop single-nucleotide polymorphism (SNP) markers based on the DM v4.03 genome sequence (PGSC, 2011) for fine mapping. Select genes from the target interval and PCR amplify 1,200 bp long fragments. Sequence fragments from R and NR parents.
2. Select SNPs that are heterozygous from R parent and homozygous from NR parent. Design new high resolution melting (HRM) markers to amplify 150 bp amplicons containing the ideal SNP and screen on the population (SNPs cause difference in PCR product melting curve). The target interval will be further narrowed down.
3. Alternatively, new HRM markers can be developed randomly on exon sequences between the target interval from DM. 150 bp small amplicons can be used for SNP screening by the LightScanner system.

Fine mapping

1. Construct 10× coverage BAC library for the responding genotype. For heterozygous diploid potato genotypes (e.g. 150,000 BAC clones for 900 Mb haploid size ×2).
2. Screen the BAC library using the co-segregating markers identified in the previous steps. Use the markers to select BAC clones that are in coupling phase with the resistance gene to create a minimum tiling path covering the region of interest. Sequence the selected BAC clones.
3. Annotate the genes in the sequenced region and select candidate genes based on their predicted function.
4. Clone the candidate genes in a binary expression vector (e.g. pK7WG2) and co-infiltrate with the matching effector in non-responding potato and *Nicotiana benthamiana* plants to confirm the recognition of the effector from the cloned resistance gene.
5. Generate stable transformants of the candidate gene into a NR potato background (e.g. cultivar Désirée) for further complementation and functional studies.

Apoplastic effector cloning and transformation of *Pichia pastoris*

We use the amino acid sequence HHHHHHVKLYPYDVPDYAAA (underlined are the 6× His and 1× HA-tag residues with spacer amino acids in between) encoded by the DNA sequence 5′-CATCATCACCATCACCACGTTAAGTTGTACCCATAC-GACGTTCCAGATTACGCTGCTGCT-3′ in N′ of the mature effector protein to generate N-terminal 6xHis-1xHA-tagged effector fusions in PichiaPink™ pPinkα-HC (see note 9).

1. Synthesize codon optimized effector genes for protein production in *Pichia pastoris* (see note 7 and 9). Six times His and HA tag as well as restriction sites have to be added (StuI in forward primer and KpnI, NaeI, FseI, or SwaI in reverse primer) to facilitate ligation into vector pPinkα-HC (secreted expression, high copy) (see note 8).
2. Digest codon optimized effector genes and the pPinkα-HC and vector with the appropriate enzymes and continue cloning as described in the manual .
3. Transform competent *E. coli* with pPinkα-HC-effector constructs. Select 5 colonies for colony PCR confirmation, plasmid isolation and sequencing.
4. Sequence 5 clones per construct with AOX5′ and AOX3′ primers. Be sure to carefully check for the correct insertion of the effector gene in the vector cassette and the absence of any mutation before and following the cloned gene.
5. Prepare 10 µg of plasmid DNA to be used for transformation by isolating plasmid DNA and digesting with a restriction enzyme that does not cut within your gene (e.g. one of *Mam* I, *Eco*N I, *Spe* I, or *Afl* II).
6. Clean up the digest by ethanol/sodium acetate precipitation and washing as described in the manual . Air-dry, and resuspend in 10 µL sterile, deionized water. Use immediately or store at -20°C.
7. Prepare electro-competent PichiaPink cells as described in the manual on the day of transformation (do not store cells at -80°C as transformation efficiency will drop significantly). Use 80 µL of cells and 10 µL of plasmid (10 µg total DNA amount) per electro-transformation.
8. After electroporation, immediately add 1 ml of sterile YPDS medium in the cuvette and incubate at 30°C for 3-4 h.
9. Plate 500 µL in PAD selection plates and incubate at 30 °C till colonies appear. Usually it takes 3 days for the first transformants to appear (see note 10).
10. Four to five days after transformation, select 10 of the most well developed, white *P. pastoris* colonies, for each construct (see note 10 and 11).

High throughput protein production screening

1. From the original transformation plates, patch 8-10 clones in a fresh PAD selection plate and incubate for 2-3 days at 30°C.
2. In a 96-deep well plate (2 ml capacity volume), add 200 µL of BMGY per well and inoculate with each clone.
3. Incubate at 28-30°C on a shaking incubator at 300 rpm for 24 h.
4. Spin down to pellet cells by centrifugation (1,500 × g for 15 min).
5. Remove supernatants with pipetting and add 300 µl of BMMY for inducing expression.

6. Incubate at 28°C in a shaking incubator at 300 rpm for 48 h.
7. Spin down to pellet cells and for each culture collect 150 µl supernatant in a clean 96-wells PCR plate.
8. To each supernatant, add 50 µl of 4× Laemmli loading buffer and heat to 95°C for 10 min.
9. Run SDS-PAGE with all clones per construct and perform western blot with anti-HA or anti-His to detect secreted recombinant proteins.
10. Select the highest producing clone for further protein production. Prepare glycerol stock of this clone by growing it in YPD for 48 h, adding sterile glycerol to 20% v/v and snap freezing in liquid nitrogen.

(a)

Effector protein	Molecular weight (kDa)	Concentration (BCA) µg/ml	Total µg CEX purified protein*
HisHA-INF1	12,72	263	1575
HisHA-SCR74-A10	8,05	351	2103
HisHA-SCR74-G1	8,02	295	1769
HisHA-SCR74-B3b	8,20	1391	8347
HisHA-EPI1	16,83	339	2035
HisHA-EPIC2B	13,84	517	3102

(b)

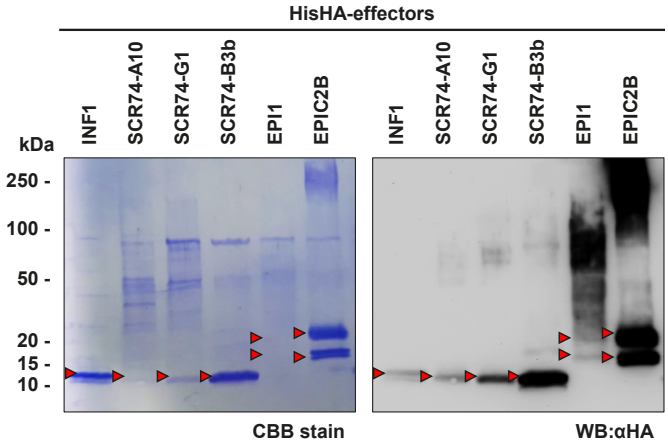


FIGURE 3 | Production of six recombinant HisHA-tagged effectors from *Phytophthora infestans* using the *Pichia pastoris* system. Six HisHA tagged effector proteins from *P. infestans* were produced in *P. pastoris* (INF1, SCR74-A10, SCR74-G1, SCR74-B3b, EPI1, EPIC2B), using a 200 ml induction culture, as described. a) Recombinant effectors were purified using cation exchange chromatography (described in **Chapter 4**). The concentration of the purified proteins was determined by BCA assay (ThermoFisher Scientific) and the total amount of protein obtained in 6 ml of elution volume is shown (*). b) SDS-PAGE of samples in a) was performed, followed by Coomassie brilliant blue (CBB) staining and western blot with anti-HA (Miltenyi Biotech).

Small scale recombinant effector production

1. Using the highest protein producing *P. pastoris* clone (from PAD selection plates or a -80°C glycerol stock), inoculate 5 ml of YPD and grow for one to two days in order to obtain a start culture.
2. Inoculate 25 ml of BMGY with 100 µL of the clone pre-culture in a 250 ml baffled flask. Grow at 28°C in a shaking incubator (250-300 rpm) until culture reaches an OD_{600} of 2-6 (usually 16–20 h).
3. Use this 25 ml culture to inoculate 1 l of BMGY in a 3 or 4 l baffled flask and grow at 28°C with vigorous shaking (250-300 rpm) until the culture reaches log phase growth (OD_{600} = 2-6) (usually 6-8 h).
4. Harvest the cells by centrifuging in sterile centrifuge bottles at 1,500–3,000 x g for 5 min at room temperature.
5. Decant the supernatant and resuspend the cell pellet in 200 ml of BMMY medium to induce expression.
6. Transfer the cell suspension in a 2 l baffled flask, cover with an air-porous tape such as AirPore™ (Qiagen, Venlo, the Netherlands) or PureLink™ Air Porous Tape (Thermo Fisher Scientific, Waltham, USA). Continue to grow at 28°C with shaking at 300 rpm.
7. Add 2 ml of 100% MetOH every 24 h to maintain induction.
8. Induce protein expression for 48-72 h.
9. Aliquot the culture in 50 ml conical tubes and centrifuge at 4,000 × g for 30 min to pellet the cells. Transfer supernatants to new 50 ml conical tubes and repeat centrifugation in order to completely clarify the supernatant.
10. Filter sterilize the supernatants through a 0.45 µm syringe filter.
11. Transfer sterilized supernatant in the Amicon Ultra 400 ml stirred cell, equipped with a membrane with a pore size (MWCO) that equals half or less the predicted effector molecular weight.
12. Concentrate the supernatant by applying 5.2 bars pressure of nitrogen gas and medium stirring speed till the volume reaches around 10-20 ml. Collect concentrate in a 50 ml tube. Supernatants can be kept for a short term at 4°C till purification or be stored at -80°C.
13. Purify recombinant apoplastic effector proteins with a method of choice (see note 12).

Infiltration of apoplastic effector proteins into leaf apoplast

1. Dilute purified tagged apoplastic effector proteins in sterile Milli-Q water at the desired molar concentration. Start by testing different molar concentrations, e.g. 10 nM to 10 µM (see note 13 and 14).
2. Use a needleless syringe to infiltrate protein solution in the lower side of a fully

expanded leaf. Use Milli-Q water, buffer or another, non-recognized protein (e.g. a cysteine mutant) as a negative control (see note 14 and 15).

3. Score responses at 3, 5 and 7 days post-infiltration, depending on the protein concentration used and the type of response (Fig. 1) (see note 4 and 5).

Notes

1. Choose young, healthy and fully developed leaves for agro-infiltrations.
2. For high-throughput screening with PVX agroinfection, use 4-5 week old plants. Those plants have big leaves to inoculate more spots than smaller leaves.
3. Eye protection should be worn during the agroinfiltration process. Change gloves when infiltrating with different strains to avoid cross contamination.
4. When scoring agroinfiltration, PVX-agroinfection or protein infiltration experiments, always compare your obtained effector responses with the controls. It is good to realize that intensity of responses also depend on transformation efficiency and sensitivity to *Agrobacterium* (and PVX) in the particular genetic background. Therefore, be sure to check for background responses to *Agrobacterium* or PVX.
5. Assay scoring can also be done at different time points, depending on the type of effector and inoculated plant genotypes. In the case of PVX-agroinfection, cell death responses usually start to appear from day 8, and are optimal at day 14. For agroinfiltration, cell death responses can appear from 2 days and reach a maximum at 5 days post infiltration. For protein infiltration, depending on the concentration used, cell death responses can appear from 2 days and reach a maximum at one week post infiltration. Keep in mind that responses to effectors are not necessarily associated with cell death (Jones and Dangl, 2006; Katagiri and Tsuda, 2010).
6. Cell death responses triggered by PRR may not be observed at temperatures below 20°C (Cheng et al., 2013).
7. Most secreted apoplastic effectors are small. Synthesizing is the best option. In addition, when synthesizing, codon optimization is possible which can lead to an increase in protein production yields. Codon optimization services can be requested at most companies.
8. ATG start codon or the yeast consensus Kozak sequence should not be included when cloning in pPinkα-HC vector as they are on the α-mating factor pre-sequence. You must add a stop codon at the 3′ of the effector gene, as it is not present in vector.
9. Tag position may affect protein recognition or function. For apoplastic effectors, a small N-terminal tag is used in most of the cases.

10. When transforming PichiaPink strains, low copy number transformants will appear on the PAD selection plates. Those will have a red-pinkish colour. It is advised not to use those for protein expression.
11. It is not necessary to screen PichiaPink tranformants by PCR. All white colonies should be positive, high copy number transformants.
12. Typically, ion exchange chromatography or immobilized metal affinity chromatography (IMAC) are used. IMAC exploits the metal binding affinity of histidine residues fused to the effector protein. Mainly nickel or cobalt coated beads are utilized for this purpose.
13. Protein concentration is important for an adequate response. Molar concentrations should be used instead of grams.
14. Do not use very high protein concentrations, as they could lead to toxic effects and therefore the experiment will be non-informative. A control for unspecific responses must be performed by including non-responding genotypes in every experiment.
15. Be sure that your protein solutions for infiltration are free of bacterial contamination. Potato plants tend to have a high background response to bacteria.

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Chapter 3

The ELR-SOBIR1 complex functions as a two-component RLK to mount defense against *Phytophthora infestans*

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abstract

The ELICITIN RESPONSE (ELR) protein from *Solanum microdontum* can recognize INF1 elicitin of *Phytophthora infestans* and trigger defense responses. ELR is a receptor-like protein (RLP) that lacks a cytoplasmic signaling domain and is anticipated to require interaction with a signaling-competent receptor-like kinase (RLK). SUPPRESSOR OF BIR1-1 (SOBIR1) has been proposed as a general interactor for RLPs involved in immunity and as such, is a potential interactor for ELR. Here we investigate whether SOBIR1 is required for response to INF1 and resistance to *P. infestans* and whether it associates with ELR. Our results show that virus-induced gene silencing (VIGS) of *SOBIR1* in *Nicotiana benthamiana* leads to loss of INF1-triggered cell death and increased susceptibility to *P. infestans*. Using genetic complementation, we found that the kinase activity of SOBIR1 is required for INF1-triggered cell death. Co-immunoprecipitation experiments showed that ELR constitutively associates with potato SOBIR1 *in planta*, forming a bipartite receptor complex. Upon INF1 elicitation, this ELR-SOBIR1 complex recruits SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3) leading to downstream signaling activation. Overall, our study shows that SOBIR1 is required for basal resistance to *P. infestans* and for INF1-triggered cell death, and functions as an adaptor kinase for ELR.

Keywords: BAK1/SERK3, cell death, ELICITIN RESPONSE (ELR) receptor-like protein (RLP), INF1 effector, late blight, pattern recognition receptor (PRR), *Phytophthora infestans*, SUPPRESSOR OF BIR1-1 (SOBIR1).

Introduction

Plants rely on cell surface, plasma membrane-spanning pattern-recognition receptors (PRRs) as a first line of apoplastic defense against microbial pathogens. PRRs recognize microbial molecules such as proteins that play a role in defense suppression (so-called effectors) or structural components, referred to as microbe-associated molecular patterns (MAMPs) (Couto and Zipfel, 2016). Two types of PRRs are recognized; those that have an intracellular kinase domain, which are receptor-like kinases (RLKs), and those without such a domain, the receptor-like proteins (RLPs). Leucine-rich repeat (LRR)-containing RLPs recognize MAMPs or effectors. However, as they lack an obvious cytoplasmic signaling domain, they are anticipated to require partner proteins for initiating defense signaling. The extent to which RLP and RLKs associate to enable immune signaling remains poorly understood.

RLPs from diverse plant families (i.e. *Brassicaceae* and *Solanaceae*), involved in immunity have been found to constitutively associate with the LRR-RLK SOBIR1 (SUPPRESSOR OF BIR1-1), forming a heterodimeric complex (Gust and Felix, 2014; Liebrand et al., 2014; Bi et al., 2016). Some examples are Ve1, Cf-2, Cf-4, Cf-9, RLP23, RLP30 and RESPONSIVENESS TO BOTRYTIS POLYGALACTURONASES1 (RBPG1, RLP42), which are all RLPs involved in pathogen perception in tomato (*Solanum lycopersicum*, *Sl*) or *Arabidopsis thaliana* (*At*) (Liebrand et al., 2013; Zhang et al., 2013; Bi et al., 2014; Zhang et al., 2014; Albert et al., 2015). SOBIR1 has been suggested to be a positive regulator of plant defense, and overexpression of *AtSOBIR1* in *Arabidopsis* resulted in a constitutive defense phenotype (Gao et al., 2009).

Both in tomato and potato (*Solanum tuberosum*, *St*), two alleles of *SOBIR1* are present, named *SOBIR1* and *SOBIR1-like*, with putatively redundant functions (Liebrand et al., 2013; Liebrand et al., 2014). In several plant species, *SOBIR1* transcripts have been found to increase upon pathogen challenge or treatments with salicylic acid or pathogen elicitors (Liebrand et al., 2014; Peng et al., 2015). Moreover, *Sl*SOBIR1 and *Sl*SOBIR1-like, and *At*SOBIR1 were found to be required for resistance against several pathogens, including fungi and the oomycetes *Hyaloperonospora arabidopsidis* and *Phytophthora parasitica* (Liebrand et al., 2014; Zhang et al., 2014; Peng et al., 2015). It has been proposed that RLP-SOBIR1 complexes are functional equivalents of genuine RLKs in the sense that SOBIR1 provides the kinase domain that is lacking from the RLP (Gust and Felix, 2014; Liebrand et al., 2014; Bi et al., 2016).

Late blight, caused by the oomycete *Phytophthora infestans*, is the most threatening disease of potato. So far, breeding for late blight resistance has been focused on the introduction of cytoplasmic resistance (*R*) genes of the nucleotide-

binding leucine-rich repeat (NLR) class. However, the fast evolving RXLR effector repertoire of *P. infestans* has been shown to promptly defeat any introduced *R* gene (Vleeshouwers et al., 2011), and resistance mechanisms based on the recognition of more conserved effectors might provide a more durable alternative (Du et al., 2015). Elicitins form a major class of conserved oomycete effectors, having MAMP features, in *Phytophthora* and *Pythium* species (Derevnina et al., 2016). Recently, the RLP ELICITIN RESPONSE (ELR) was identified in the wild potato species *Solanum microdontum* (*Sm*). ELR specifically recognizes elicitins of *Phytophthora* spp., and was shown to quantitatively enhance resistance against *P. infestans* in cultivated potato (Du et al., 2015). ELR localizes at the plasma membrane, similar to other RLPs involved in development and immunity (Du et al., 2015). Unlike other MAMP-triggered responses, recognition of elicitins results in a swift cell death response in *Solanum* and *Nicotiana* species (Chaparro-Garcia et al., 2011; Du et al., 2015).

Independent of whether the PRR is an LRR-RLP or LRR-RLK, it has been shown that upon elicitor perception there is recruitment of members of the SERK family of LRR-RLKs by the activated PRR (Chinchilla et al., 2007; Albert et al., 2015; Postma et al., 2016). The most well-studied member of this family is BAK1 (BRI1-ASSOCIATED KINASE 1), also known as SERK3 (SOMATIC EMPBRYOGENESIS RECEPTOR KINASE 3). Similar to SOBIR1, two SERK3 alleles are recognized in *Solanum*; SERK3a and SERK3b, with presumably overlapping functions as well (Peng and Kaloshian, 2014). *Nicotiana benthamiana* (*Nb*) SERK3 was identified as a component required for INF1-induced cell death (Chaparro-Garcia et al., 2011). In line with this, ELR was found to associate with SERK3/BAK1, and this association was found to be stabilized upon INF1 elicitation (Du et al., 2015). However, whether other co-regulatory RLKs mediate response to INF1 is unknown.

In this study, we employed VIGS and genetic complementation to test whether SOBIR1 is required for resistance to *P. infestans* and INF1-induced cell death. We found that SOBIR1 is required for resistance of *N. benthamiana* to various *P. infestans* isolates and is indispensable for INF1-triggered cell death. By co-immunoprecipitation assays we found that SOBIR1 constitutively associates with ELR *in planta*, in contrast to the enhanced interaction with SERK3. Furthermore, we demonstrate that the kinase domain of SOBIR1 is required for cell death induction by INF1, while it is not required for interaction with ELR. Overall we provide

evidence that ELR functions in a tri-partite complex with SOBIR1 and SERK3 in order to trigger downstream signaling upon elicitin perception. Additionally, we show that SOBIR1 supports ELR function, which, along with potential other RLPs also requiring SOBIR1 for their function, mediates resistance to *P. infestans*.

Results

SOBIR1 contributes to defense against *Phytophthora infestans*

Since SOBIR1 is required for the function of several RLPs and for basal resistance against pathogens, including *P. parasitica* (Peng et al., 2015), we hypothesized that SOBIR1 could be involved in plant defense against *P. infestans*. To test this, we employed a tobacco rattle virus (TRV)-mediated virus-induced gene silencing (VIGS) approach. *N. benthamiana* plants were agro-inoculated with *A. tumefaciens* carrying the TRV-*NbSOBIR1/-like* construct that has previously been shown to knock-down the expression of *NbSOBIR1* and *NbSOBIR1-like* (Liebrand et al., 2013). TRV-*GUS* (serving as a negative control) and TRV-*PDS* (targeting phytoene desaturase to monitor the onset of silencing), were included in the experiment (Kumagai et al., 1995; Liu et al., 2002).

Quantitative RT-PCRs confirmed that *NbSOBIR1* expression was significantly reduced in the TRV-*NbSOBIR1/-like*-inoculated plants at three weeks post inoculation, while *NbSOBIR1-like* was found, as expected, to be merely expressed (Fig. S1) (Liebrand et al., 2013). Leaves were taken from the silenced plants and spot-inoculated with the fluorescent *P. infestans* isolate 88069td (expressing tdTomato red fluorescent protein). Lesion growth was visually examined five days after inoculation and we observed consistently larger lesions on the TRV-*NbSOBIR1/-like*-inoculated leaves as compared to plants inoculated with TRV-*GUS* (Figs. 1a, S2). Molecular quantification of *P. infestans* biomass on inoculated *N. benthamiana* leaves, by measuring the abundance of *Pi*β-tubulin and *NbEF1*α as an internal standard, confirmed that a much higher pathogen biomass was detected in TRV-*NbSOBIR1/-like*-inoculated leaves (Fig. 1b). Inoculation with two isolates currently causing late blight epidemics in the UK and the Netherlands, UK3928A and Katshaar, respectively, resulted in a similar finding (Figs. 1b, S2). This result indicates that the observed increase in plant susceptibility upon *NbSOBIR1/-like* silencing is not isolate-specific. Furthermore, these data show that SOBIR1 and SOBIR1-like clearly contribute to resistance against *P. infestans*.

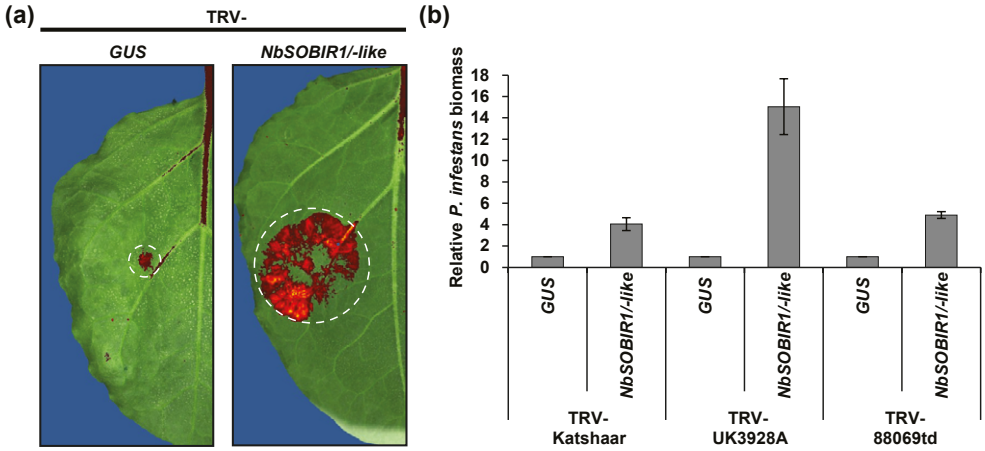

FIGURE 1 | SOBIR1 is required for defense responses against *Phytophthora infestans*. Four week-old *Nicotiana benthamiana* plants, inoculated with recombinant TRV carrying either an *NbSOBIR1/-like* or *GUS* insert, were inoculated with zoospores obtained from the three *P. infestans* isolates Katshaar, UK3928A, or 88069td. (a) Fluorescent/visible overlay image of mycelial growth of isolate 88069td expressing tdTomato red fluorescent protein on TRV-inoculated *N. benthamiana* at 5 dpi. (b) Relative biomass quantification of *P. infestans* isolates on TRV-inoculated *N. benthamiana* leaves at 5 dpi. Quantification was performed by RT-qPCR and by comparing the amplification of *P. infestans* β-*tubulin* to *N. benthamiana EF1*α that was used as an internal standard. Error bars indicate the standard deviation of two technical replicates of one representative experiment. The experiment was repeated three times and gave similar results.

SOBIR1 is involved in INF1-induced cell death in *N. benthamiana*

INF1 and other elicitins are known to cause a *Nb*SERK3-dependent cell death in *N. benthamiana* (Kamoun et al., 1997; Chaparro-Garcia et al., 2011). To investigate whether elicitin-triggered defense signaling in *N. benthamiana* also requires *NbSOBIR1*, we conducted a VIGS experiment. For this, *N. benthamiana* plants were agro-inoculated with TRV-*NbSOBIR1/-like*. Agro-inoculation with TRV-*NbSERK3a/b* and TRV-*GUS* were included as positive and negative controls, respectively, while TRV-*PDS* was included as a visual control for the onset of silencing. At three weeks after the inoculation with TRV, plants were agro-infiltrated to express INF1 or empty vector (EV). After four days, the expected INF1-induced cell-death was evident in the TRV-*GUS*-inoculated plants, but significantly decreased cell death was detected in leaves of TRV-*NbSOBIR1/-like*- and TRV–*NbSERK3a/b*-inoculated *N. benthamiana* plants (Fig. 2a, b). Similar loss of INF1-triggered cell death was observed when the same *GUS*- and *NbSOBIR1/-like*-silenced leaves were infiltrated with 1 μM of purified INF1 protein (Fig. S3a, b). These data show that *NbSOBIR1* is required for the INF1-mediated cell death response in *N. benthamiana*.

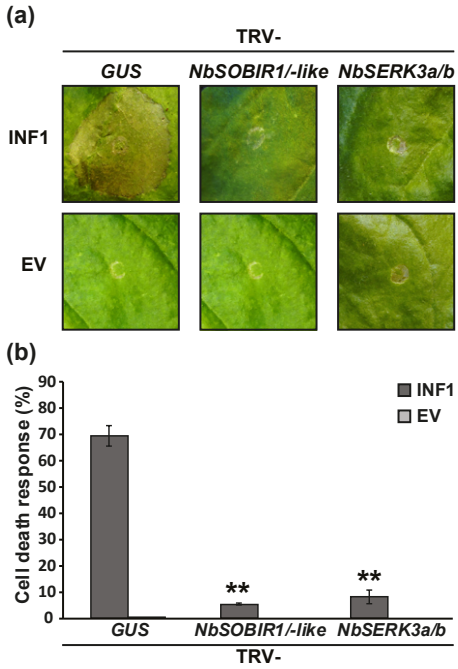

FIGURE 2 | SOBIR1 is required for the cell death response triggered by INF1 elicitin in *Nicotiana benthamiana*. Four week-old *N. benthamiana* plants, inoculated with TRV-*NbSOBIR1/-like*, TRV-*GUS* (negative control) or TRV-*NbSERK3a/b* (positive control), were agro-infiltrated with INF1 or empty vector (EV) at the onset of *PDS* silencing (not shown). (a) Representative pictures of treated leaves at 4 dpi. (b) Cell death quantification of treated leaves at 4 dpi. Error bars indicate the standard error of three biological repeats (n = 64). Asterisks indicate significance at $p<0.01$ (Student's t-test).

3

Synthetic SOBIR1 from *S. microdontum* can complement SOBIR1 from *N. benthamiana*

SOBIR1 is highly conserved in plants, but some degree of diversity occurs (Liebrand et al., 2014). We used PCR-based cloning to identify the homologs of SOBIR1 in *S. microdontum*, which is the source of ELR. We found that *Sm*SOBIR1 and *Sm*SOBIR1-like homologs share 97.9% and 99% amino acid similarity to *St*StSOBIR1 (Sotub06g029250.1.1) and *St*SOBIR1-like (Sotub03g023250.1.1), respectively (Fig. S4). To test whether *Sm*SOBIR1 can complement for INF1-induced cell death in *NbSOBIR1-/like* silenced *N. benthamiana* plants, we generated a synthetic version that encodes the original (WT) *Sm*SOBIR1 protein sequence from *S. microdontum* (*SmSOBIR1syn*^{WT}), but which is not targeted by the TRV-*NbSOBIR1/-like* VIGS construct used in VIGS experiments with *N. benthamiana* (Liebrand et al., 2013) (Figs S5a, S6). To verify that the synthetic gene drives expression of the expected protein, we performed a western blot that confirmed the presence of Myc-tagged *Sm*SOBIR1syn^{WT} in plants silenced for *NbSOBIR1/-like* (Fig. S5b). Subsequently, we performed a cell-death complementation experiment. For this, we silenced *N. benthamiana* plants by agro-inoculation with TRV-*NbSOBIR1/-like* and took along TRV-*GUS* as a negative control, and subsequently transiently co-expressed *Sm*SOBIR1 (control) or the *Sm*SOBIR1syn^{WT} with INF1. We found that INF1-triggered cell death was not affected in the TRV-*GUS*-inoculated plants, while in

the TRV-*NbSOBIR1/-like*-inoculated plants INF1-triggered cell death was severely compromised. Interestingly, *Sm*SOBIR1syn^{WT} co-expression with INF1 restored the cell death response (Fig. 3). This observation indicates that *Sm*SOBIR1syn^{WT} complements for the loss of *Nb*SOBIR1/-like in *Nb*SOBIR1/-like-silenced plants.

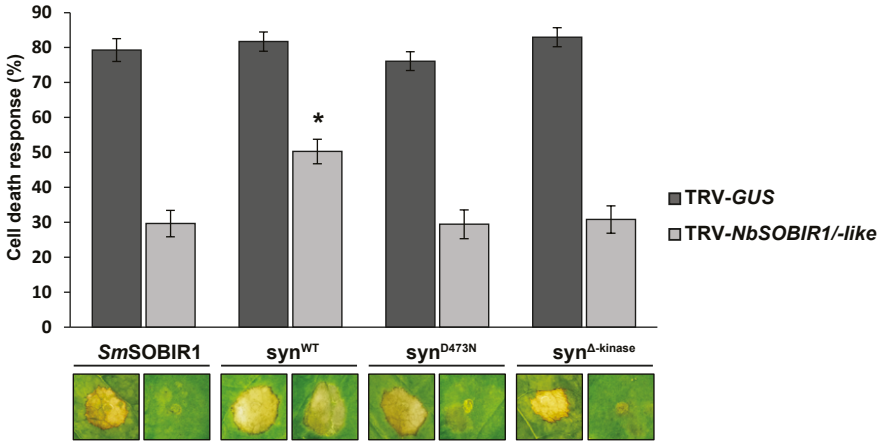

FIGURE 3 | Synthetic versions of *SmSOBIR1* complement the loss of INF1-triggered cell death in TRV-*NbSOBIR1/-like*-inoculated *Nicotiana benthamiana*. *Sm*SOBIR1 wild type (WT) (control), *Sm*SOBIR1syn^{WT}, *Sm*SOBIR1syn^{D473N} or *Sm*SOBIR1syn$^{\Delta\text{-kinase}}$, were transiently co-expressed in TRV-*GUS* or TRV-*NbSOBIR1/-like*-inoculated plants, together with INF1. Cell death was scored at 5 dpi and representative pictures are shown. Results are an average of three independent biological repeats. The asterisk indicates significance at $p<0.05$ (One-way ANOVA, LSD test).

SOBIR1 requires a functional kinase domain for mediating INF1-induced cell death

Next, we investigated whether kinase activity of SOBIR1 is required for cell death induction by INF1. We cloned different versions of *SmSOBIR1syn,* in which the core catalytic aspartate (D) of the conserved RD kinase motif is substituted by an asparagine (N) residue (*Sm*SOBIR1syn^{D473N}); a mutation that causes loss of kinase activity for all tested so-called "RD" RLKs tested so far (Schwessinger et al., 2011; Liebrand et al., 2013). In addition, we generated a *Sm*SOBIR1syn mutant that completely lacks the kinase domain (with a deletion of amino acids 333 to 625; *Sm*SOBIR1syn$^{\Delta\text{-kinase}}$)(Fig. S5a). Similar to *Sm*SOBIR1syn^{WT}, by using western blotting, we confirmed that the synthetic *SmSOBIR1* mutant versions drive production of the expected protein variants in plants silenced for *NbSOBIR1/-like* (Fig. S5b). Next, we again proceeded to a cell-death complementation experiment by co-expressing the kinase-inactive *Sm*SOBIR1syn mutants with INF1 in *NbSOBIR1/-like*-silenced *N. benthamiana* plants. We found that, unlike *Sm*SOBIR1syn^{WT}, both *Sm*SOBIR1syn^{D473N} and *Sm*SOBIR1syn$^{\Delta\text{-kinase}}$ failed to restore the INF1-triggered cell

death response, indicating that the kinase activity of SOBIR1 is required for INF1-triggered cell death (Fig. 3).

ELR associates with the co-regulatory RLK SOBIR1

In order to investigate whether ELR associates with *Sm*SOBIR1/-like from *S. microdontum*, we performed co-immunoprecipitation experiments. We generated constructs for transiently expressing C-terminally Myc epitope-tagged *Sm*SOBIR1 and *Sm*SOBIR1-like and co-expressed them with ELR-eGFP in *N. benthamiana*. The RLP Cf-4 was included as a positive control, and the RLK FLS2 and eGFP alone were used as negative controls (Liebrand et al., 2013). As shown in Fig. 4, immunopurification of ELR-eGFP, Cf-4-eGFP and FLS-2-GFP by using GFP affinity beads and subsequent detection of co-purifying *Sm*SOBIR1-myc and *Sm*SOBIR1-like-myc, indicates that, like Cf-4, ELR associates *in planta* with *Sm*SOBIR1 and *Sm*SOBIR1-like, whereas no interaction of the two SOBIR1 homologs with FLS2 and eGFP was observed.

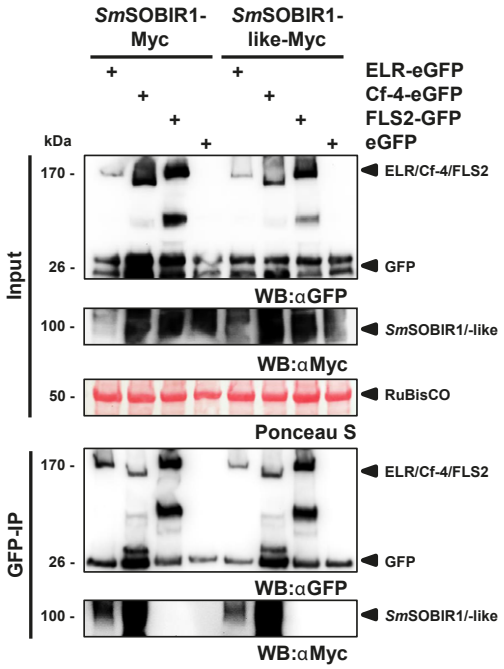

FIGURE 4 | ELR forms a complex with *Sm*SOBIR1 and *Sm*SOBIR1-like. *Sm*SOBIR1-myc and *Sm*SOBIR1-like-myc were transiently co-expressed in *Nicotiana benthamiana* leaves, together with ELR-eGFP, Cf-4-eGFP (positive control), FLS2-GFP or eGFP (negative controls), as indicated. Total protein was extracted and was subjected to immunoprecipitation using GFP_TrapMA beads to capture ELR, Cf-4 and FLS2. The immunopurified proteins were detected with anti-GFP, while the interaction with *Sm*SOBIR1-Myc or *Sm*SOBIR1-like-Myc was assessed with anti-Myc. Ponceau S staining of RuBisCO indicates equal protein loading. This figure is representative for three biological repeats.

The *Sm*SOBIR1 kinase domain is not required for interaction with ELR

To test whether the kinase domain of SOBIR1 is required for interaction with ELR, we co-expressed ELR with either an empty vector, *Sm*SOBIR1syn^{WT} or the *Sm*SOBIR1syn versions either lacking kinase activity or lacking the kinase domain (*Sm*SOBIR1syn^{D473N} and *Sm*SOBIR1syn$^{\Delta\text{-kinase}}$, respectively), in *N. benthamiana*. Our results show that SOBIR1 kinase activity and the kinase domain itself are not required for its interaction with ELR, similar to what has been shown for the interaction between Cf-4 and SOBIR1, as in all cases the interaction with ELR remains intact (Fig. S7a) (Bi et al., 2016).

In addition to this, we noted an ELR-stabilizing effect when SOBIR1 is co-expressed with this RLP. To confirm this observation, we performed a similar experiment as above. For this, ELR and the different *Sm*SOBIR1 variants were transiently co-expressed in *N. benthamiana*, total protein was extracted at two days after agro-infiltration, and ELR accumulation was determined by western blotting and signal intensity quantification. In three independent experiments, we found that co-expression of the *Sm*SOBIR1 variants, all accumulating at similar levels and interacting with ELR (Figs. S5b, S7a), led to an increase in the amounts of ELR accumulating, as compared to co-expression with the empty vector (Fig. S8). *Sm*SOBIR1 co-expression with ELR was found to result in an up to 13-fold increase in ELR accumulation. Moreover, both the kinase-active and kinase-inactive versions of SOBIR1, in addition to *Sm*SOBIR1syn completely lacking the kinase domain, have a stabilizing effect on ELR, though a bit lower as compared to *Sm*SOBIR1syn. This indicates that the stabilization effect is likely not directly related to kinase activity of SOBIR1, but is rather based on ELR interaction with SOBIR1.

Summarizing, these results show that the interaction between ELR and SOBIR1 is stabilizing the complex. Moreover, ELR association with SOBIR1 likely requires the extracellular-juxtamembrane, transmembrane and intracellular-juxtamembrane domains of ELR, as proposed for other RLPs (Fig. S8b) (Gust and Felix, 2014; Bi et al., 2016).

ELR functions in a tri-partite complex with SOBIR1 and SERK3

It was previously shown that ELR associates with SERK3 and that this association is specifically enhanced by INF1 elicitation. In order to investigate whether the observed constitutive interaction of ELR with SOBIR1 is affected by INF1, we co-expressed ELR with SOBIR1 and SERK3 in *N. benthamiana*. Two days after agroinfiltration, we infiltrated purified INF1 protein or the buffer in which INF1 was dissolved, and checked whether ELR interacted with SOBIR1 and SERK3 through co-immunoprecipitation and western blotting (Fig. 5). We found that,

upon 15 min elicitation with 1 μM INF1 protein (see Fig. S3b), ELR interaction with SERK3 is enhanced, as expected (Fig. 5) (Du et al., 2015). On the other hand, ELR interaction with SOBIR1 was not affected by INF1 elicitation, indicating that the interaction is constitutive and remains intact. These data show that ELR is present as a constitutive two-component RLK with SOBIR1, and forms a tri-partite ELR-SOBIR1-SERK3 complex upon INF1 perception.

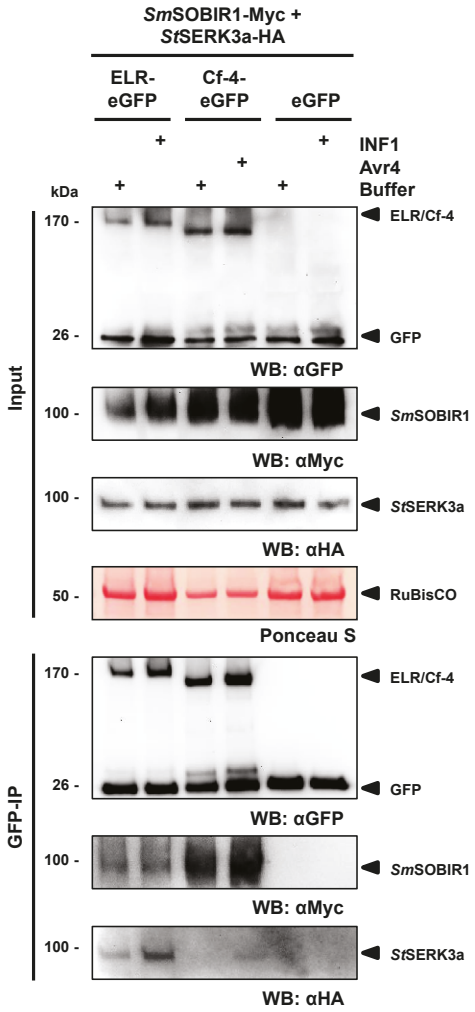

FIGURE 5 | ELR forms a constitutive complex with SOBIR1, while it interacts with SERK3a in an INF1-inducible manner. *Sm*SOBIR1-myc and *St*SERK3a-HA were transiently co-expressed in *Nicotiana benthamiana* leaves, together with ELR-eGFP, Cf-4-eGFP or eGFP, as indicated. Elicitation was performed at three days post infiltration with 1 μM purified INF1 protein (for ELR and eGFP) or 1 μM purified Avr4 (for Cf-4). Total protein was extracted and was subjected to immunoprecipitation using GFP_TrapMA beads to capture ELR, Cf-4 and eGFP respectively. The immunopurified proteins were detected with anti-GFP, while the interaction with *Sm*SOBIR1-Myc or *St*SERK3a-HA, was assessed with anti-Myc or anti-HA, respectively. Ponceau S staining of RuBisCO indicates equal protein loading. This figure is representative for three biological repeats.

Discussion

In this study we show that basal resistance of *N. benthamiana* to *P. infestans* requires *Nb*SOBIR1. This is evident since *N. benthamiana* plants silenced for *NbSOBIR1* showed larger lesions and increased *P. infestans* biomass, for a range of different isolates. Our results are in line with the recent finding that resistance against *P. parasitica* of tomato requires SOBIR1 (Peng et al., 2015). Overall, our data support the importance of SOBIR1 in basal defense against pathogens, likely by supporting ELR function and potentially that of other LRR-RLPs as well (Liebrand et al., 2014).

ELR carries a short cytoplasmic tail without any obvious signaling domain and as such, it was hypothesized that additional partner proteins are mediating signal transduction. Indeed, SERK3/BAK1, a common interactor of LRR-containing PRRs, has been found to associate with ELR (Du et al., 2015). Recently, the LRR-RLK SOBIR1 has been shown to constitutively associate with a multitude of RLPs (Gust and Felix, 2014; Liebrand et al., 2014; Zhang et al., 2014; Albert et al., 2015; Bi et al., 2016). In this study we provide evidence that SOBIR1 is also required as a co-regulatory RLK for the response to the elicitin INF1. By gene silencing we have demonstrated that the cell death response to INF1 in *N. benthamiana* requires SOBIR1, similar to what has been described for ParA1 (Peng et al., 2015). In addition, by employing genetic complementation assays using synthetic *SmSOBIR1*, we found that the INF1-triggered cell death response requires an active *Sm*SOBIR1 kinase domain. This indicates that the SOBIR1 kinase domain actively takes part in downstream defense signaling initiated by INF1, which is in line with previous studies on ParA1, and Avr4 in Cf-4-expressing plants (Liebrand et al., 2013; Peng et al., 2015). However, the PRRs responsible for elicitin-triggered responses in tomato cv. Summer Sweet and *N. benthamiana* have not been cloned. Tomato is known to have a close ELR homolog, however, no obvious homologs are found in *N. benthamiana*. Therefore, it remains unknown whether those responses are due to the presence of a putative functional ELR orthologue (Peng et al., 2015).

Biochemical evidence from this work supports the observation that the INF1 receptor physically associates with SOBIR1. Using co-immunoprecipitation, we found that ELR associates *in planta* with both *Sm*SOBIR1 and its close homolog *Sm*SOBIR1-like and a stabilized complex is formed. This association is constitutive, since it does not require INF1 and remains unaltered upon INF1 elicitation. ELR thus behaves similar to other RLPs involved in immunity, such as Ve1, Cf-2, Cf-4, Cf-9, RLP23, RLP30 and RLP42 (Liebrand et al., 2013; Zhang et al., 2013; Bi et al., 2014; Zhang et al., 2014; Albert et al., 2015). Moreover, we have consistently observed a stabilization effect of ELR when SOBIR1 was co-expressed. Although a different setup was used, this finding is complementary to the observation of reduced Cf-4

and Ve1 protein levels when *SOBIR1* was silenced (Liebrand et al., 2013). For Cf-4 it was concluded that SOBIR1 potentially acts as a scaffold protein (Bi et al., 2016), in addition to being required for downstream signaling (Liebrand et al., 2013). For both ELR and Cf-4, kinase activity of SOBIR1 is not required for stabilization, which is in agreement with the hypothesis that the GxxxG dimerization motif present in the trans-membrane domains, in addition to the juxta membrane domains of ELR and SOBIR1, are involved in their interaction as was proposed for many RLP-type LRR receptors (Gust and Felix, 2014; Bi et al., 2016).

ELR has been shown to localize at the plasma membrane (Du et al., 2015). Therefore, it can be assumed that interaction with SOBIR1/SERK3 is also occurring at this location, similar to what was observed for Cf-4 (Postma et al., 2016). *C. fulvum* Avr4-induced endocytosis of SOBIR1 in Cf-4-expressing plants has been recently shown in *N. benthamiana* (Postma et al., 2016). Therefore, it is likely that INF1 elicitation also causes endocytosis of the ELR-SOBIR1 complex. In agreement with this hypothesis, the elicitins cryptogein (from *P. cryptogea*) and ParA1 (from *P. parasitica*), have been shown to induce clathrin-mediated endocytosis of SOBIR1 (Leborgne-Castel et al., 2008; Peng et al., 2015). Future studies should address whether this endocytosis is SERK3-dependent as well (Postma et al., 2016).

Studies on elicitin recognition in *N. benthamiana* have revealed the E3 ligase CYS, MET, PRO, AND GLY PROTEIN 1 (CMPG1) to be an important component for the cell death response triggered by INF1 (Bos et al., 2010). CMPG1 acts as a hub for signaling downstream of both MAMP and effector perception, as it is required for the Cf-, Pto- and CELLULOSE-BINDING ELICITOR LECTIN (CBEL)-mediated cell death response as well (Gilroy et al., 2011). Despite the lack of molecular evidence showing interaction of CMPG1 with PRRs, we anticipate that the E3 ligase activity of CMPG1 modulates the first steps downstream of effector perception, possibly through promoting ubiquitination (Gilroy et al., 2011). SOBIR1 seems to be heavily modified as it migrates as a smear on SDS gels as revealed by western blots (i.e. as shown in SOBIR1 bands in Figs. 4, 5), however the type of modifications and their biological relevance have not been reported yet. *P. infestans* is known to secrete AVR3a, an RXLR effector which suppresses INF1-triggered cell death. AVR3a is suppressing MAMP-triggered responses in at least two ways; either by binding to CMPG1 or by blocking receptor endocytosis via association with DYNAMIN-RELATED PROTEIN 2 (DRP2), a protein involved in cellular trafficking (Bos et al., 2010; Chaparro-Garcia et al., 2015). With the discovery that ELR functions in a complex with SERK3 and SOBIR1, it is now appropriate to test whether CMPG1 is involved in trafficking of activated ELR-SOBIR1 and whether AVR3a is interfering in this process.

Recently, the cloning of ELR gave insight into the molecular mechanisms involved in apoplastic elicitin perception (Du et al., 2015; Derevnina et al., 2016). Understanding how RLPs such as ELR function is a major step towards their deployment as resistance genes in plant breeding. With our work we show that elicitin recognition and basal defense in *Solanaceae* requires SOBIR1 which supports ELR function and immunity to *P. infestans*. ELR functions thus similar to Cf-4- and Cf-9- mediated recognition of Avr4 and Avr9, respectively, and the downstream responses also appear to be similar (Liebrand et al., 2013; Postma et al., 2016). We propose that, the ELR-SOBIR1 complex functions as a two component RLK that recruits SERK3 in an INF1-dependent manner. This tripartite complex formation is reminiscent of the well-characterized RLKs FLS2 and EFR which, upon elicitation with their respective ligands flg22 and elf18, are associating with SERK3/BAK1 (Zipfel et al., 2006; Chinchilla et al., 2007; Boller and Felix, 2009). Since PRRs contribute to quantitative resistance and thus offer a lower selection pressure, stacking of ELR with other PRRs such as RLP23 could lead to a more durable resistance against the devastating pathogen *P. infestans* (Albert et al., 2015; Du et al., 2015).

Materials and methods

Plant material and growth conditions

Nicotiana benthamiana plants were grown from seeds and maintained in climate controlled greenhouse compartments at 22/18 °C and 16/8 h light day/night regime at 70% relative humidity. All protein expression and cell death assays were performed under these conditions.

Production and purification of INF1

INF1 was produced from a stationary culture of *P. infestans* strain IPO-C, as previously described (Chaparro-Garcia et al., 2011), with some minor modifications. Briefly, *P. infestans* was grown for 4-5 weeks in liquid Plich medium. The mycelium was removed by passing through filter paper and the culture medium was snap-frozen and freeze-dried. The resulting powder was dissolved in 100 ml of 10 mM Tris-HCl, 10 mM NaCl (pH 7.4) buffer. The solution was then dialyzed overnight (3.5 kDa cut off, Spectrum RC dialysis tubing), against the same buffer, at 4°C. The resulting INF1-containing solution was loaded onto a 10 mM Tris-HCl (pH 7.4)-equilibrated Q Sepharose Fast-Flow column (GE Healthcare). Column was washed with three column volumes with the same equilibration buffer. Next, the column was eluted with a linear gradient of 0 - 500 mM NaCl in 10 mM Tris-HCl (pH 7.4) and fractions

of 10 ml were collected. The presence of INF1 in the different fractions was assayed by SDS-PAGE, followed by CBB or silver staining. Protein concentration was estimated by BCA assay (Thermo-Fisher Scientific). Purified INF1 was infiltrated in *N. benthamiana* leaves to confirm cell-death inducing activity. Avr4 protein from *Cladosporium fulvum* has been produced previously in *Pichia pastoris* (van den Burg et al., 2001).

Binary vectors for *Agrobacterium tumefaciens*-mediated transient transformation

Construction of pBin-KS-*p35s::ELR-eGFP*, pBin-KS-*p35s::Cf-4-eGFP*, pCAMBIA2300–*pAtFLS2::SlFLS2-GFP* and pTRV1 has been described (Liu et al., 2002; Liebrand et al., 2012; Liebrand et al., 2013; Du et al., 2015). For cloning of novel constructs, target gene sequences were amplified from cDNA using Phusion proofreading polymerase (Thermo Fisher Scientific), employing primers mentioned in Supplementary Table 1(Table S1). The *SmSOBIR1* and *SmSOBIR1-like* genes, as well as a *PDS* VIGS fragment (targeting phytoene desaturase and used as a control for gene silencing) were amplified from *S. microdontum* genotype 360-1 (source of ELR) cDNA.

A synthetic (syn) construct of *Sm*SOBIR1 carrying a D473N mutation (*Sm*SOBIR1syn^{D473N}) and synonymous nucleotide changes to enable expression in *TRV-NbSOBIR1/-like* VIGSed *N. benthamiana* was synthesized (Genscript) (Figs. S4a, S5). Using overlap extension PCR, the wild type version (*Sm*SOBIR1syn^{WT}) was reconstituted as described in Liebrand et al. (2013) (see Table S2, Fig. S4). A version of *Sm*SOBIR1syn (nucleotides 1 - 996) lacking the kinase domain (*Sm*SOBIR1syn$^{\Delta\text{-kinase}}$) was amplified using *Sm*SOBIR1syn^{D473N} as a template.

A *GUS* VIGS fragment was amplified from the commercial vector pENTR-*GUS* (Invitrogen). *SmSOBIR1*, *SmSOBIR1-like*, *Sm*SOBIR1syn as well as the VIGS fragments for *GUS* and *SmPDS* were cloned in pENTR/D-TOPO vector (Invitrogen). The inserts of all entry clones were checked by sequencing and transferred to destination vectors by means of an LR reaction using LR-Clonase II enzyme mix (Invitrogen), resulting in the following constructs: pGWB20-*p35s::SmSOBIR1-myc*, pGWB20-*p35s::SmSOBIR1-like-myc*, pGWB20-*p35s::SmSOBIR1syn^{WT}-myc*, pGWB20-*p35s::SmSOBIR1syn^{D473N}-myc*, pGWB20-*p35s::SmSOBIR1syn$^{\Delta\text{-kinase}}$-myc* (-myc refers to C-terminal 10x Myc), pTRV2-*GUS* and pTRV2-*PDS* (Table S2).

Co-immunopurification and immunoblotting

The proteins under study were transiently expressed in young, fully-expanded leaves of 3 week-old *N. benthamiana* plants using *Agrobacterium*-mediated transient transformation (agro-infiltration). At 2 d post agro-infiltration, leaves were collected, snap-frozen in liquid nitrogen and ground to a fine powder. Protein extraction was performed in modified RIPA buffer, containing IGEPAL CA-630 as a sole detergent and supplemented with EDTA-free protease inhibitor cocktail (Roche), as described previously (Liebrand et al., 2012; Liebrand et al., 2013). Briefly, modified RIPA buffer was added at a ratio of 2 ml/g of frozen ground plant material and mixed. The mixture was incubated at room temperature with occasional mixing, until all frozen material was thawed. Then, 2 ml of protein extract was centrifuged for 30 min at 13000 × g to pellet plant debris. Clarified extracts, from which input samples were collected, were incubated with 15 µl RIPA-equilibrated GFP-Trap_MA beads (50 % slurry, Chromotek) for 1 h at 4˚C. Beads were then washed 5 times with RIPA, after which they were incubated at 95˚C for 5 min in 100 µl 2x Laemli SDS buffer for 10 min, while 150 µl of input sample was mixed with 50 µl 4x Laemli SDS buffer and also incubated at at 95˚C for 5 min. In order to dissolve protein aggregates prior to SDS-PAGE, samples were mixed 1:1 with 8 M urea and incubated at room temperature for 10-20 min. The samples were then again incubated at 95˚C for 5 min, centrifuged at 13000 × g and proteins present in 35 µl of the supernatant were separated by SDS-PAGE using TGX 4-20% gradient gels (BioRad). Subsequently, proteins were transferred to a PVDF membrane using a MiniProtean wet transfer system (BioRad), following standard procedures. Blots to be incubated with anti-GFP antibodies were blocked in Tris-buffered saline with 0.05% Tween20 (TBS-T), containing 3% bovine serum albumin (BSA), while blots to be incubated with αMyc and αHA were blocked with TBS-T containing 5% skimmed milk. The antibodies were added in TBS-T solution at the following dilutions: αGFP-HRP 1.5:5000 (Miltenyi Biotech), αHA-HRP 1.5:5000 (Miltenyi Biotech), αMyc-HRP 2.5:5000 (Santa Cruz-biotech). For chemo-luminescent signal development, SuperSignal West-Femto substrate (ThermoFisher Scientific) was used. Imaging of western blots was done using a G-BOX system (Syngene).

VIGS assays

VIGS experiments were performed using wildtype *N. benthamiana* plants, as described (Liebrand et al., 2013). Briefly, 2 week-old *N. benthamiana* seedlings were inoculated by agro-infiltration (agro-inoculation) with 1:1 mixtures of pTRV1 (Liu et al., 2002) in combination with pTRV2*::NbSOBIR1/-like* (Liebrand et al., 2013), pTRV2*::NbSERK3a/b* (Heese et al., 2007; Chaparro-Garcia et al., 2011), pTRV2*::GUS*, or pTRV2*::PDS*, at a final OD_{600} of 0.5 using a needleless syringe.

Agrobacterium transient cell death assays

For INF1 cell death induction assays, young, fully expanded leaves of 4-5 week-old TRV-*GUS*- or TRV-*NbSOBIR1/-like*-inoculated *N. benthamiana* plants (i.e. 2-3 weeks after VIGS) were used. VIGSed leaves were agroinfiltrated with pCB302-3-*p35s-INF1* and pCB302-3-*p35s-EV* (empty vector) (Du et al., 2015), at an OD_{600} of 0.5. Leaves were visually examined for INF1-triggered cell-death at 4 d post-agroinfiltration. For genetic complementation assays using *Sm*SOBIR1syn variants, VIGSed plants were co-infiltrated with 1:1 mixtures of *Agrobacterium* carrying pCB302-3-*p35s-INF1*, in combination with one of the following constructs: pGWB20-*p35s::SmSOBIR1-myc*, pGWB20-*p35s::SmSOBIR1syn*^{WT}-*myc*, pGWB20-*p35s::SmSOBIR1syn*^{D473N}-*myc* or pGWB20-*p35s::SmSOBIR1syn*$^{\Delta\text{-kinase}}$-*myc*, at a final OD_{600} of 0.5. Infiltrated leaves were observed for INF1-induced cell death at 5 d post-infiltration. In all cases, the percentage of cell death was quantified using scores of 0%, 25%, 50%, 75% and 100%, based on visual observation of the infiltrated area showing cell death when compared to the total area, as described previously (Du et al., 2014).

Inoculations with *P. infestans*

Detached leaf assays with *P. infestans* on VIGSed *N. benthamiana* were performed as described (Vleeshouwers et al., 1999). Briefly, leaves were placed with their petioles into tap water-saturated floral foam (Oasis) with the abaxial side facing upwards. *P. infestans* zoospore suspensions were prepared as described previously (Vleeshouwers et al., 1999) and inoculations using these spores were performed twice per leaf, by pipetting 10 µl droplets of a suspension of 1×10^5 zoospores/ml (in tap water) onto the abaxial side. At 5 dpi, pictures were taken under normal or long wave UV-light. UV photos of the fluorescent isolate 88069td (expressing tdTomato red fluorescent protein, a fluorescent protein consisting of a tandem dimer of a dsRed monomeric mutant) (Whisson et al., 2007) were taken using the PathoScreen system (PhenoVation).

RNA extraction and quantitative PCR analyses

RNA was isolated from TRV-inoculated *N. benthamiana* plants at 3 week post inoculation. RNA extraction was performed using the RNAplant easy kit (Qiagen) and cDNA was synthesized on 1 µg of total RNA using Superscript II (Invitrogen), according to manufacturer's instructions. *NbSOBIR1* expression was quantified by RT-qPCR by using the expression of *N. benthamiana ELONGATION FACTOR 1*α (*EF1*α) as a reference (Nicot et al., 2005). Quantitative RT-PCRs were performed on a CFX96 Real-Time System (BioRad). Gene expression data were normalized to the

expression of *NbEF1*α and the $2^{-\Delta\Delta Ct}$ method was used for data analysis (Livak and Schmittgen, 2001). See Table S1 for the primers that were used.

P. infestans biomass quantifications on infected leaves were performed using qPCR. Leaf discs (30 mm in diameter) were excised from the inoculation spots of each leaf at 5 dpi. Per treatment, leaf material was pooled, snap frozen and ground to a fine powder, independently for each biological repeat. Genomic DNA was extracted using the DNeasy Plant kit (Qiagen) and *P. infestans* biomass was quantified in a similar way as for gene expression, by comparing the relative abundance of *P. infestans* β-*tubulin* to that of *N. benthamiana EF1*α (see Table S1).

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Author contributions

E.D., M.H.A.J., S.K. and V.G.A.A.V. planned and designed the research. E.D. and D.W., performed experiments and analyzed the data. E.D., V.G.A.A.V., R.G.F.V. and M.H.A.J., wrote the manuscript.

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Supplementary information

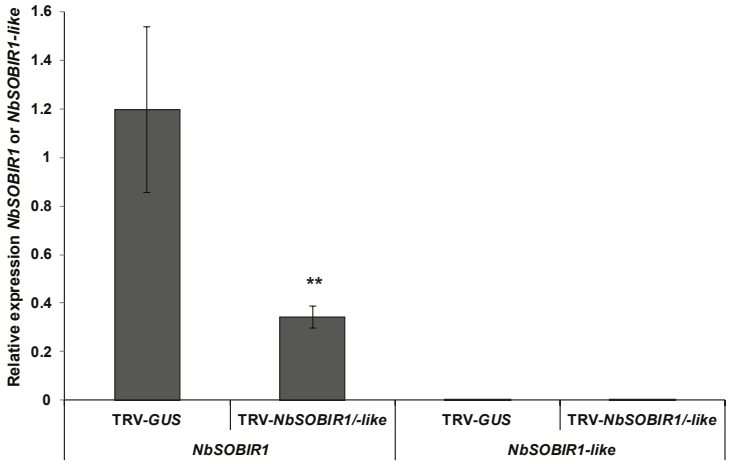

FIGURE S1 | *NbSOBIR1* is successfully silenced in TRV-*NbSOBIR1/-like*-inoculated *Nicotiana benthamiana*. *N. benthamiana* plants were inoculated with TRV-*GUS* or TRV-*NbSOBIR1/-like* and the relative expression of *NbSOBIR1* was determined at three weeks post inoculation. Quantitative RT-PCR data for *NbSOBIR1* were normalized to the expression of the *Nb*EF1α gene and a relative quantification was performed. Error bars indicate the standard deviation of three biological repeats. Asterisks indicate significance at p<0.01 (One-way ANOVA, LSD test).

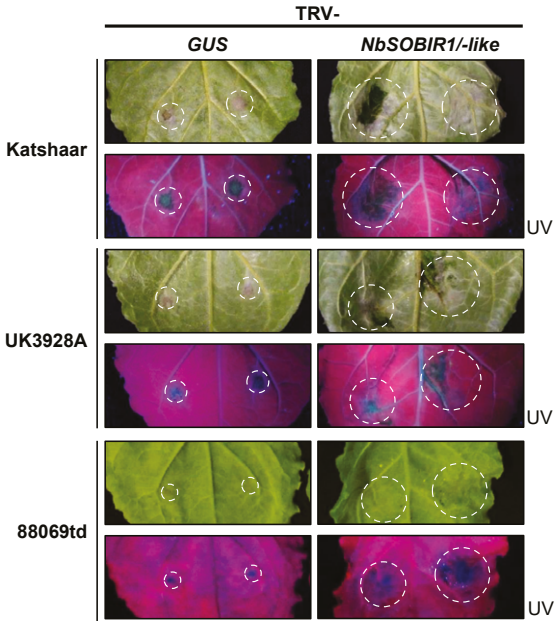

FIGURE S2 | SOBIR1 is required for resistance of *Nicotiana benthamiana* to *Phytophthora infestans*. Four week-old *Nicotiana benthamiana* plants, inoculated with TRV-*GUS* or TRV-*NbSOBIR1/-like* (Liebrand et al., 2013), were inoculated with zoospores obtained from three different *P. infestans* isolates (Katshaar, UK3928A and 88069td). Images of representative leaves were taken at 5 days post inoculation (dpi) under normal or UV light.

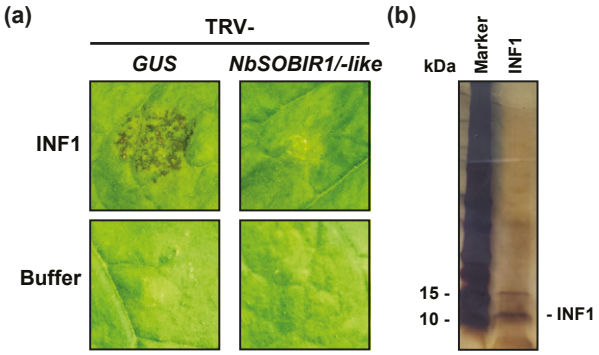

FIGURE S3 | SOBIR1 is required for the cell death response triggered by INF1 protein. Four week-old *Nicotiana benthamiana* plants, inoculated with TRV-*GUS* or TRV-*NbSOBIR1/-like* (Liebrand et al., 2013), were infiltrated with 1 μM INF1 protein. (a) Representative pictures from the INF1 protein infiltration taken at 4 days post infiltration. (b) SDS-PAGE and silver staining of the INF1 protein purified from *Phytophthora infestans* culture filtrate using anion exchange.

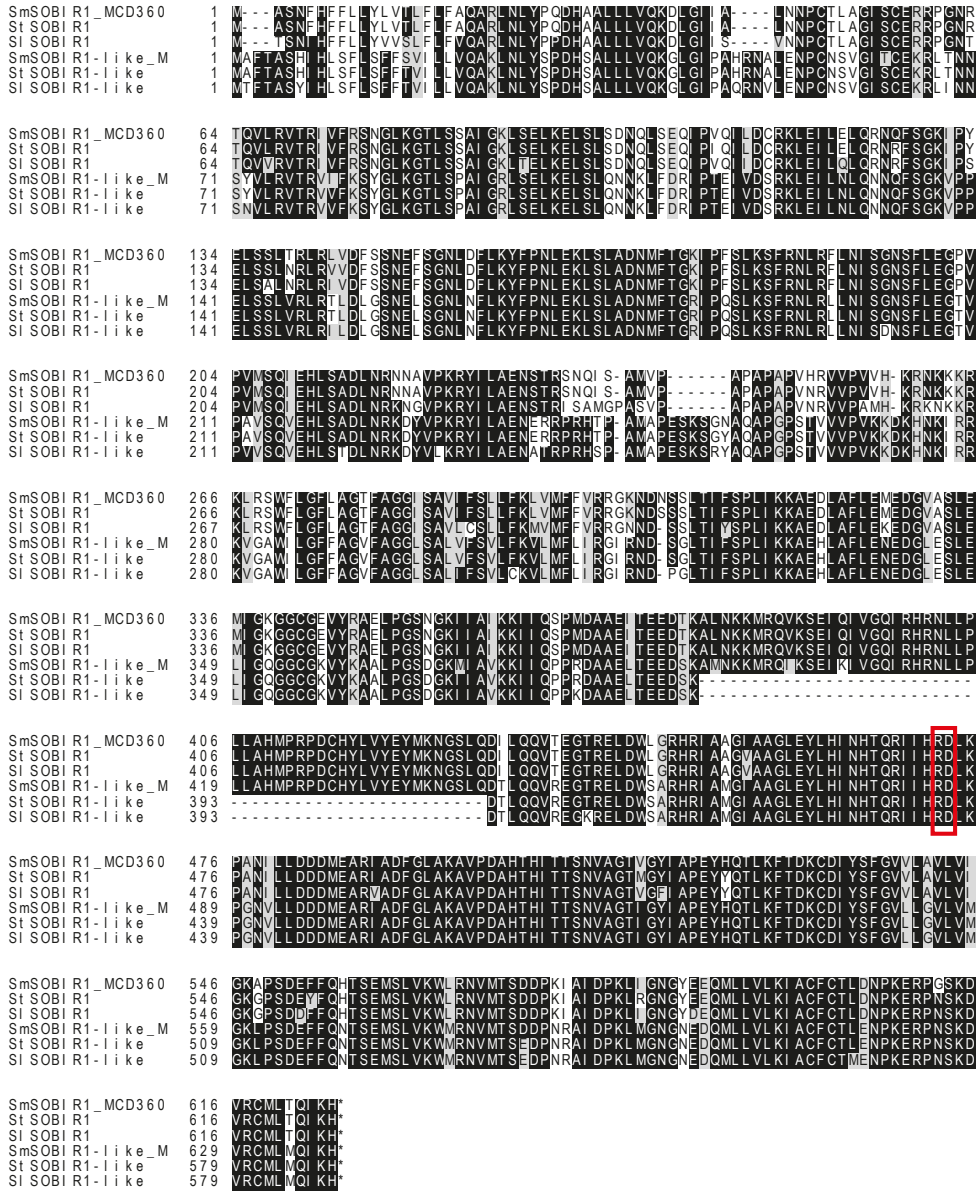

FIGURE S4 | Alignments of *Sm*SOBIR1 and *Sm*SOBIR1-like with *St*SOBIR1, *St*SOBIR1-like, *Sl*SOBIR1 and *Sl*SOBIR1-like. Amino acid sequence alignment of SOBIR1 was created using COBALT, and BOXSHADE was used to visualize conservation. Residues highlighted in black are identical in >50% of the six sequences, while residues highlighted in grey are residues with similar properties. The RD motif is highlighted by a red rectangle.

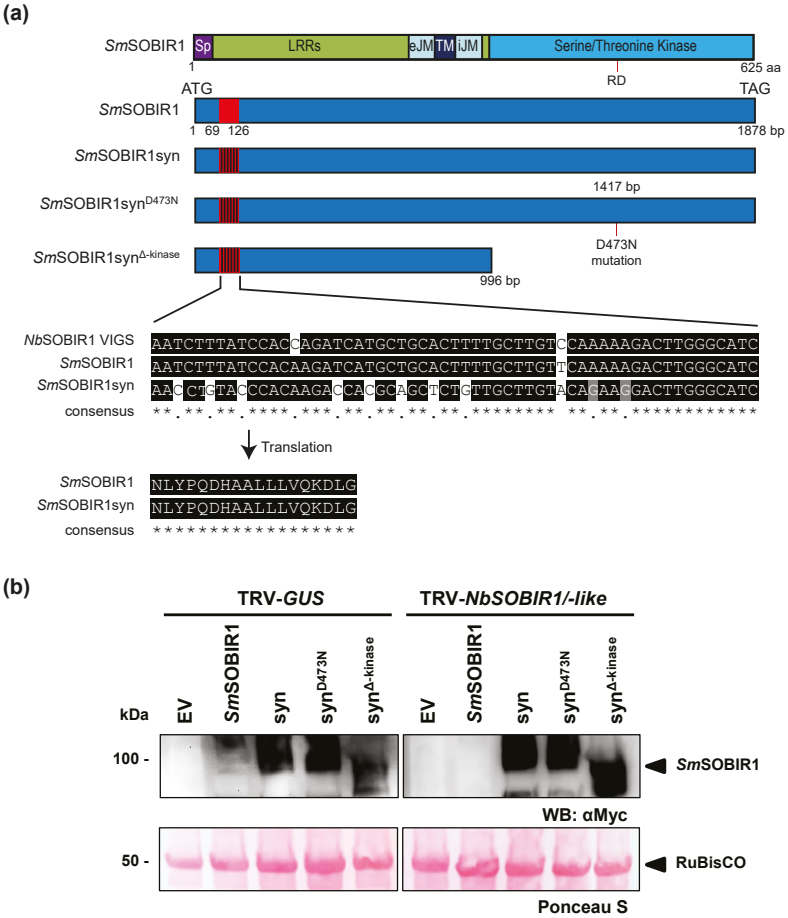

FIGURE S5 | Designing of synthetic versions of *SmSOBIR1* (*Sm*SOBIR1syn) and *in planta* protein detection. (a) Schematic overview of the synthetic constructs of *Sm*SOBIR1 used in this study. In red is the *SmSOBIR1* sequence that is targeted by the TRV-*NbSOBIR1/-like* construct. Protein (top) and gene models are scaled to represent the actual domain positions. Alignments show the nucleotide differences between *Sm*SOBIR1 and *Sm*SOBIR1syn^{WT} sequences, while the translated amino acid sequence remains identical. (b) Western blot showing the detection of *Sm*SOBIR1syn variants in TRV-*GUS* or TRV-*NbSOBIR1/-like*-inoculated plants. Wild type *Sm*SOBIR1 (WT) and empty vector (EV) were used as controls.

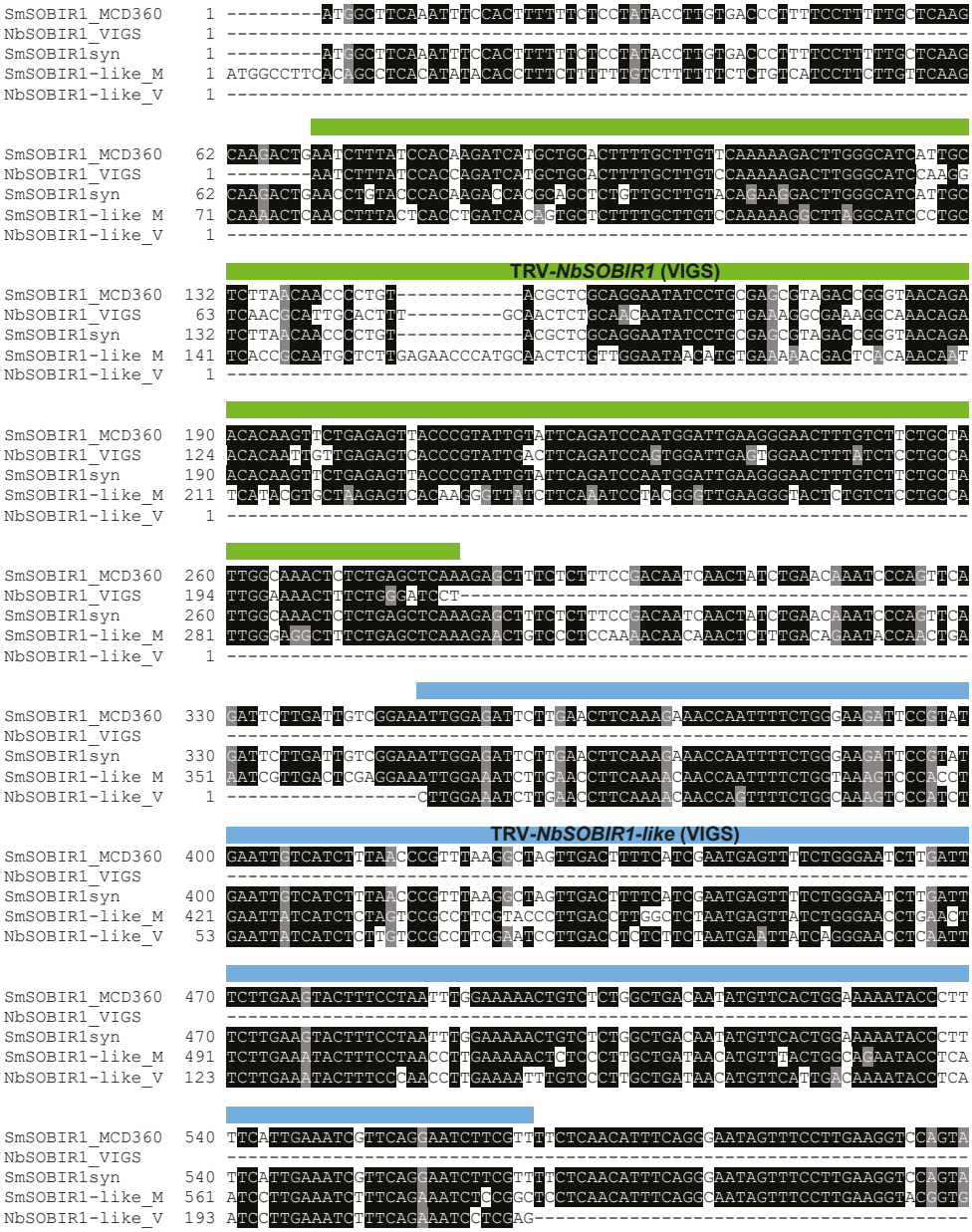

FIGURE S6 | DNA alignment of *SmSOBIR1*, *SmSOBIR1syn^{WT}* and *SmSOBIR1-like* with the TRV-*NbSOBIR1/-like* VIGS fragments. DNA sequence alignment of SOBIR1 was created using MUSCLE, and BOXSHADE was used to visualize conservation. Nucleotides highlighted in black are identical in all sequences while residues highlighted in grey are residues identical in >50% of the sequences. The positions of *SmSOBIR1* that are targeted by the TRV-*NbSOBIR1/-like* construct are highlighted with a green or blue line, respectively, above the sequence.

```
SmSOBIR1_MCD360   610 CCTGTCATGAGTCAAATTGAGCATTTATCAGCAGATTTGAATCGAAACAATGCCGTTCCCAAACGTTACA
NbSOBIR1_VIGS         ------------------------------------------------------------------------
SmSOBIR1syn       610 CCTGTCATGAGTCAAATTGAGCATTTATCAGCAGATTTGAATCGAAACAATGCCGTTCCCAAACGTTACA
SmSOBIR1-like_M   631 CCTGCCCTGAGTCAACTTGAGCACTTATCAGCAGACTTAAATCGAAAAGATTATGTTCCTAAGCGTTACA
NbSOBIR1-like_V       ------------------------------------------------------------------------

SmSOBIR1_MCD360   680 TTCTTGCTGAGAATTCAACAAGGTCAAATCAGATATCTGCAATGGTGCCTGCTCCAGCTCCAGCACCGGT
NbSOBIR1_VIGS         ------------------------------------------------------------------------
SmSOBIR1syn       680 TTCTTGCTGAGAATTCAACAAGGTCAAATCAGATATCTGCAATGGTGCCTGCTCCAGCTCCAGCACCGGT
SmSOBIR1-like_M   701 TTCTTGCTGAGAACGAAAGAAGGCCAAGACACACCCCTGCAATGGCACCAGAGTCCCAATCAGGAAATGC
NbSOBIR1-like_V       ------------------------------------------------------------------------

SmSOBIR1 MCD360   750 CCATCGTG-----------------TTGTACCGG---TGGTGCATAAACGTAACAAGAAGAAAAGGAAG
NbSOBIR1_VIGS         ------------------------------------------------------------------------
SmSOBIR1syn       750 CCATCGTG-----------------TTGTACCGG---TGGTGCATAAACGTAACAAGAAGAAAAGGAAG
SmSOBIR1-like_M   771 CCAAGCTCCAGGACCTAGTACAGTTGTAGTACCAGTAAAAAAAGACAAACACAACAAGATCAGAAGGAAG
NbSOBIR1 like_V       ------------------------------------------------------------------------

SmSOBIR1_MCD360   799 TTAAGATGTTGGTTTCTTGGTTTCCTAGCTGGAACTTTTGCTGGGGGTATATCTGCTGTGATCTTTTCGT
NbSOBIR1_VIGS         ------------------------------------------------------------------------
SmSOBIR1syn       799 TTAAGATGTTGGTTTCTTGGTTTCCTAGCTGGAACTTTTGCTGGGGGTATATCTGCTGTGATCTTTTCGT
SmSOBIR1-like_M   841 GTAGGCGCCTGGATTCTGGGATTGTTTGCTGGAGTTTTCGCTGGGGGCTTATGGGCCTTGGTCTTCTCCG
NbSOBIR1-like_V       ------------------------------------------------------------------------

SmSOBIR1_MCD360   869 TGCTATTCAAGCTCGTAATGTTCTTCGTAAGAAGGGGAAAGAACGATAATTCAAGTTTAACGATATTTAG
NbSOBIR1_VIGS         ------------------------------------------------------------------------
SmSOBIR1syn       869 TGCTATTCAAGCTCGTAATGTTCTTCGTAAGAAGGGGAAAGAACGATAATTCAAGTTTAACGATATTTAG
SmSOBIR1-like_M   911 TCCTCTTCAAGGTGCTTATGTTTTTAATC---AGAGGGATCAGAATGATTCAGGCTTAACAATTTTCAG
NbSOBIR1-like_V       ------------------------------------------------------------------------

SmSOBIR1_MCD360   939 TCCGTTGATCAAGAAAGCGGAGGACTTGGCTTTCTTAGAGATGGAAGATGGAGTAGCATCACTTGAAATG
NbSOBIR1_VIGS         ------------------------------------------------------------------------
SmSOBIR1syn       939 TCCGTTGATCAAGAAAGCGGAGGACTTGGCTTTCTTAGAGATGGAAGATGGAGTAGCATCACTTGAAATG
SmSOBIR1-like_M   978 TCCATTGATTAAGAAAGCCGAGCACTTGGCCTTTCTGGAGAACGAAGATGGATTGGAATCACTAGAACTC
NbSOBIR1-like_V       ------------------------------------------------------------------------

SmSOBIR1_MCD360  1009 ATTGGAAAAGGTGGATGTGGAGAAGTTTATAGAGCCGAGTTACGAGGGAGTAATGGGAAGATTATAGCTA
NbSOBIR1_VIGS         ------------------------------------------------------------------------
SmSOBIR1syn      1009 ATTGGAAAAGGTGGATGTGGAGAAGTTTATAGAGCCGAGTTACGAGGGAGTAATGGGAAGATTATAGCTA
SmSOBIR1-like_M  1048 ATTGGGCAAGGTGGATGTGGAAAAGTTTACAAAGCTGCGTTACCTGGAAGTGACGGAAAGATGATAGCCG
NbSOBIR1-like_V       ------------------------------------------------------------------------

SmSOBIR1_MCD360  1079 TAAAGAAGATTATACAATCCCCAATGGATGCTGCAGAGATCACGGAGGAAGATACTAAGGGGTTGAACAA
NbSOBIR1_VIGS         ------------------------------------------------------------------------
SmSOBIR1syn      1079 TAAAGAAGATTATACAATCCCCAATGGATGCTGCAGAGATCACGGAGGAAGATACTAAGGGGTTGAACAA
SmSOBIR1-like_M  1118 TAAAGAAGATCATACAACCACCAAGGGATGCTGCAGAACTCACTGAGGAAGATAGCAAGGGTATGAATAA
NbSOBIR1-like_V       ------------------------------------------------------------------------

SmSOBIR1_MCD360  1149 GAAAATGCGTCAAGTTAAATCAGAAATTCAAATTGTAGGTCAAATCAGACACCGGAATCTGCTTCCATTA
NbSOBIR1_VIGS         ------------------------------------------------------------------------
SmSOBIR1syn      1149 GAAAATGCGTCAAGTTAAATCAGAAATTCAAATTGTAGGTCAAATCAGACACCGGAATCTGCTTCCATTA
SmSOBIR1-like_M  1188 GAAAATGCGCCAGATTAAATCAGAAATCAAAATTGTAGGTCAAATCAGACACCGGAATTTGCTTCCCCTA
NbSOBIR1-like_V       ------------------------------------------------------------------------

SmSOBIR1_MCD360  1219 CTGGCGCATATGCCAAGGCCAGACTGTCATTACTTGGTGTATGAATATATGAAAAATGGGAGTTTACAGG
NbSOBIR1_VIGS         ------------------------------------------------------------------------
SmSOBIR1syn      1219 CTGGCGCATATGCCAAGGCCAGACTGTCATTACTTGGTGTATGAATATATGAAAAATGGGAGTTTACAGG
SmSOBIR1-like_M  1258 CTGGCACATATGCCAAGACCAGACTGCCATTACTTGGTCTATGAGTACATGAAAAATGGGAGCTTACAGG
NbSOBIR1-like_V       ------------------------------------------------------------------------
```

FIGURE S6 | *Continued*

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SmSOBIR1_MCD360 1289 ATATCCTGCAGCAAGTCACAGAAGGCACAAGAGAATTAGATTGGTTGGGACGACACAGAATTGCAGCGGG
NbSOBIR1_VIGS        ------------------------------------------------------------------------
SmSOBIR1syn     1289 ATATCCTGCAGCAAGTCACAGAAGGCACAAGAGAATTAGATTGGTTGGGACGACACAGAATTGCAGCGGG
SmSOBIR1-like_M 1328 ATACCCTGCAGCAAGTCAGAGAGGGGACACGGGAAGTAGATTGGTCGGGACGTCACCGAATTGCAATGGG
NbSOBIR1-like_V      ------------------------------------------------------------------------

SmSOBIR1_MCD360 1359 GATAGCTGCTGGTCTTGAGTATCTCCATATAAACCATACTCAACGCATAATTCACAGAGATCTAAAGCCA
NbSOBIR1_VIGS        ------------------------------------------------------------------------
SmSOBIR1syn     1359 GATAGCTGCTGGTCTTGAGTATCTCCATATAAACCATACTCAACGCATAATTCACAGAGATCTAAAGCCA
SmSOBIR1-like_M 1398 AATAGCTGCTGGACTGGAGTATCTCCATATAAATCATACTCAGCCTATAATTCACAGAGATCTAAAGCCA
NbSOBIR1-like_V      ------------------------------------------------------------------------

SmSOBIR1_MCD360 1429 GCAAATATCCTACTTGATGATGACATGGAAGCTCGAATAGGTGATTTTGGGCTTGCAAAGGGAGTTCCAG
NbSOBIR1_VIGS        ------------------------------------------------------------------------
SmSOBIR1syn     1429 GCAAATATCCTACTTGATGATGACATGGAAGCTCGAATAGGTGATTTTGGGCTTGCAAAGGGAGTTCCAG
SmSOBIR1-like_M 1468 GGCAATGTCCTGCTTGATGATGACATGGAAGCTCGAATTGGAGATTTTGGCCTTGCAAAGGGTGTGCCAG
NbSOBIR1-like_V      ------------------------------------------------------------------------

SmSOBIR1_MCD360 1499 ATGCTCATACACATATTACGACTTCAAATGTTGCAGGAACTGTGGGATATATTGCACCAGAATATCATCA
NbSOBIR1_VIGS        ------------------------------------------------------------------------
SmSOBIR1syn     1499 ATGCTCATACACATATTACGACTTCAAATGTTGCAGGAACTGTGGGATATATTGCACCAGAATATCATCA
SmSOBIR1-like_M 1538 ATGCTCATACACATATTACAACTTCAAATGTGGCAGGAACTATAGGATACATGCGCTCCAGAATATCATCA
NbSOBIR1-like_V      ------------------------------------------------------------------------

SmSOBIR1_MCD360 1569 GACACTGAAGTTTACAGACAAGTGTGATATATACAGCTTCGGTGTGGTGCTACCTGTGTTGGTTATCGGA
NbSOBIR1_VIGS        ------------------------------------------------------------------------
SmSOBIR1syn     1569 GACACTGAAGTTTACAGACAAGTGTGATATATACAGCTTCGGTGTGGTGCTACCTGTGTTGGTTATCGGA
SmSOBIR1-like_M 1608 GACACTAAAGTTCACTGATAAGTGTGATATATACAGCTTCGGGGTGCTTCTAGGCGTGCTAGTTATGGGA
NbSOBIR1-like_V      ------------------------------------------------------------------------

SmSOBIR1_MCD360 1639 AAGGCTCCATCGGATGAATTTTTCCAACATACTTCTGAGATGAGTTTAGTTAAGTGGCTGAGAAATGTAA
NbSOBIR1_VIGS        ------------------------------------------------------------------------
SmSOBIR1syn     1639 AAGGCTCCATCGGATGAATTTTTCCAACATACTTCTGAGATGAGTTTAGTTAAGTGGCTGAGAAATGTAA
SmSOBIR1-like_M 1678 AAGCTTCCATCTGATGAGTTCTTCCAGAACACTTCTGAGATGAGTTTAGTGAAATGGATGAGAAATGTCA
NbSOBIR1-like_V      ------------------------------------------------------------------------

SmSOBIR1_MCD360 1709 TGACTTCTGATGATCCTAAAATAGCAATTGATCCTAAGCTGATAGGAAATGGATATGAGGAGCAAATGCT
NbSOBIR1_VIGS        ------------------------------------------------------------------------
SmSOBIR1syn     1709 TGACTTCTGATGATCCTAAAATAGCAATTGATCCTAAGCTGATAGGAAATGGATATGAGGAGCAAATGCT
SmSOBIR1-like_M 1748 TGACTTCTGATGATCGAAAGAGAGCAATTGATCCAAAGCTGATGGGTAATGGAAATGAGGAGCAAATGCT
NbSOBIR1-like_V      ------------------------------------------------------------------------

SmSOBIR1_MCD360 1779 TTTGGTTCTCAAGATAGCTTGCTTTTGTACTCTCGAGAATCCAAAGGAGAGGCCTGGCAGTAAGGATGTT
NbSOBIR1_VIGS        ------------------------------------------------------------------------
SmSOBIR1syn     1779 TTTGGTTCTCAAGATAGCTTGCTTTTGTACTCTCGAGAATCCAAAGGAGAGGCCTGGCAGTAAGGATGTT
SmSOBIR1-like_M 1818 TTTGGTTCTCAAGATTGCCTGCTTTTGTACCCTCGAGAATCCTAAGGAGAGGCCTAATAGTAAGGATGTT
NbSOBIR1-like_V      ------------------------------------------------------------------------

SmSOBIR1 MCD360 1849 AGGTGCATGTTAACTCAGATCAAGCATTAG
NbSOBIR1 VIGS        ------------------------------
SmSOBIR1syn     1849 AGGTGCATGTTAACTCAGATCAAGCATTAG
SmSOBIR1-like_M 1888 AGGTGCATGTTGATGCAAATCAAGCATTAA
NbSOBIR1-like_V      ------------------------------
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FIGURE S6 | *Continued*

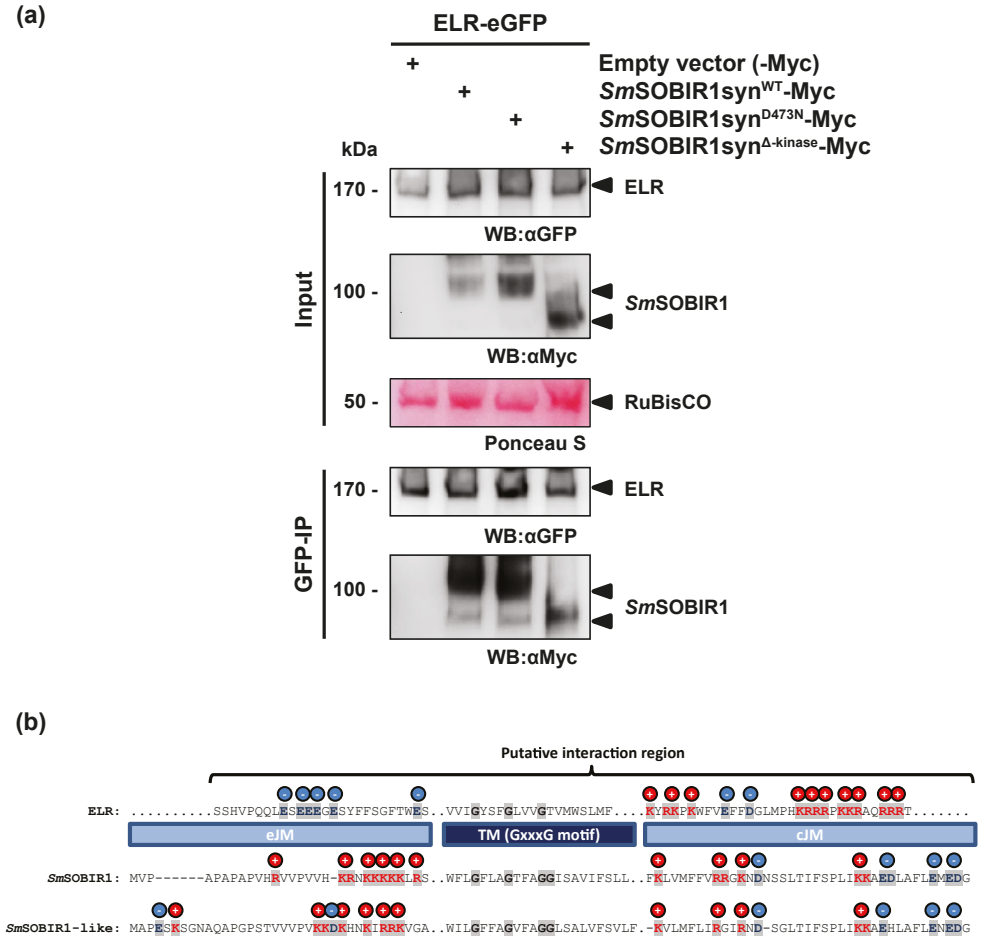
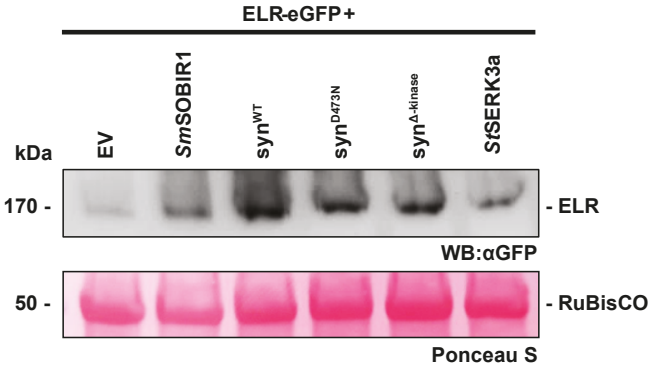


FIGURE S7 | Kinase activity of SOBIR1 and its kinase domain are not required for its association with ELR. (a) ELR-eGFP was transiently co-expressed in *Nicotiana benthamiana* in combination with empty vector (EV) or *Sm*SOBIR1syn variants (*Sm*SOBIR1syn^{WT}, *Sm*SOBIR1syn^{D473N} or *Sm*SOBIR1syn$^{\Delta\text{-kinase}}$). Total protein was extracted and was subjected to immunoprecipitation using GFP_TrapMA beads to capture ELR. The immunopurified proteins were detected with anti-GFP, while the interaction with *Sm*SOBIR1syn variants was assessed with anti-Myc. Ponceau S staining of RuBisCO indicates equal protein loading. This figure is representative for three biological repeats. (b) Schematic representation of the putative interaction site between the extracellular-juxtamembrane (eJM), transmembrane (TM) and intracellular-juxtamembrane (iJM) domains of ELR and *Sm*SOBIR1 based on Gust and Felix (2014) and Bi et al. (2016). Charged amino acids are indicated by highlighting and a symbol according to charge (+ for positive or – for negative charge). The GxxxG motif in the TM is also highlighted.



	ELR-eGFP +					
Relative band intensity	EV	*Sm*SOBIR1 -Myc	*Sm*SOBIR1syn -Myc	*Sm*SOBIR1syn ^{D473N}-Myc	*Sm*SOBIR1syn $^{\Delta\text{-kinase}}$-Myc	*St*SERK3a -HA
Experiment 1	1.0×	4.6×	13.0×	6.4×	5.7×	1.9×
Experiment 2	1.0×	-	12.2×	-	-	-
Experiment 3	1.0×	-	-	6.2×	-	-

FIGURE S8 | *Sm*SOBIR1 stabilizes ELR independently of its kinase activity. ELR-eGFP was transiently co-expressed in *Nicotiana benthamiana* with empty vector (EV), *Sm*SOBIR1, *Sm*SOBIR1synWT, *Sm*SOBIR1syn^{D473N} or *Sm*SOBIR1syn$^{\Delta\text{-kinase}}$. Total protein was extracted and western blotting was performed with anti-GFP to visualize ELR accumulation. Quantification of ELR band intensity was performed with imageJ. Relative band intensity results were obtained by comparing the signal intensity of the SOBIR1 and SERK3a agroinfiltrations to the EV agroinfiltration of each experiment. Ponceau S staining of RuBisCO indicates equal protein loading. Data from three independent experiments are shown.

Table S1. Constructs used in this study.

Purpose	Construct	Binary vector	Notes	*A. tumefaciens* strain	References
Cell death assays	INF1	pCB302-3		AGL1	(Du et al., 2015)
	Empty vector	pCB302-3		AGL1	(Du et al., 2015)
	Empty-Myc	pGWB20	Empty vector C′ 10× Myc	AGL1	This study
Protein fusions	ELR-eGFP	pBin-KS-GWY-35s-eGFP	C′ eGFP	C58C1	(Du et al., 2015)
	Cf-4-eGFP	pBin-KS-GWY-35s-eGFP	C′ eGFP	C58C1	(Liebrand et al., 2012)
	*St*SERK3a-HA	pGWB14	C′ 3× HA	GV3101	(Du et al., 2015)
	Empty-eGFP	pBin-KS-GWY-35s-eGFP	Empty vector, C′ eGFP	AGL1	This study
	*Sm*SOBIR1-Myc	pGWB20	C′ 10× Myc	AGL1	This study
	*Sm*SOBIR1-like-Myc	pGWB20	C′ 10× Myc	AGL1	This study
	*Sm*SOBIR1syn-Myc	pGWB20	C′ 10× Myc	AGL1	This study
	*Sm*SOBIR1syn^{D473N}-Myc	pGWB20	C′ 10× Myc	AGL1	This study
	*Sm*SOBIR1syn$^{\Delta-kinase}$-Myc	pGWB20	C′ 10× Myc	AGL1	This study
Gene silencing (VIGS)	pTRV1		TRV-VIGS	GV3101	(Liu et al., 2002)
	pTRV2-*GUS*		TRV-VIGS	AGL1	This study
	pTRV2-*StPDS*		TRV-VIGS	AGL1	This study
	pTRV2-*NbSERK3a/3b*		TRV-VIGS	C58C1	(Heese et al., 2007)
	pTRV2-*NbSOBIR1/-like*		TRV-VIGS	C58C1	(Liebrand et al., 2013)

Table S2. Primers used in this study.

Primer name	Purpose	Sequence 5′→3′	References
StSOBIR Fwd	Cloning	CACCATGGCTTCAAATTTCCACTTTTTTCT	This study
StSOBIR Rev	Cloning	ATGCTTGATCTGAGTTAACATGCA	This study
StSOBIR-like Fwd	Cloning	CACCATGGCCTTCACAGCCTCACA	This study
StSOBIR-like Rev	Cloning	ATGCTTGATCTGCATCAACATGC	This study
GUS 1-240 VIGS Fwd	Cloning	CACCCATGGTCCGTCCTGTAGAAA	This study
GUS 1-240 VIGS Rev	Cloning	GCCCAACCTTTCGGTATAAA	This study
SmSOBIR1$^{\Delta\text{-kinase}}$ Rev	Cloning	TGCTACTCCATCTTCCATCTCT	This study
Sm/NbPDS VIGS Fwd	Cloning	CACCAACTAAACCATTGGAGATTGTTATTG	This study
Sm/NbPDS VIGS Rev	Cloning	TTAATCCCTAATTCTCCAAACAGG	This study
NbSOBIR1 qRT-PCR Fwd	qPCR	CTTAGAAAAACTCTCTTTAGC	(Liebrand et al., 2013)
NbSOBIR1 qRT-PCR Rev	qPCR	TATGGATTGGAGTGACATTATG	(Liebrand et al., 2013)
NbSOBIR1-like qRT-PCR Fwd	qPCR	GCAATTGTAGTACCAGTACAC	(Liebrand et al., 2013)
NbSOBIR1-like qRT-PCR Rev	qPCR	AATCAATGGACTGAAAAC	(Liebrand et al., 2013)
*Pi*β-*tubulin* qPCR Fwd	qPCR	GGTCGTGGAGCCCTATAACG	This study
*Pi*β-*tubulin* qPCR Rev	qPCR	GTCACCATAAGTGGGGGTGG	This study
*St/NbEF1*α qPCR Fwd	qPCR	TGACCAAGATTGACAGGCGT	This study
*St/NbEF1*α qPCR Rev	qPCR	GCAAAACGACCCAATGGTGG	This study

Chapter 4

ELR binds elicitins from *Phytophthora* species to trigger SOBIR1/SERK3-mediated cell death

Emmanouil Domazakis
Jan Lochman
Richard G. F. Visser
Matthieu H. A. J. Joosten
Vivianne G. A. A. Vleeshouwers

abstract

- Several pairs of pattern-recognition receptors (PRRs) with apoplastic ligands have been described but in many cases, the actual evidence for ligand binding is lacking. The receptor-like protein (RLP) ELR causes cell death with various elicitins of *Phytophthora* species and in this study, we investigated whether ELR can associate with elicitins.
- We performed *in planta* and *in vitro* co-immunoprecipitation of ELR with several affinity-tagged elicitins as well as *in planta* transient co-expression assays.
- We found that ELR physically associates with elicitins INF1, ParA1 and β-CRY, which leads to cell death induction. ELR also triggers cell death with INF2A and CRY2. Remarkably, a C'-tagged INF1 and β-CRY cross-linked dimer (β-CRY^{DIM}) were unable to induce cell death, despite of their binding to ELR.
- Co-immunoprecipitation experiments showed that C'-tagged INF1 prevents association of ELR with its co-receptors SOBIR1 and SERK3 that are required for the active receptor complex formation, and β-CRY^{DIM} caused inhibition of ELR- association with SOBIR1 but not with SERK3.
- Together, we demonstrate that ELR is a genuine PRR that binds elicitins of *Phytophthora* species. Moreover, we provide the first report of ligand-mediated inhibition of ELR-SOBIR1-SERK3 signaling complex formation *in vivo*. This mechanistic uncoupling could find use in plant immune signaling studies.

Introduction

A major part of a plant's defense against microbes depends on the effective detection of microbe-associated molecular patterns (MAMPs). MAMPs are recognized in the plant apoplast, typically by membrane-localized pattern recognition receptors (PRRs) (Couto and Zipfel, 2016). All plant PRRs that have been identified so far contain a variable number of leucine-rich repeats (LRR), lysine motifs or lectin domains in the ecto-domain, a transmembrane domain and may contain a kinase domain. LRR-type receptor-like protein (RLPs) and receptor-like kinases (RLKs) predominantly recognize proteinaceous MAMPs or effectors. Despite the increasing number of PRRs discovered in the past 20 years, for most of them it has not been determined whether they are indeed true receptors or just involved in the complex (Dodds and Rathjen, 2010; Zipfel, 2014; Boutrot and Zipfel, 2017; Tang et al., 2017). Especially for RLPs, evidence of binding for MAMPs/effectors has been reported only for fungal endo-polygalacturonases and ethylene-induced xylanase and the widely conserved pattern nlp20, which bind to RLP42/RBPG1, LeEIX2 and RLP23, respectively (Ron and Avni, 2004; Zhang et al., 2014; Albert et al., 2015; Boutrot and Zipfel, 2017). So far, binding of oomycete-specific proteins to a host PRR has not been reported.

Elicitins are a family of structurally conserved extracellular proteins specific to *Phytophthora* and *Pythium* (Ponchet et al., 1999). Elicitins have features of oomycete MAMPs, as they have been shown to induce localized cell death, salicylic acid-, jasmonic acid- and ethylene- dependent accumulation as well as systemic resistance (SR) on several responding plant genotypes across various families (Vleeshouwers et al., 2006; Derevnina et al., 2016). The Solanaceaous *Nicotiana benthamiana, N. tabacum* and *S. microdontum* mount a specific cell death response to INF1 elicitin (Kamoun et al., 1998; Huitema et al., 2005; Vleeshouwers et al., 2006; Du et al., 2015). All elicitins share a highly conserved 98-amino-acid elicitin domain, which contains six cysteine (Cys) residues at conserved positions for the formation of disulphide bridges (Jiang et al., 2006; Derevnina et al., 2016). Elicitins of the canonical class ELI-1 consist of a signal peptide and the elicitin domain only. All other elicitin classes possess an additional C'-terminal extension, which is of variable length and is putatively related to oomycete plasma membrane or cell wall anchoring (Qutob et al., 2003).

ELR, a RLP from the wild potato *Solanum microdontum* specifically responds to various elicitins of diverse *Phytophthora* species (Du et al., 2015). The recognition spectrum ranges from elicitins belonging to elicitin class ELI-1 (e.g. INF1, RAMA1, ParA1, CRY2), ELI-2 (e.g. INF2A) and ELI-4 elicitins (e.g. INF5, INF6) (Jiang et al., 2006; Du et al., 2015) (Fig. S1). Elicitins do not seem to share a conserved stretch

of amino acids, as it is found in bacterial flagellin, EF-Tu or NLPs (Felix et al., 1999; Kunze et al., 2004; Bohm et al., 2014), but rather the conserved structure of elicitins is likely recognized by ELR (Derevnina et al., 2016). In line with this, elicitins that are recognized by ELR share rather low amino acid similarities, ranging from 45-65% (Du et al., 2015).

ELR is lacking a cytoplasmic kinase domain and thus incapable of downstream signaling. Recently it was shown that ELR physically associates with SOBIR1 in a ligand-independent manner, and associates with SERK3 upon induction with elicitins (Du et al., 2015; Domazakis et al., 2018). Both SOBIR1 and SERK3 are required for ELR function and for basal resistance to pathogens including *Phytophthora infestans* (Chaparro-Garcia et al., 2011; Liebrand et al., 2014; Peng et al., 2015; Domazakis et al., 2018).

In this study, we provide evidence that ELR physically associates with INF1 elicitin using *in planta* and *in vitro* setups. We tested diverse additional elicitins of different *Phytophthora* species and correlated physical interaction with the receptor with the occurrence of ELR-mediated cell death responses. Our data render ELR as a genuine PRR, which associates with various members of the elicitin family of *Phytophthora* species, however, its association with downstream interactors depends on the oligomeric state of elicitins.

Materials and methods

Plant materials

Nicotiana benthamiana plants used were derived from seeds. *Solanum hjertingii* genotype 349-3 (HJT349-3) plants were clonally propagated *in vitro* on Murashige and Skoog (MS) medium supplemented with 20% w/v sucrose as described (Murashige and Skoog, 1962; Du et al., 2015). Seedlings of *N. benthamiana* as well as well-rooted HJT349-3 plantlets were transferred to pots with disinfected soil in a climate-regulated greenhouse compartment (22/18°C and 16/8 h light day/night regime at 70% relative humidity) where they were grown for 3 weeks prior to agroinfiltration experiments. The transient protein expression and cell death assays were performed under the same conditions.

Binary vectors for *Agrobacterium tumefaciens*-mediated transient transformation

Construction of pBin-KS-*p35s:ELR-eGFP*, pBin-KS-*p35s:Cf-4-eGFP*, pBin-KS-*p35s:eGFP*, pCB302-3-*p35s:INF1*, pGWB20-*p35s-SmSOBIR1-Myc*, pGWB14-*p35s-StSERK3A-HA* and pTRV1 has been described (Du et al., 2015; Liebrand et

al., 2013; Liu et al., 2002). Elicitin constructs lacking the native signal peptide and carrying PR1Sp (tobacco PR1 signal peptide) and two N'-HA-tags were synthesized in pUC57 (Genscript). Those elicitins included INF1, INF1^{C23S}, ParA1, β-CRY and PYU1 and the elicitin domains of INF2A (a.a. 21-118), INF2B (a.a. 21-118), CRY2 (a.a. 21-118) and INL1 (a.a. 19-104) (elicitin domains according to (Jiang et al., 2006)) (Figs. S1a, b, S2a, b). Genes were amplified using Phusion DNA polymerase (Thermo Fisher Scientific) and were cloned in pENTR/D-TOPO. Then the genes were recombined to pK7WG2 binary vector (Karimi et al., 2002) by LR reaction (Invitrogen). To obtain C'-HA-tagged INF1, the native ORF lacking a stop codon was amplified with its native signal peptide from pGR106-INF1 vector in a similar way (Du et al., 2015). pENTR-INF1 was recombined to pGWB14 vector (Nakagawa et al., 2007) to obtain the C'-HA-tagged fusion construct pGWB14:*p35s-INF1-3HA* (Fig. S2a). Final constructs were transformed in *A. tumefaciens* stain AGL1.

Agrobacterium transient cell death assays

For elicitin cell death induction assays, young, fully expanded leaves of 3 wk-old *N. benthamiana* or *N. tabaccum* SR1 plants were agroinfiltrated with pK7WG2-empty vector (EV), pCB132:*p35s-INF1*, pK7WG2:*p35s-PR1Sp2HA-elicitins* constructs or pGWB14:*p35s -INF1-3HA* at an OD_{600} of 0.2. Similarly, *Solanum hjertingii* 349-3 plants were co-infiltrated with 1:1 mixtures of *A. tumefaciens* carrying pK7WG2:*p35s-ELR* (OD_{600}=0.15) with pK7WG2-EV, pCB132:*p35s-INF1* or pK7WG2:*p35s-PR1Sp2HA-elicitins* constructs (OD_{600}=0.1). Leaves were visually examined for cell-death at 3-5 dpi. Cell death quantifications were performed as described previously (Du and Vleeshouwers, 2014).

Co-immunoprecipitations and immunoblotting

In planta protein interactions were performed by transient expression in young fully-expanded leaves of 3 wk-old *N. benthamiana* using *Agrobacterium*-mediated transformation followed by co-immunoprecipitation. At 2 d post infiltration, leaves were collected, snap-frozen in liquid nitrogen and ground to a fine powder. Protein extraction, co-immunoprecipitation and western blot with αGFP-HRP and αHA-HRP was performed as described (Domazakis et al., 2018). For detection of lined ubiquitin, the αUb-FK2-HRP was used (Enzo Life Sciences) (detects K^{29}-, K^{48}-, and K^{63}-linked mono- and polyubiquitinylated proteins) at a dilution of 2:5000.

Protein infiltration assays

Protein infiltrations for co-receptor competition assays with β-CRY, or the β-CRY covalently cross-linked dimer (β-CRY^{DIM}) (Uhlíková et al., 2016) were performed

by using 100 nM of each protein was infiltrated in *N. benthamiana* leaves. Then the infiltrated leaves were incubated for one hour. Subsequently leaf material was collected for protein extraction and immunoprecipitation. For β-CRY and β-CRY^{DIM} cell death assays in *N. tabacum*, 20 nM of purified protein was used. Elicitations with INF1 derived from *P. infestans* were performed by infiltrating a buffer or 1 μM purified INF1 protein and incubating for 15 min, as described (Domazakis et al., 2018).

Production and purification of recombinant proteins

Recombinant His-HA-INF1 and His-HA-SCR74 (variant B3b, NCBI accession AY723717.1, (Liu et al., 2005)) were synthesized and codon optimized for expression in *Pichia pastoris*. Codon optimized genes were designed to carry N'-His-HA tags as described previously (Domazakis et al., 2017) (Fig. S2b). Genes were synthesized with flanking restriction sites for *Stu*I and *Kpn*I to facilitate cloning and were obtained in pUC57 vector (GenScript). Genes were cloned in frame with the α-mating factor in pPinkα-HC vector (Invitrogen) enabling protein secretion. *P. pastoris* PichiaPink™ strain 1 (Invitrogen) was transformed by electroporation and high protein producing clones were selected using a high throughput screening method (Domazakis et al., 2017). For protein expression, yeast cultures were first grown in 1 l of BMGY at 28˚C, 250 rpm shaking, till OD_{600} = 2-6 in a 3 l conical flask. Cells were pelleted by centrifugation and were resuspended in 200 ml of BMMY containing 1% MetOH at 28˚C, 300 rpm shacking, for induction in a 2 l conical flask. Induction was maintained for 48 hrs with extra MetOH to 1% v/v added every 24 hrs. After 48 hrs, the cultures were harvested and the cells were removed by centrifugation. Supernatants were filter sterilized through a 0.22 μm syringe filter. A pressurized Amicon stirred cell concentrator (Millipore) combined with a 3 kDa exclusion filter was used to concentrate the supernatants to a volume of ~10 ml. Purification was performed using cation exchange. A column was assembled with SP sepharose. Beads were equilibrated with 10 volumes of acetic acid buffer (50 mM acetic acid, pH 4.5). Concentrated yeast supernatants containing effectors were diluted five times in acetic acid buffer and were slowly loaded to the column. Column was then washed with five volumes of acetic acid buffer and proteins were eluted by applying five volumes of elution buffer (10 mM Tris, 500 mM NaCl, pH = 8) and collecting several fractions. Protein containing fractions were obtained by measuring A280 absorbance, SDS-PAGE and western blotting. Protein concentrations were determined using BCA assay (Thermo Fisher Scientific).

In vitro binding assay

A. tumefaciens strains carrying ELR-eGFP or Cf-4-eGFP were infiltrated in *N. benthamiana* at an OD_{600} of 1 and 0.8, respectively. Two days after agroinfiltration, leaves were collected and were snap frozen in liquid nitrogen. Leaves were ground to a fine powder and RIPA extraction buffer was added at 2 ml/g of material. Then the extract was diluted at a ratio of 1:3 in binding buffer (100 mM MES, 10 mM NaCl, 3 mM $MgCl_2$, pH=6 (Saur et al., 2016)) and 2 ml was applied to the His-HA-INF1- or His-HA-SCR74- bound beads. The mixture was incubated for 1 hr at 4°C. Co-immunoprecipitation was performed as described above.

Results and discussion

ELR associates with INF1 *in planta*

To study whether ELR associates with INF1 in co-immunoprecipitation experiments, we generated both N′- and C′-tagged versions of INF1. N′-tagged INF1 was designed with a tobacco pathogenesis-related protein 1 (PR1) signal peptide (Sp) and a 2×HA tag (PR1Sp-2HA-INF1), while C′-tagged INF1 was cloned with its native signal peptide, fused to 3×HA (Fig. S2a). To test whether ELR associates with INF1 *in planta*, we co-expressed the HA-tagged versions of INF1 with ELR-eGFP (Du et al., 2015), and used Cf-4-eGFP or eGFP as positive and negative controls for ELR-eGFP, respectively. In addition, we included a cysteine mutant of INF1 (PR1Sp-2HA-INF1^{C23S}), lacking in structure one of the three disulfide bridges, which leads to decreased cell death (Kamoun et al., 1997), as another negative control for INF1. After performing co-immunoprecipitation, we found that ELR specifically immunopurifies with INF1. ELR associated with both N′- and C′-tagged INF1, indicating that tagging position does not affect the interaction with the receptor (Fig. 1a). As expected, Cf-4 and eGFP were not able to associate with any tagged INF1, whereas the INF1^{C23S} mutant did show severely reduced interaction with ELR in line with the reduced cell death phenotype (Kamoun et al., 1997). Interestingly, we noticed that INF1 migrates as a double band in the total protein extract, while after immunoprecipitation of ELR, only a single band is found (Fig. 1a, inputs). We consider that the upper band in INF1 corresponds to the unprocessed version of the protein while the lower band, which associates with ELR, corresponds to the mature protein form. In sum, these data show that ELR specifically associates with INF1 *in planta* and that this interaction occurs with the mature form of the effector, likely in the plant apoplast.

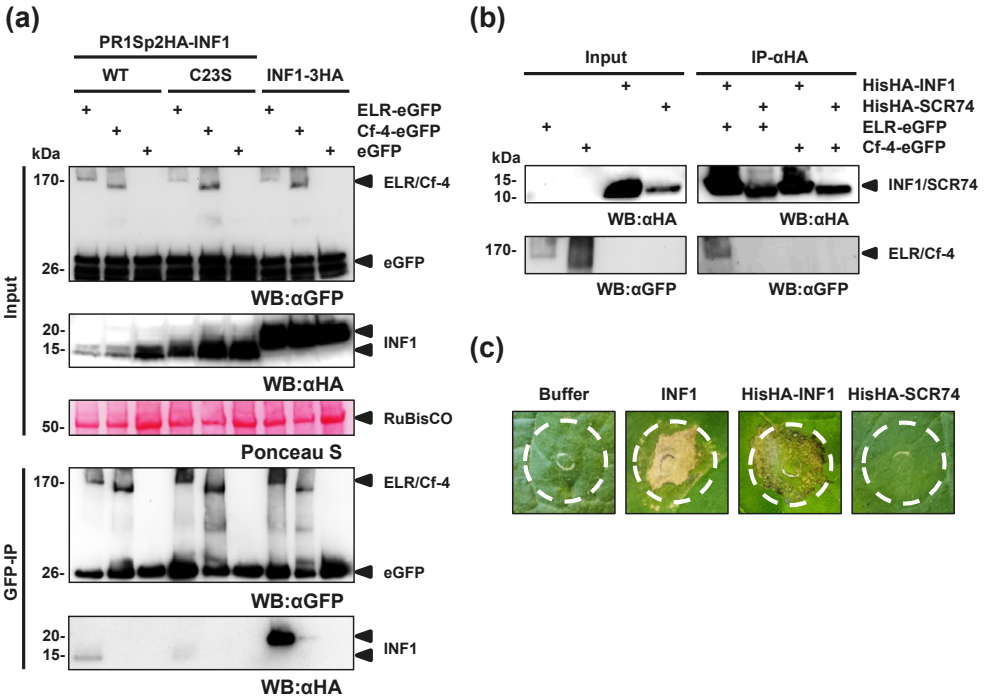


FIGURE 1 | ELR associates with INF1 elicitin *in planta* and under *in vitro* conditions. (a) N-terminal HA-tagged INF1 wild type (PR1Sp-2HA-INF1^{WT}), the INF1^{C23S} cysteine mutant (PR1Sp-2HA-INF1^{C23S}) or C-terminal HA-tagged INF1 (INF1-3HA) were co-expressed with ELR-eGFP, Cf-4-eGFP or GFP in *Nicotiana benthamiana*. Total protein was extracted at 2 days post infiltration and was subjected to immunoprecipitation using anti-GFP beads to capture ELR-eGFP, Cf-4-eGFP and GFP. The immunopurified proteins were detected with anti-GFP, while their interaction with INF1 was assessed with anti-HA antibodies. Ponceau S staining of RuBisCO indicates equal protein loading. Results are representative of three biological repeats. (b) Purified recombinant HisHA-INF1 or HisHA-Scr74 were incubated at an saturating amount (500 pmol each) with anti-HA beads for 30 min. Subsequently, beads were washed and a diluted ELR-eGFP- or Cf-4-eGFP-containing extract (from transiently transformed *N. benthamiana*) was incubated with the beads. After a 1 h incubation, samples were separated with SDS-PAGE and western blotting was performed. The immunopurified effector proteins HisHA-INF1 and HisHA-SCR74 were detected with anti-HA antibodies, while the interaction with ELR was assessed with anti-GFP. Ponceau S or CBB staining indicate equal loading. Results are representative of two biological repeats. (c) Functionality test of 1 μM recombinant HisHA-INF1 and HisHA-Scr74 proteins in *N. benthamiana*, compared to INF1 produced in *P. infestans* culture filtrate.

ELR associates with INF1 *in vitro*

To further validate the association between ELR and INF1, we designed an *in vitro* binding assay with purified INF1 protein. His-HA tagged INF1 as well as an unrelated protein SCR74 were produced in *Pichia pastoris* (Liu et al., 2005; Domazakis et al., 2017) and purified using cation exchange. To test for association, ELR-eGFP and Cf-4-eGFP receptors were transiently expressed in *N. benthamiana* as described above and were tested for association with effector proteins. First,

the His-HA-INF1 and His-HA-SCR74 proteins were immuno-absorbed in anti-HA beads at saturating amounts. Diluted *N. benthamiana* extract containing either ELR-eGFP or Cf-4-eGFP was applied to the beads in binding buffer. After incubation and washing, we found that ELR-eGFP is specifically being captured from the plant extract by INF1 but not by the control protein SCR74 (Fig. 1b). In contrast, Cf-4-eGFP was not associating with either of these effectors, as expected. As a control, we confirmed that the recombinant His-HA-INF1 is recognized in *N. benthamiana* similar to INF1 from *P. infestans*, as expected, while His-HA-SCR74 is not recognized (Fig. 1c). These data show that ELR specifically associates with INF1 and complements the *in planta* observations.

AVR3a does not affect binding of ELR to INF1 but alters protein ubiquitination

Several cytoplasmic effectors from *Phytophthora sojae* and *P. infestans* are able to suppress INF1 response (Bos et al., 2006; Oh et al., 2009; Wang et al., 2011; Zheng et al., 2014; Derevnina et al., 2016). Among the most studied RxLR effectors with such function is AVR3a. We hypothesized that the (cytoplasmic) AVR3a protein is unlikely to cause inhibition of ELR binding with INF1 since that occurs in the apoplast. To test this hypothesis, we co-expressed ELR with INF1 in *N. benthamiana*, together with untagged AVR3a^{KI} and AVR3a^{EM}, that represent the avirulence and virulence allele of AVR3a, respectively (Bos et al., 2009). After co-immunoprecipitation, we found that co-expression with either AVR3a allele does not affect binding of ELR to INF1, as expected (Fig. S3). However a reduction in the accumulation of both ELR and INF1 proteins could be observed.

AVR3a is known to interact and inhibit the E3 ubiquitin ligase CMPG1 and to interact with DRP2, a protein involved in receptor-mediated protein endocytosis (Bos et al., 2010; Chaparro-Garcia et al., 2015). In both cases, AVR3a seems to interfere with receptor internalization, where ubiquitination is an important component. To test what is the effect of AVR3a on ELR ubiquitination, we performed western blot with an antibody recognizing linked ubiquitin. We found that AVR3a^{KI} and AVR3a^{EM} are both causing a profound decrease in total protein ubiquitination (Fig. S3, inputs) which is also observed after immunoprecipitation of ELR. Interestingly, similar results were reported previously where AVR3a^{KI} was found to decrease smearing (indication of ubiquitination) of observed western blot bands when co-expressed with the RLKs FLS2, EFR and BAK1 (see Fig. A2.3., (Chaparro-Garcia, 2012)). From those preliminary findings we can hypothesize that AVR3a is not inhibiting interaction of ELR with INF1, but rather plays a role in inhibiting downstream signaling by affecting ubiquitination (Bos et al., 2010; Chaparro-Garcia et al., 2015).

ELR associates *in planta* with ELI-1 elicitins

In contrast to other PRRs that recognize small epitopes of their cognate MAMP and often do not lead to cell death, ELR seems to recognize some structural domain of elicitins and can induce cell death in plants (Du et al., 2015). For studying whether the cell death inducing activity by the various elicitins is associated with their binding ability to ELR, we generated affinity tagged versions of diverse elicitin domains from various *Phytophthora* and one *Pythium* species. More specifically, N'-HA-tagged elicitin domains of INF1, INF2A, INF2B, INL1 from *P. infestans*, ParA1 from *P. parasitica*, CRY2 from *P. cryptogea* and PYU1 from *Pythium ultimum*. The tested elicitins belong to two different classes, i.e. ELI-1 and ELI-2 with INL1 (class ELL) and PYU1 serving as outgroups (Fig. S1a, b, c) and share 23-94% amino acid identity to INF1. Subsequent agro-coinfiltrations were performed with ELR in *Solanum hjertingii*, a wild *Solanum* genotype that does not respond to elicitins (Fig. 2a, b). We found that the tagged elicitins INF1, ParA1, CRY2 (ELI-1) and INF2A (ELI-2) induced significant cell death when co-expressed with ELR at 3 days post-infiltration, as expected (Du et al., 2015) (Fig. 2a, b). To further confirm the cell death inducting activity of those elicitins, we agroinfiltrated them in *Nicotiana tabacum*, a species well known for responding to elicitins (Ricci et al., 1989; Huitema et al., 2005; Hoeberichts et al., 2013). In tobacco, additional cell death is found for INF2B compared to ELR, similar to what has been described (Huitema et al., 2005). In addition, the INF1^{C23S} mutant induced cell death in this plant, as a possible result of relatively high concentration of *in planta* expressed protein together with higher sensitivity of *Nicotiana* plants to elicitins (Vleeshouwers et al., 2006) (Fig. S4).

To investigate whether elicitin-induced cell death activity is associated with their binding ability with ELR, we performed an *in planta* co-immunoprecipitation experiment of ELR-eGFP with the diverse tagged elicitins. N'-HA-tagged INF1 and INF1^{C23S} were used as positive and negative controls, respectively (Fig. 2c). We found that, besides INF1, ELR interacts with ParA1 and β-CRY, which also belongs to the same ELI-1 class (Fig. S1c). CRY2 and INF2A (Fig. S1a, b) that share a lower amino acid identities to INF1 (74% and 52%, respectively), may exhibit lower affinity to ELR and remain below the detection threshold of our assay, however *in planta*, these elicitins trigger significant cell death when co-expressed with the receptor (Du et al., 2015) (Fig. 2a, b). Overall, these data confirm that ELR is able to recognize and associate with elicitins beyond *P. infestans* and is supported by co-immunoprecipitation experiments for three ELI-I elicitins.

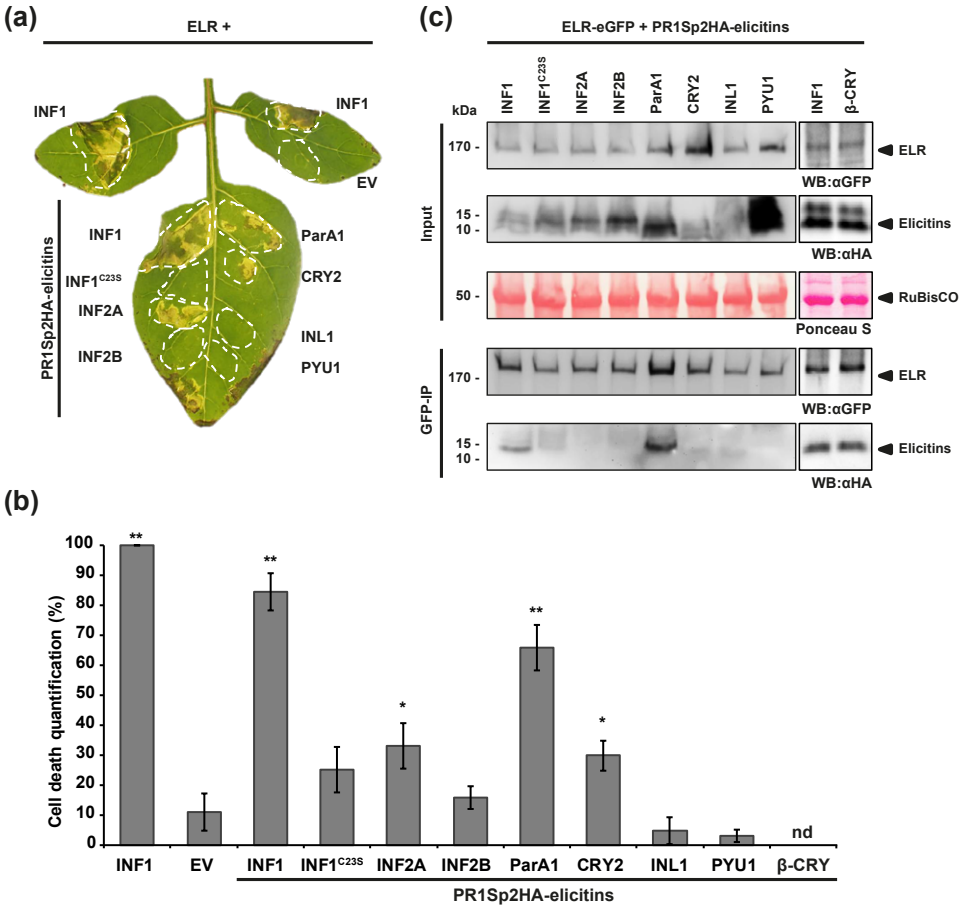


FIGURE 2 | ELR recognizes diverse elicitins and associates *in planta* with elicitins ParA1 and β-CRY from *Phytophthora parasitica* and *P. cryptogea*, respectively. Seven diverse elicitins (ParA1, CRY2, β-CRY, INF2A, INF2B, INL1 and PYU1) were tested for recognition by ELR together with INF1 and INF^{C23S} as controls. (a) N-terminal HA-tagged elicitin recognition in *Solanum hjertinjii* 349-5 upon their transient co-expression with ELR (data not available for β-CRY). (b) quantification of cell death phenotypes from experiment (a). (c) Co-immunoprecipitation of ELR with N-terminal HA-tagged elicitins. Total protein was extracted at 2 days post infiltration and was subjected to immunoprecipitation using anti-GFP beads to capture ELR. The immunopurified proteins were detected with anti-GFP, while the interaction with elicitins was assessed with anti-HA antibodies. Ponceau S staining of RuBisCO indicates equal protein loading. Results are representative of three biological repeats.

Binding to ELR and cell death induction can be uncoupled

Adding affinity tags to proteins is known to sometimes affect their function or interaction with other proteins. For example, C′-HA tagging of BAK1 showed decreased reactive oxygen species (ROS) signaling potency while it did not affect association with FLS2 (Ntoukakis et al., 2011; Schwessinger et al., 2011). As described above, agroinfiltration of N′-terminal tagged elicitins led to cell death

but the C′-tagged INF1-3HA did not (Fig. S5a, b, c). This finding was unexpected, since this INF1-3HA version is still able to associate with ELR (Figs. 1, S5d). Since 3×HA tag is relatively small, we hypothesized that a potential oligomerization may be happening to INF1-3HA that is leading to increased size and is thereby preventing association with downstream interactors. In line with this, elicitins cinnamomin (87% identity to INF1) (Rodrigues et al., 2006) and β-CRY (79% identity to INF1) (Uhlíková et al., 2016) can dimerize through helix A interactions (Fig. S6d), and elicitins POD-1 and POD-2 can form heterohexamers (Takenaka et al., 2011). An important role in the POD-1/POD-2 heterohexamer structure stabilization has a C′ extension terminated by Cys residue (Takenaka et al., 2011), which was reminiscent of the extra Cys in the 3HA-tag of INF1-3HA (Fig. S6a, b, c). Indeed, in contrast to PR1Sp2HA-INF1, the majority of INF1-3HA protein migrates as a band of about 120 kDa under non-reducing SDS-PAGE after agroinfiltration in *N. benthamiana,* which may correspond to the INF1 hexamer form (Fig. S6b, c). Under reduction conditions, the band of 20 kDa is observed, as expected.

We have shown that ELR associates with both SERK3 and SOBIR1 (Domazakis et al., 2018). To test whether formation of INF1-3HA polymer affects the interaction of co-receptors downstream of ELR binding, we simultaneously co-expressed ELR N′- and C′-tagged INF1 together with SOBIR1 and SERK3. Cf-4 was used as a negative control. After co-immunoprecipitation with aGFP, we found that in all cases, ELR was able to interact with both INF1 versions, as expected (Figs. 1, S5d). Intriguingly, upon co-expression of ELR with INF1-3HA, interaction with SOBIR1 was severely decreased while interaction with SERK3 was almost completely abolished. In contrast, when PR1Sp2HA-INF1 was used, interaction of ELR with both SOBIR1 and SERK3 was evident, as expected (Fig. S5d). From this we conclude that C′-3HA-tagged INF1 binding to ELR is indeed inhibiting the formation of the signaling potent ELR-SOBIR1-SERK3 complex (Domazakis et al., 2018).

Elicitin dimerization leads to reduced ELR-mediated cell death

ELI-1 β-CRY elicitin can form dimers, which cause less cell death than the monomers when infiltrated in tobacco (Gooley et al., 1998; Ponchet et al., 1999). More specifically, a chemically cross-linked dimer of β-CRY (β-CRY^{DIM}) was largely compromised in induction of cell death (Uhlíková et al., 2016). Hence, to strengthen our conclusions with C′-3HA-tagged INF1, we tested whether the β-CRY^{DIM} loss of cell death induction with ELR is due to co-receptor competition as well. To test this, we co-expressed ELR together with SOBIR1 and SERK3 in *N. benthamiana* leaves and two days later, we infiltrated leaves with β-CRY and β-CRY^{DIM} purified proteins. After co-immunoprecipitation, we found that, similarly to INF1-3HA, treatment with β-CRY^{DIM} abolished the interaction of ELR with SOBIR1 whereas the

interaction with SERK3 remained unchanged (Fig. S7a). This potentially explains the observed accumulation of *PR1* and *PR5* transcripts (Fig. S7b, c, d). Indeed, from studies on FLS2 it is known that ROS burst and MAPK3/6 activation are two independent early events (Xu et al., 2014). SOBIR1 is clearly connected to ROS production, endocytosis and activation of cell death in *Arabidopsis* (Gao et al., 2009) and tomato (Peng et al., 2015) and loss of its interaction with ELR is expected to negatively affect cell death induction upon INF1 perception. Those observations further support the previous conclusion with C'-3HA-tagged INF1, and are in agreement with the notion that both SOBIR1 and SERK3 are required to obtain a signal reaching the threshold for cell death (Mur et al., 2008; Chaparro-Garcia et al., 2011; Adachi et al., 2015; Domazakis et al., 2018). However, the difference in the competition rates for SERK3 and SOBIR1 likely depends on the elicitin oligomer size or it is due to their binding in distinct parts of ELR.

The ω-loop region of elicitins is likely involved in ELR binding

Elicitin dimerization leaves the ω-loop region found opposite of helix A exposed. Previously, within ω-loop an important role of residue Leu41 in the binding process to the potential partner in plant responsible for defense response was proved (Dokládal et al., 2012). Interestingly, the less active proteins INF2A and INF2B (compare to INF1, ParA1, CRY2, β-CRY) contain within the ω-loop region at position 43 an Asp residue, instead of Thr (Fig. S6e). This fact could influence protein-binding activity to ELR and lead to weakened interaction. We thus hypothesize that the potential binding site of elicitins to ELR is located in this ω-loop region.

Binding of INF1 to ELR triggers changes in co-receptor accumulation and phosphorylation dynamics

Binding of ligands to PRRs is known to trigger spatio-temporal changes in PRR/co-receptor complexes (Bücherl et al., 2017). To study the effect of INF1 on the ELR/SOBIR1/SERK3 complex, we performed agro-coinfiltration of ELR and co-receptors in *N. benthamiana*, followed by INF1 elicitation. When ELR and SOBIR1 were co-expressed, a stabilization of ELR was observed, as expected (Domazakis et al., 2018). However, INF1 elicitation led to a rapid decrease in the accumulation of both ELR and SOBIR1 (Fig. S8a, b). This effect was not evident when ELR was co-expressed with both SOBIR1 and SERK3. In contrast, when ELR was co-expressed with SERK3 alone, there was no significant change in its accumulation upon INF1 elicitation (Fig. S8a, b).

For monitoring the phosphorylation status of the elicitin perception complex, ELR, SOBIR1 and SERK3 were co-expressed and samples were collected with or without INF1 elicitation. Immunoprecipitations were performed for capturing ELR, SOBIR1 or SERK3 and samples were separated in a special gel (Phos-Tag) which causes phosphorylated proteins to migrate slower (Kinoshita et al., 2006). Using this method, we found that ELR is not phosphorylated, as expected by its lack of kinase domain. SERK3 on the other hand showed that it is constitutively phosphorylated in two states, with INF1 elicitation inducing one state over the other (Fig. S8b). For SOBIR1, we also found that it is phosphorylated, likely in two stages as well. However, INF1 elicitation resulted in a severe decrease in its accumulation, and the phosphorylated bands could not be discriminated. We conclude that INF1 elicitation causes changes in the dynamics of the ELR complex accumulation and co-receptor phosphorylation status. This is in line with previous findings where the formation of SOBIR1-containing endosomes upon elicitation with ParA1 elicitin was described (Peng et al., 2015). We thus hypothesize that the rapid decrease in ELR and SOBIR1 accumulation upon INF1 elicitation is due to the formation of endosomes, which are insoluble in our assays while SERK3 is likely not present in these vesicles, remaining soluble.

Conclusion

Reverse genetics using effector screens led to the identification of ELR, however, evidence of ELR functioning as a genuine PRR was lacking so far. In this study we demonstrated that ELR is able to bind elicitins. In addition, we have shown that only monomeric elicitins can trigger cell death upon binding to ELR, as elicitin dimers/hexamers are not allowing the normal formation of an ELR-SOBIR1-SERK3 complex. To our knowledge, this is the first report of a co-receptor inhibition by an effector protein and highlights the importance of performing functional tests for any recombinant fusion protein. This mechanistic uncoupling of ELR from co-receptors could be potentially utilized in plant immune signaling studies. For example, by inhibiting ELR/SOBIR1/SERK3 (with INF1-3HA) or ELR/SOBIR1 (with β-CRY^{DIM}) complex formation, studies on RLPs-mediated signaling could be facilitated. Finally, the use of immobilized INF1 on a matrix to identify ELR suggests the potential of using immobilized effectors for identifying their respective PRRs using proteomics in transformation-recalcitrant plant species (Boutrot and Zipfel, 2017).

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Supplementary information

(a)

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INF1        1 ----TTCTTSQQTVAYVALVSLLSDTSFNQCST-DSGYSYLTATSLPTTEQYKLMCASTA
ParA1       1 ----TTCTTTQQTAAYVALVSLLSDTSFNQCST-DSGYSYLTATSLPTTEQYKLMCASTA
β-CRY       1 ----TACTATQQTAAYKTLVSLLSDASFNQCST-DSGYSYLTAKALPTTAQYKLMCASTA
CRY2        1 ----TACTTQQTAAYVALVSLLSESFFSTCAS-DSGYSYLTATALPTTAQYELMCASTA
INF2A       1 ----ETCSPTDQTTAYSTLASVLLLSSFQGCAD-DSGFSLLYSTALPDDDQYVKMCASDN
INF2B       1 ----ETCSPTDQTTAYSTLASVLLLSSFQGCAD-DSGFSLLYSTALPDDAQYVKMCASDN
INL1        1 ----ASCDVT-------SLQPLLLSSDTTTCAT-DSGYT-VTSLATPTDAEMDVMCTSTA
PYU1        1 YDEVTECPAIE----ELKLAPLAANPNLSVCQD-ASGNQYLPPVCYPTDACRALMCVHPE
consensus   1       *. . .. ..  * .... . .  *   .**....   ..*.  .. .**..

INF1       56 CKFMINKIVSLNAPDCPLTVPTSGLVLNVYSYANGFSSTCASL
ParA1      56 CKFMINKIVTLNPPDCPLTVPTSGLVLNVFTYANGFSSTCASL
β-CRY      56 CNFMIKKIVTLNPPNCPLTVPTSGLVLNVYSYANGFSNKCSSL
CRY2       56 CQEMIEEIIALNPPDCPLTVPTSGLVLNVYEYANDFASTCASL
INF2A      56 CKSLIESVASLNPPNCPLTVPTSGLVLNVVDLTSGFSEKCSSS
INF2B      56 CKSLIESVAGLNPPNCPLTVPTSGLVLNVVDLTSGFSKKCSSS
INL1       48 CQSVLSQLETLAPSECTL-----GTFALYADLITPLSNHCAGA
PYU1       56 CFNLIDAIKALNVSDCVLVF--GDVKLNVKKLIEEFEPSCF--
consensus  61 * ...  .  *... *.*..........      ..  * .
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(b)

Elicitin	INF1	ParA1	β-CRY	CRY2	INF2A	INF2B	INL1	PYU1
INF1	ID							
ParA1	94%	ID						
β-CRY	81%	83%	ID					
CRY2	74%	77%	73%	ID				
INF2A	52%	53%	56%	52%	ID			
INF2B	51%	53%	57%	53%	97%	ID		
INL1	27%	29%	32%	33%	31%	32%	ID	
PYU1	30%	30%	31%	32%	30%	31%	23%	ID

(c)

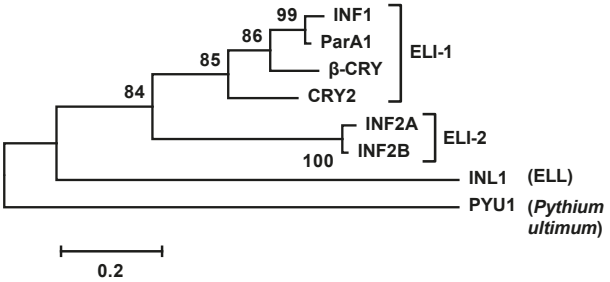

FIGURE S1 | Characteristics of elicitins used in this study. (a) The elicitin domains of eight elicitins from *Phytophthora* sp. and *Pythium ultimum* were aligned using COBALT, and BOXSHADE was used to visualize conservation. Residues identical in all sequences are highlighted in black while those identical in >80% of the sequences are highlighted in grey. (b) Percentages of amino acid sequence identity of the elicitin domain between the different elicitins. (c) Neighbor-joining phylogenetic tree was created based on the 98 amino acid elicitin domain using MEGA 5.1. Bootstrap support values (1000 replicates) above 50% are given next to branches.

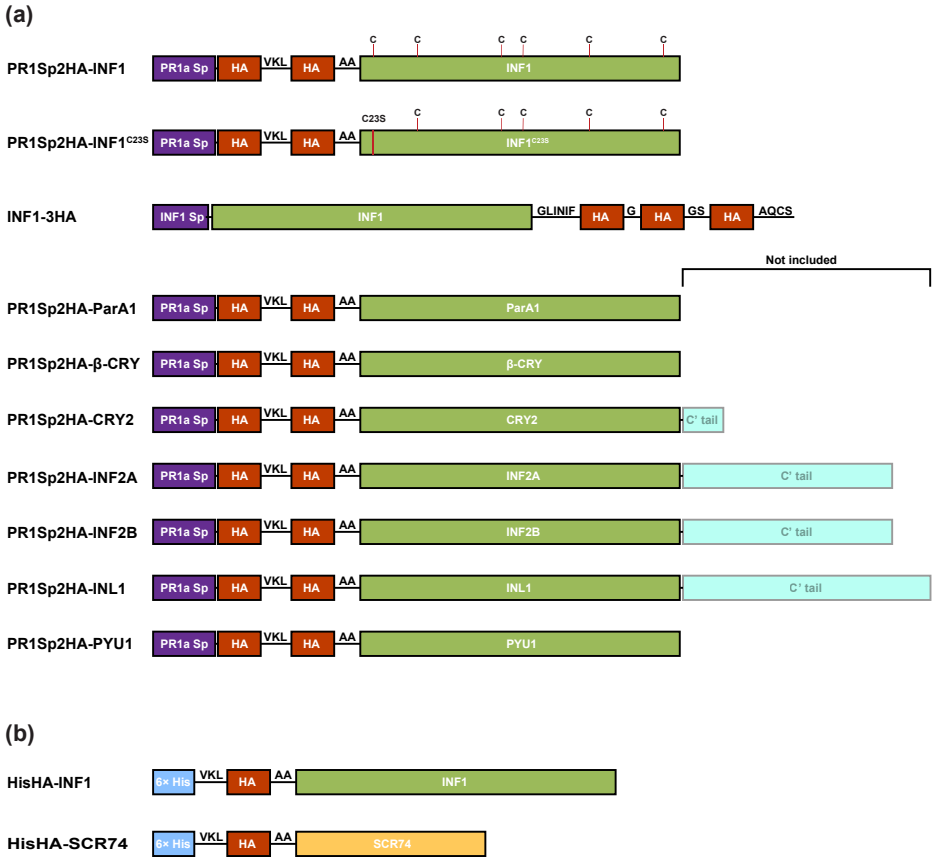

FIGURE S2 | Overview of affinity tagged elicitins used in this study. Effector constructs used for (a) transient expression *in planta* using *Agrobacterium tumefaciens* or for (b) recombinant protein expression in *Pichia pastoris*. Pathogenesis-related protein 1a (PR1a) or native INF1 signal peptides (Sp) were used while effectors were carrying hemagglutinin (HA) or histidine (His) tags, as indicated.

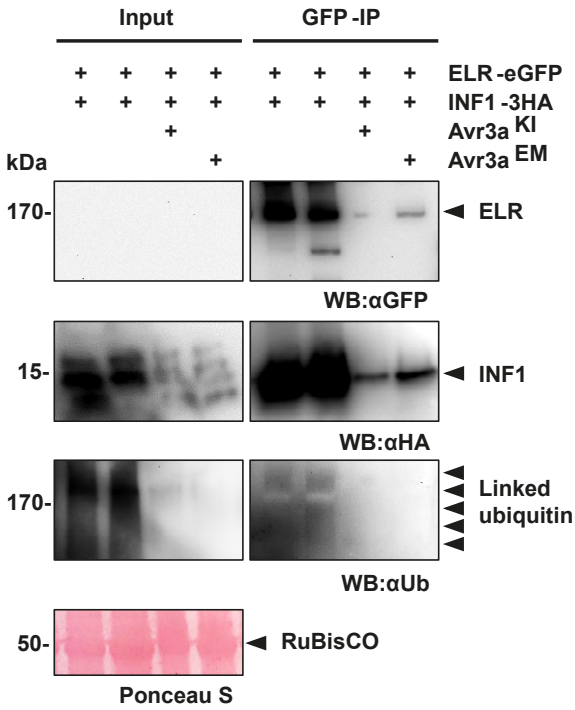

FIGURE S3 | Avr3a co-expression affects protein ubiquitination but not ELR binding with INF1. ELR-eGFP was co-expressed with INF1-3HA and Avr3a^{KI} or Avr3a^{EM}in *Nicotiana benthamiana*. Total protein was extracted at 2 days post infiltration and was subjected to immunoprecipitation using anti-GFP beads to capture ELR-eGFP. The immunopurified ELR was detected with anti-GFP, while its interaction with INF1 was assessed with anti-HA antibodies. Linked ubiquitin was detected with anti-Ub antibody. Ponceau S staining of RuBisCO indicates equal protein loading. Results are preliminary, from one experiment.

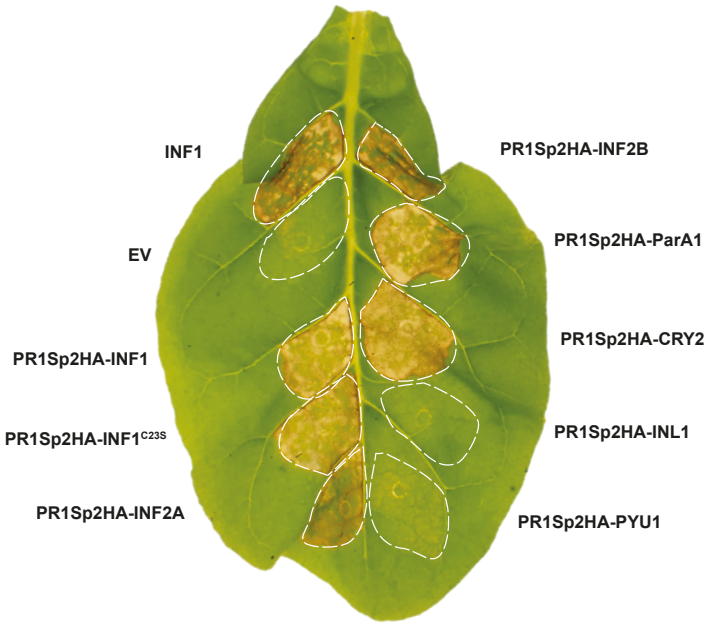

FIGURE S4 | N-terminal-HA-tagged elicitins are recognized in tobacco. N-terminal-HA-tagged elicitins (PR1Sp-2HA-) were transiently expressed in *Nicotiana tabacum* SR1 for testing for cell death induction. Non-tagged INF1 and empty vector (EV) were used as controls. A representative leaf, photographed at 3 dpi, is shown.

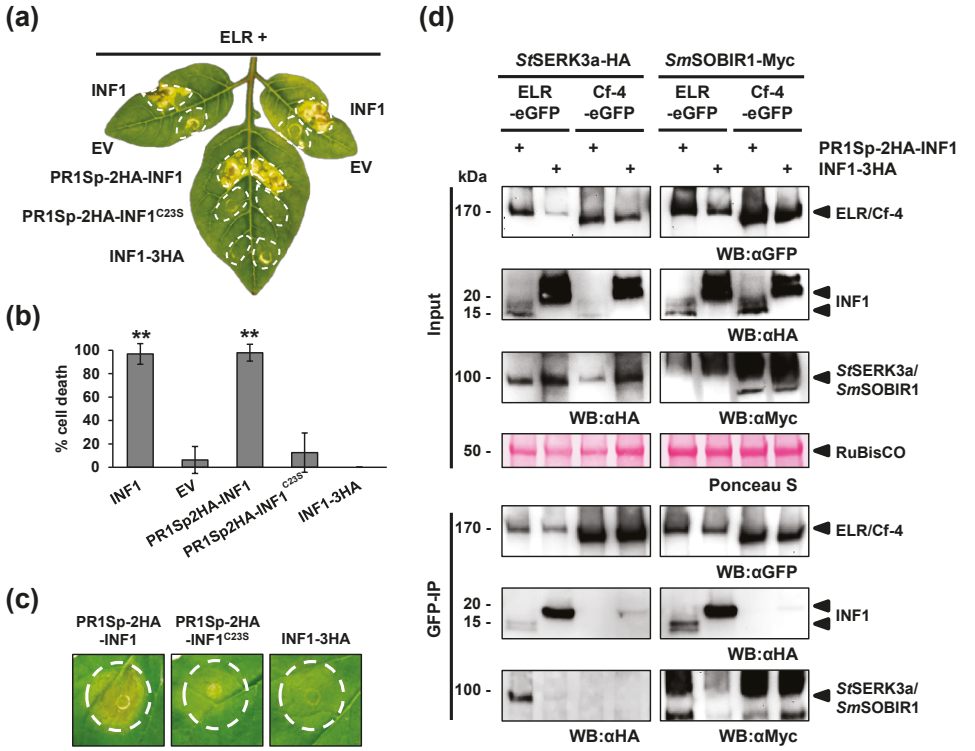

FIGURE S5 | C-terminal 3HA tagging of INF1 causes Inhibition of the interaction between ELR, SOBIR1 and SERK3, abolishing cell death induction. (a, b) N-terminal HA tagged PR1Sp-2HA-INF1 and the PR1Sp-2HA-INF1^{C23S} mutant or C-terminal HA-tagged INF1 was transiently expressed in *Solanum hjertingii* 349-3 (together with ELR) or (c) in *N. benthamiana*. Photos of cell death responses were obtained at 3 dpi (*S. hjertinjii*) or 5 dpi (*N. benthamiana*). (d) N-terminal (PR1Sp-2HA-INF1) and C-terminal (INF1-3HA) HA-tagged INF1 was transiently co-expressed with ELR and *St*SERK3a or with ELR and *Sm*SOBIR1 in *Nicotiana benthamiana*. Cf-4 was used as a negative control. Total protein was extracted and was subjected to immunoprecipitation using anti GFP beads to capture ELR-eGFP and Cf-4-eGFP. The immunopurified proteins were detected with anti-GFP, while the interaction with *St*SERK3a-HA, *Sm*SOBIR1-Myc or HA-tagged INF1, as a result of co-immunoprecipitation of the latter proteins with the eGFP-tagged proteins, was assessed with anti-HA or anti-Myc, respectively. Ponceau S staining of RuBisCO indicates equal protein loading.

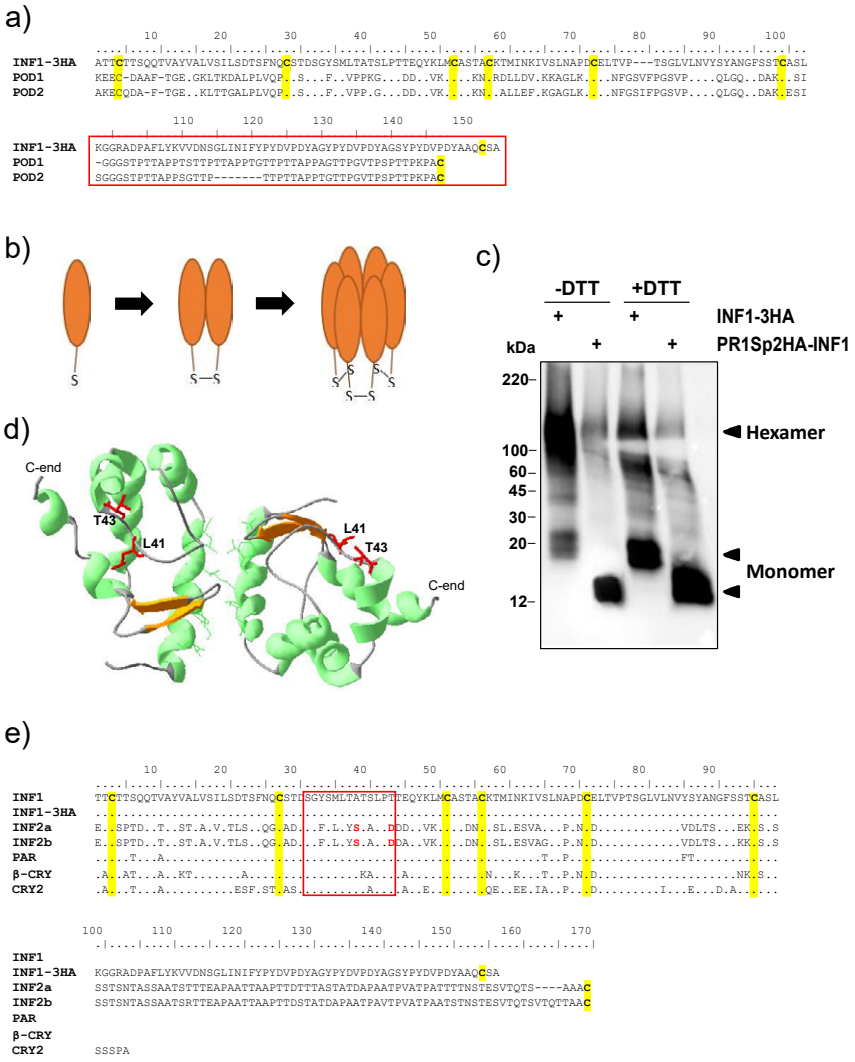

FIGURE S6 | Oligomerization of INF1-3HA and structure motifs likely involved in interaction with ELR. (a) Sequence alignment of INF1-3HA protein with elicitins POD1 and POD2 from *Pythium oligandrum*. C-terminal tails are framed in red. (b) Model of INF1-3HA oligomerization with disulfide bonds between the Cys7 residues. (c) N-terminal (PR1Sp2HA-INF1) and C-terminal (INF1-3HA) HA-tagged INF1 was transiently expressed in *Nicotiana benthamiana*. Total protein was extracted and subjected to SDS-PAGE under reducing (+DTT) and non-reducing (-DTT) conditions. INF1-3HA protein was detected with anti-HA. (d) The structure of cinnamomin dimer loaded with sterol visualized using the VMD program (Humphrey et al., 1996). The surface-exposed residues located in omega loop region (Leu41, Thr43), are shown in red. (e) Sequence alignment of seven elicitins used in this study. The ω-loop region is framed in red while the residues with assumed role in the interaction with ELR are marked in red as well.

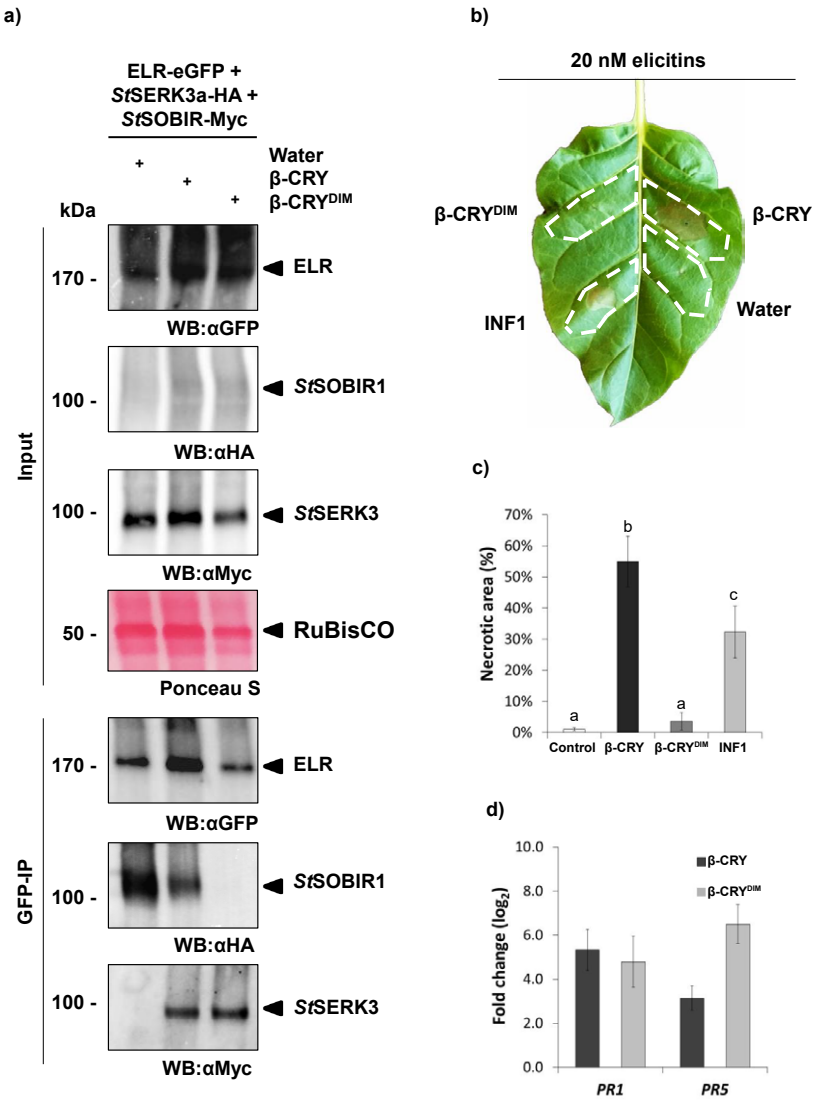

FIGURE S7 | A covalently cross-linked dimer of β-CRY causes inhibition of the interaction between ELR and SOBIR1 and suppresses the elicitin-induced cell death. (a) Water, β-CRY or β-CRY covalently cross-linked dimer (β-CRY^{DIM}) (Uhlíková et al., 2016) were infiltrated into *Nicotiana benthamiana* leaves transiently expressing *St*SERK3a-HA, *St*SOBIR1-Myc and ELR-eGFP. Immunoprecipitation was performed using anti GFP beads to capture ELR-eGFP. Immunopurified ELR was detected with anti-GFP, while the interaction of ELR with *St*SERK3a-HA or *St*SOBIR1-Myc, as a result of co-immunoprecipitation of the latter proteins with eGFP-tagged ELR, was assessed with anti-HA or anti-Myc antibodies, respectively. Ponceau S staining of RuBisCO indicates equal protein loading. (b, c) Water and 20 nM proteins β-CRY or β-CRY^{DIM} crosslinked dimer and INF1) were infiltrated into the *Nicotiana tabacum* leaves. A representative leaf, photographed at 2 d post treatment, is shown together with analysis of necrotic area relative to the total area of the infiltrated sector. In (c) each bar represents the standard error of four replicates from three different experiments. A student's t-test with $p \leq 0.01$ was used to determine whether differences were statistically significant. Different letters denote a significant

difference. (d) Effect of 20 nM β-CRY and β-CRY^{DIM} proteins on the accumulation of transcripts for *PR1a* and *PR5* in *N. tabacum* leaves. Gene expression relative to a water treated control was calculated at 48 hours after protein infiltration using the ΔΔCt method (Livak and Schmittgen, 2001). The values given in the graphs are the $\log_2$ values of relative quantification. Error bars indicate standard deviation. A greater than two-fold change in transcript accumulation was taken as significant.

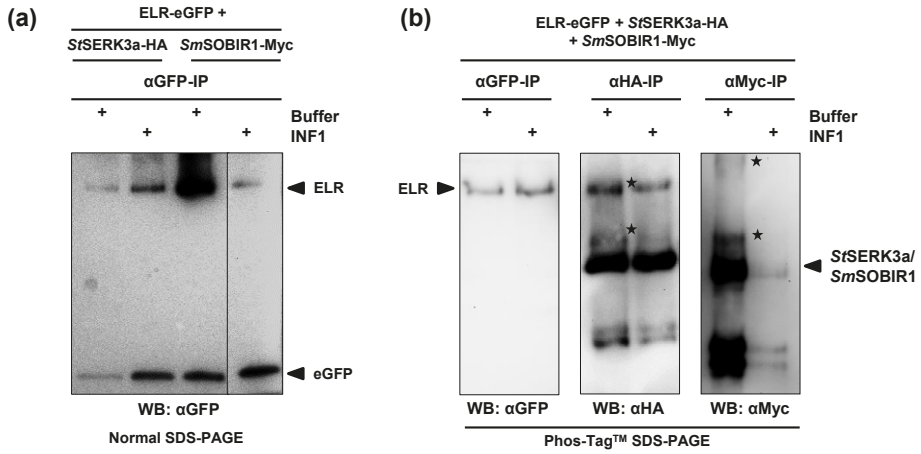

FIGURE S8 | Protein accumulation and phosphorylation dynamics of the ELR-SOBIR1-SERK3a complex upon elicitation by INF1. ELR-eGFP was transiently co-expressed in *Nicotiana benthamiana* with *St*SERK3a-HA or *Sm*SOBIR1-Myc, or both. *N. benthamiana* leaves were elicited with INF1 or buffer, and after 15 min the leaves were analyzed. (a) Total protein was extracted and was subjected to immunoprecipitation using anti-GFP beads to capture ELR. The immunopurified proteins were separated by SDS-PAGE and western blot was performed with anti-GFP to detect ELR. (b) Total protein was extracted and was subjected to immunoprecipitation using anti-GFP, anti-HA or anti-Myc beads to capture ELR-eGFP, *St*SERK3a-HA or *Sm*SOBIR1-Myc, respectively, under conditions to preserve the protein phosphorylation status (Phospho-STOP). The immunopurified proteins were separated by Phos-Tag™ SDS-PAGE and western blot was performed with anti-GFP, anti-HA or anti-Myc to detect ELR-eGFP, *St*SERK3a-HA or *Sm*SOBIR1-Myc, respectively. Asterisks (*) indicate the potential phosphorylated bands of *St*SERK3a or *Sm*SOBIR1.

Chapter 5

Targeted mutagenesis of ELR in wild *Solanum* species using CRISPR/Cas9

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abstract

ELR encodes for a receptor-like protein (RLP) which recognizes INF1 and other elicitins and enhances resistance to *P. infestans* when overexpressed in cultivated potato. However, in potato it has so far been impossible to estimate what the resistance contribution of a single RLP is to a pathogen in its native genetic background. In this study, we used CRISPR/Cas9-mediated genome editing to target *ELR* in wild *Solanum*. A construct containing two single-guide RNAs was developed, targeting the DNA region between LRR3-6 which is found specifically in functional *ELR* orthologs. Using a transient expression assay in *Nicotiana benthamiana*, we found that the construct was effective in creating mutations in *ELR*. This finding prompted us into attempting stable transformation of *S. microdontum*, *S. microdontum* subsp. *gigantophyllum*, *S. edinense*, *S. papita*, *S. phureja* and *S. chacoense*. While we were unable to obtain transformants of *S. microdontum*, we obtained a few positive transformants in each of the other species. These transformants were subsequently screened for altered responses to INF1 using PVX agroinfection. Transformants with decreased INF1 response were found in *S. papita* and *S. edinense* and were screened for the presence of *ELR* mutations. Using enrichment approaches, heterozygous *ELR* mutations were detected in these transformants which could explain the reduction of INF1-triggered cell death. Results from this study indicate that the CRISPR/Cas9 as a method of targeted genome editing on PRRs is possible in several different wild *Solanum* species. However, to obtain homozygous mutants, either a high number of transformants need to be screened or crossing experiments with positive transformants should be carried out.

Introduction

Potato is the third most important food crop in the world and the most important non-grain crop (Barrell et al., 2013). Hence, the production of potato tubers is playing an important role in the modern diet of almost all people worldwide. In most countries, the major threat to potato cultivation is late blight caused by the oomycete *Phytophthora infestans*, notorious for causing the Irish potato famine in the mid-18th century. Breeding for disease resistance against this pathogen has become the main subject in developing new potato varieties. So far, breeding efforts to obtain late blight-resistant potato cultivars were focused on the introduction of resistance (*R*) genes, encoded by nucleotide-binding, leucine-rich repeat (NLR) type of immune receptors (Vleeshouwers et al., 2011a).

NLR genes encode cytoplasmic receptors which upon recognition of the cognate avirulence (Avr) gene products of pathogens provide a strong, often qualitative, resistance. However, virulent pathogen races evolve quickly, evading the recognition from these receptors (Haas et al., 2009; Raffaele et al., 2010b; Vleeshouwers et al., 2011a). This selection, in combination with the highly evolving nature of *P. infestans* led to the so called 'breaking' of most introduced NLR genes so far. Despite the large number of *NLRs* cloned so far, any single introduction in potato cultivars failed to provide sustained pathogen control indicating that either multiple NLRs should be introduced simultaneously and/or that alternative layers of resistance should be explored.

Pattern recognition receptors (PRRs) have been reported as the first line of recognition of self- or microbe-derived molecules being microbe associated molecular patterns (MAMPs) or apoplastic effectors. This recognition triggers a defense response which in many cases results in quantitative resistance against pathogens (Jones and Dangl, 2006). Recent studies have investigated whether immunity that is triggered upon perception of apoplastic pathogen elicitors can enhance late blight resistance. Previous work in our group led to the identification of ELR (Du et al., 2015), a PRR that recognizes several elicitins, a highly conserved family of secreted effectors of *Phytophthora* and *Pythium* spp. *ELR* was cloned from the wild potato species *S. microdontum*. It encodes a receptor-like protein (RLP) which provides broad spectrum recognition of various elicitins in potato. ELR is a 1094 amino acid long protein which consists of seven major domains typical for RLPs. Domain A contains a 23 amino acid long signal peptide, domain B contains a Cys-rich N-terminus, domain C is divided in three subdomains, of which C1 and C3 are leucine rich repeats (LRRs) and C2 is a non-LRR island. Domain D is a spacer, domain F is a transmembrane domain while domain G is a short cytoplasmic domain. When ELR was transformed into cultivated potato, it

was found to confer partial resistance to *P. infestans* in laboratory infection assays, likely by mounting elicitin-triggered cell death (Du et al., 2015).

Functional characterization of genes by reverse genetics is an important part of modern molecular biology. Despite numerous successful interfamily transfers of PRRs into crops including potato, by means of stable overexpression, gene functional studies based on actual mutants were only possible in *Arabidopsis* or other diploid model systems. For potato, this was due to the polyploidy and heterozygosity of cultivated potato *S. tuberosum* (2n = 4× = 48) as well as other wild *Solanum* spp. (PGSC, 2011), which renders random mutagenesis approaches such as ethyl methanesulfonate (EMS), T-DNA insertion or irradiation very difficult. However, recent developments in genome editing technologies such as clustered regularly interspaced short palindromic repeats (CRISPR) and transcription activator-like effector nuclease (TALEN) have enabled the easier generation of mutants in many non-model crops including polyploids such as potato and wheat (Belhaj et al., 2013; Wang et al., 2014; Butler et al., 2015; Wang et al., 2015). Specifically, the application of CRISPR/Cas9 technology for plant genome editing has been developing rapidly in the last couple of years and holds great promise for genome manipulation of polyploid crops.

Previous work in our group has identified 32 *Solanum* spp. (Rietman, 2011) that show a cell death response upon PVX agroinfection with INF1. Eight functional *ELR* homologs have been cloned from *S. edinense*, *S. papita*, *S. phureja*, *S. microdontum* subsp. *gigantophyllum* and *S. chacoense*, which are resistant to *P. infestans* and show response to INF1 (Du, 2014; Vleeshouwers et al., 2011b). Several paralogs of ELR are found in the genome of cultivated potato. Interestingly, these homologs lack a stretch of 108 amino acids, which form the LRR3-6 in ELR and do not seem to respond to INF1 (Du et al., 2015).

In this study, we aimed to investigate whether CRISPR/CRISPR-associated protein 9 (Cas9) can be used for inducing targeted mutations in *Solanum* and serve as a tool for gene functional analysis. As a model, we have selected to knock-out the *ELR* gene. The species *S. microdontum* (source of *ELR*) has been reported to be recalcitrant to transformation with *Agrobacterium tumefaciens* (Rakosy-Tican L, 2004). Nonetheless, we attempted transformation of this species but did also include additional species carrying a functional *ELR* like *S. edinense*, *S. papita*, *S. phureja*, *S. microdontum* subsp. *gigantophyllum* and *S. chacoense* (Du, 2014). If successful, this approach will be used to develop wild potato mutants of *ELR* orthologues to determine the contribution of ELR to resistance against *P. infestans* in the native background.

Materials and methods

Plant materials

Solanum genotypes were clonally propagated *in vitro* on Murashige and Skoog (MS) medium supplemented with 20% w/v sucrose as described (Murashige and Skoog, 1962; Du et al., 2015). For experiments, top shoots were transferred to fresh growth medium at 25°C. After allowing 1-2 weeks (wks) for rooting, plantlets were transferred to jiffy pots with disinfected soil in a climate-regulated greenhouse compartment (22/18°C and 16/8 h light day/night regime at 70% relative humidity. Supplemental light of 100 W/m^2 was applied when the natural light intensity was below a 150 W/m^2 threshold). Two wks later, the plants were transferred to bigger pots and allowed to grow for an additional 2 wks (till ready for PVX-agroinfection) under the same conditions.

Nicotiana benthamiana plants used in this study were derived from seeds and were maintained under the same greenhouse growth conditions as mentioned above.

CRISPR/Cas9 cloning

The *ELR*-targeting construct used for CRISPR/Cas9 mutagenesis has been developed using the Golden Gate (GG) method (Weber et al., 2011) as following. *ELR*-specific single guide RNA 1 (sgRNA1-CCGATGGAGAAATTTGAGGCATC) and 2 (sgRNA2-CCTTGTTAACAATTTCTTTTATC) were PCR amplified using primers *ELR*sgRNA1 *Bsa*I Fwd or *ELR*sgRNA2 *Bsa*I Fwd, respectively with sgRNA BsaI Rev (Table 2) with plasmid gRNA_GFP_T1 as a template (Mali et al., 2013). sgRNA1 PCR product was cloned in pICH47751 and simultaneously placed behind the *Arabidopsis thaliana At*U6p promoter found in the level 0 module vector pICSL01009*::pAtU6* (Nekrasov et al., 2013) with a cut-ligation protocol using BsaI and T4-ligase (Thermo Fisher Scientific). sgRNA2 was cloned in pICH47761 and similarly fused to *pAtU6* as sgRNA1 using the same cut-ligation procedure (Weber et al., 2011). Those level 1 constructs were verified by sequencing using primer sgRNA *Bsa*I Rev (Table 2). The human codon optimized, GG domesticated hCas9 level 0 module, pICH47742*::p35s::Cas9-TNOS* was combined with pICH47732*::pNOS::BAR-TOCS* (*BAR* confers phosphinothricin-PPT resistance in plant), pICH47751*::pAtU6::sgRNA1*, pICH47761*::pAtU6::sgRNA2* and linker pICH41780 and cloned in pAGM4723 in a single cut-ligation reaction with *Bbs*I and T4-ligase (Thermo Fisher Scientific) (Weber et al., 2011). pAGM4723 was a gift from Sylvestre Marillonnet (Addgene plasmid # 48015). The resulting binary vector pAGM4723*::pNOS:BAR-p35s:hCas9-pAtU6::sgRNA1-pAtU6::sgRNA2* was

confirmed by *Hind*III (Thermo Fisher Scientific) digestion and was transformed into *Agrobacterium tumefaciens* strain AGL1.

Plant genomic DNA extraction

Genomic DNA (gDNA) was isolated from leaflets of *in vitro* grown potato plants. Approximately 100-150 mg of leaf tissue was collected in 2 ml tubes and snap frozen in liquid nitrogen. Two 3 mm tungsten carbide beads (Qiagen) were added to each frozen leaf sample before grinding using TissueLyser II (Qiagen) or the RETSCH machine (RETSCH, Germany) using manufacturer instructions. Then gDNA was isolated using the microprep method (Fulton et al., 1995; Jo et al., 2014). Dried gDNA pellets were resolved in MilliQ water. DNA concentrations were determined with Nanodrop (Thermo Fisher Scientific).

Nicotiana benthamiana transient CRISPR/Cas9 efficiency testing

Transient co-expressions were performed as described previously (Du et al., 2014; Domazakis et al., 2017). *A. tumefaciens* strains carrying pBin-KS-*p35s::ELR-eGFP* (Du et al., 2015) and pAGM4723*::pNOS:BAR-p35s:hCas9-pAtU6::sgRNA1-pAtU6::sgRNA2* suspension cultures in infiltration medium were mixed 1:1 at a final OD_{600} = 1 each. The leaves of 4 wk-old *N. benthamiana* plants were infiltrated using a needleless syringe. Soon after agroinfiltration, leaf disks from the treated area were collected and labeled as the 0 h sample (negative control treatment, not containing any induced mutations). Then, 48 hrs later, leaf disks from the infiltration site were also collected and were labelled as the 48 hrs sample. Genomic DNA (gDNA) extraction was performed as described above and samples were subjected to mutation enrichment using restriction digestion, similar to what has been described (Nekrasov et al., 2013). Briefly, 100 - 500 ng of isolated gDNA from both 0 hr and 48 hr treatments were digested with *Rsa*I for at least 5 hrs at 37°C, as indicated in fig. 2. This enzyme cuts only once between the selected *ELR* fragment amplified by primers *ELR* 190 Fwd and *ELR* 728 Rev. PCR was performed with Phusion polymerase (Thermo Fisher Scientific) on 50 ng from each sample before and after *Rsa*I digestion followed by cloning of the divergent band in pCR-BluntII-TOPO vector (Thermo Fisher Scientific). Plasmid DNA was isolated from several *Escherichia coli* and bi-directional Sanger sequencing was performed using commercial M13 forward and reverse primers.

Agrobacterium-mediated transformation of *Solanum*

Wild *Solanum* transformation was performed using internodal explants, as described previously (Fillatti et al., 1987; Hoekema et al., 1989; Beaujean et al., 1998). Transformed shoots were regenerated in MS media containing 4-10 µg/ml PPT for

selection while 1 mg/l zeatine riboside was used for shoot induction instead of normal zeatine. Apparent regenerated shoots from each callus were transferred to fresh MS media with PPT selection for further growth and rooting. Each regenerant was given a code and was propagated individually (Fig. 3b). Upon sufficient rooting and growth *in vitro*, plantlets were transferred to greenhouse for phenotyping and PVX agroinfection screening and were tested by PCR for the presence of the pAGM4723*::pNOS:BAR-p35s:hCas9-pAtU6::sgRNA1-pAtU6::sgRNA2* cassette using primers Cas9_6F and RB-F1 (Table 2) and DreamTaq polymerase (Thermo Fisher Scientific). Primers *StEF1*α qPCR Fwd and *StEF1*α qPCR Rev were used as an internal control for DNA quality and *ELR* 190 Fwd and *ELR* 1206 Rev were used as internal controls for the presence of ELR (Table 2).

PVX agroinfection

PVX agroinfection was carried out as described previously (Du et al., 2014; Domazakis et al., 2017). *A. tumefaciens* GV3101 strains carrying pGR106-*INF1*, pGR106 empty vector (negative control) or pGR106-*CRN2* (positive control encoding crinkling and necrosis 2 (CRN2), a nonspecific cell death inducer (Torto et al., 2003) were used in the experiments (Du et al., 2015). PVX agroinfection was performed on fully expanded leaves of 3-4 wk-old plants growing in the greenhouse by toothpick inoculation. Cell death was scored at 14 dpi.

Results

Design of *ELR*-targeting CRISPR/Cas9 and off-target prediction

Since the functionality of ELR homologs has been linked to the presence of four additional LRRs (LRR 3-6) in the C1 domain of ELR (Du 2014, Du et al., 2015), this region was selected for designing the CRISPR/Cas9 sgRNAs (Fig. 1). Namely, two sgRNAs were designed based on *ELR* from *S. microdontum* 360-1. The sgRNA2 was selected containing a restriction site (*Hpa*I) to facilitate mutation detection using digestion and PCR (Belhaj et al., 2013; Nekrasov et al., 2013; Zischewski et al., 2017). Theoretical off-target predictions using Cas-OFFinder (Bae et al., 2014) and the latest version of the potato genome (*S. tuberosum* DM16R44 PGSC v4.03) indicated that the sgRNAs are specific since there is no other region with 100% match. When allowing for two mismatches, a total of 12 off-targets could be found for sgRNA1 and only one for sgRNA2 (Table 1). Among those, eight putative off-targets are found in non-gene regions while four are found on ORFs belonging to three genes PGSC0003DMG400015351, PGSC0003DMG400002910 (2×) and PGSC0003DMG400002887, the latter two being proteins putatively involved in signaling (Table 1).

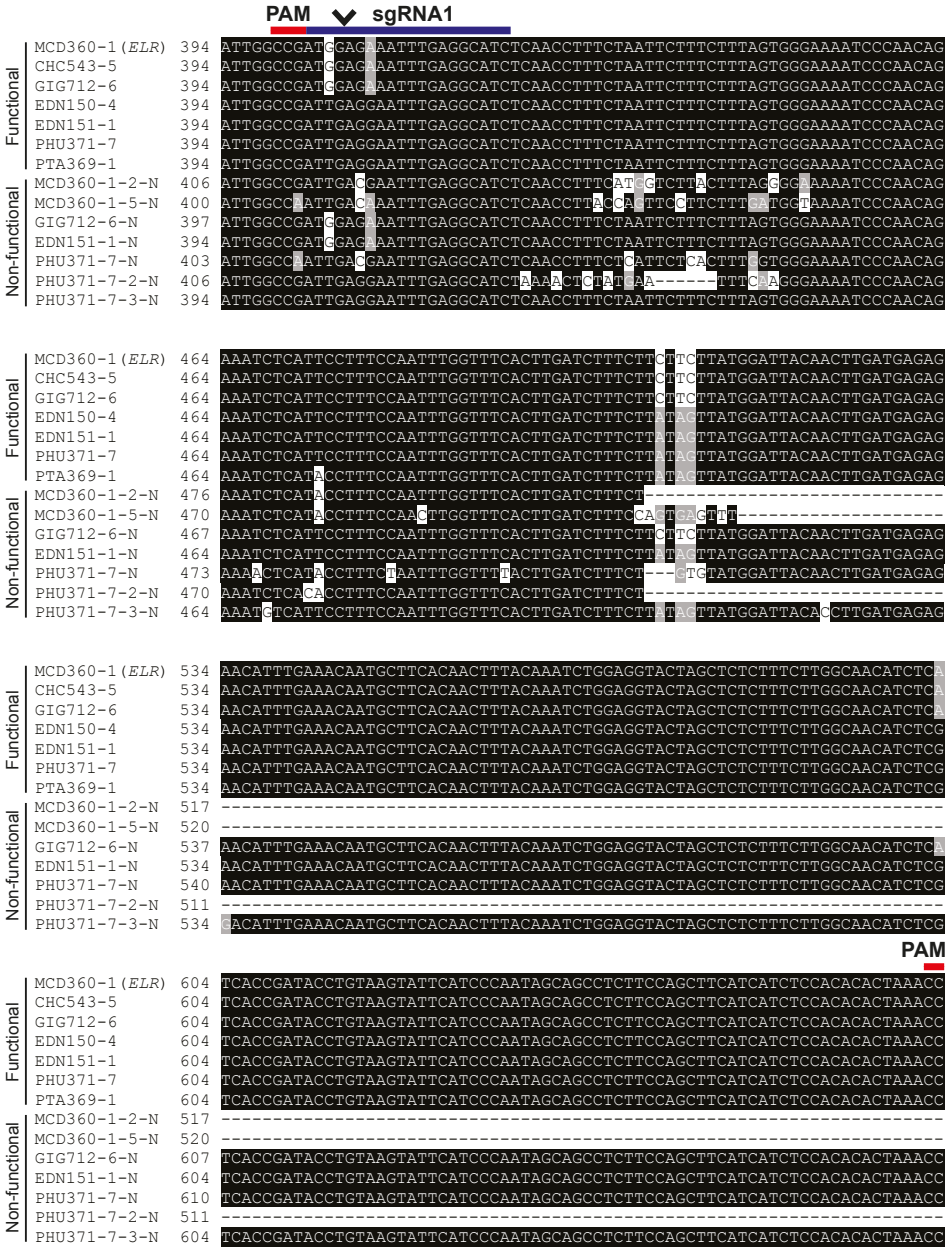


FIGURE 1 | Design of *ELR*-targeting sgRNAs for CRISPR/Cas9-induced mutagenesis. DNA sequence alignment of cloned functional and non-functional *ELR* homologs was created using COBALT and BOXSHADE was used to visualize conservation. The positions of sgRNA1 (3′ – 5′) and sgRNA2 (3′ – 5′) as well as the PAM motifs (3′ – 5′) are indicated. The red rectangle indicates the cutting position of *Hpa*I restriction endonuclease.

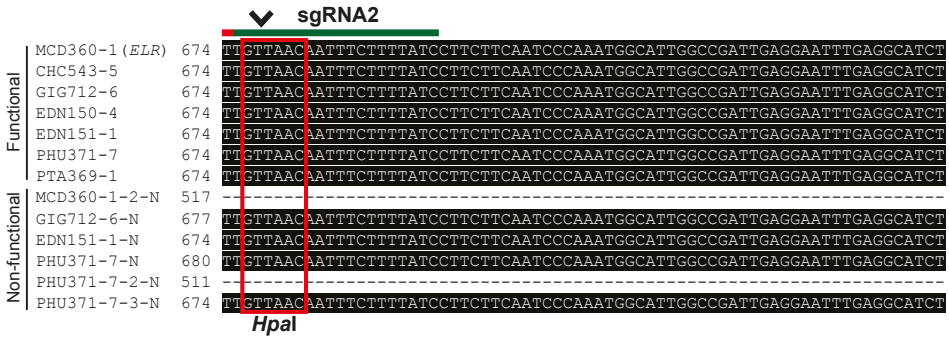


FIGURE 1 | *Continued.*

TABLE 1 | Prediction of ELR-targetting CRISPR/Cas9 off-targets in potato (PGSG v4.03) using Cas-OFFinder (Bae et al., 2014).

	sgRNA (+PAM) 5' → 3'	Chromosome	Position	Direction	Annotation
sgRNA1	GATGCCTCAAATTTCTCCATNGG				
	GATGCtTCAAATTTCaCCATTGG	ST4.03ch01	6053146	+	-
	GATGCtTCAgATTTCTCCATTGG	ST4.03ch01	57264338	-	-
	GATGCCTCAAATTTtTCaATCGG	ST4.03ch09	51860363	+	-
	GATGCCTCAAATTcCTCaATCGG	ST4.03ch12	717179	-	Conserved gene of unknown function[1]
	GATGCCTCAAATTcCTCaATCGG	ST4.03ch12	681383	-	-
	GATGCCTCAAATTcCTCaATCGG	ST4.03ch12	2677409	+	Cf-2.1[2]
	GATGCCTCAAATTcCTCaATTGG	ST4.03ch12	2546193	+	-
	GATGCCTCAAATTTgTCaATTGG	ST4.03ch12	2651873	+	-
	GATGCCTCAAATTcCTCaATCGG	ST4.03ch12	2631306	+	-
	GATGCCTCAAATTcCTCaATCGG	ST4.03ch12	2651552	+	-
	GATGCCTCAAATTcCTCaATCGG	ST4.03ch12	2903277	-	Serine/threonine protein kinase 1[3]
	GATGCCTCAAATTcCTCaATCGG	ST4.03ch12	2902941	-	Serine/threonine protein kinase 1[3]
sgRNA2	GATAAAAGAAATTGTTAACANGG				
	GATtAAAGAAATTGTTAACAAGG	ST4.03ch12	2676839	+	Cf-2.1[2]

[1]PGSC0003DMG400015351
[2]PGSC0003DMG400002910
[3]PGSC0003DMG400002887

Trial testing of CRISPR/Cas9 in *Nicotiana benthamiana*

To examine whether the designed CRISPR/Cas9 construct with two sgRNAs is able to induce the expected mutation on *ELR*, we developed a transient screening assay in *N. benthamiana*. A combination of *A. tumefaciens* carrying a vector containing *ELR* (pK7WG2-*ELR*) was co-infiltrated with the CRISPR/Cas9 construct in fully developed *N. benthamiana* leaves. As a negative control, samples from the infiltrated area were collected immediately following infiltration. At 48 h post agroinfiltration, leaf material was collected. Genomic DNA was extracted from each sample and was subjected to PCR amplification using primers *ELR* 190 Fwd and *ELR* 728 Rev, with an expected product of 539 bp. We hypothesized that if both sgRNAs are active, a deletion will occur in *ELR* with a size of approximately 273 bp. Since CRISPR/Cas9 mutations are much more rare compared to the unchanged plasmid DNA introduced in the plant, an enrichment approach would be required to obtain possible mutations. Mutation enrichment (Belhaj et al., 2013; Zischewski et al., 2017) was performed by a prolonged digestion with *Rsa*I, a restriction enzyme cutting between the two sgRNA-targeted sites (Fig. 2b). Upon PCR, we found a distinct smaller band in the digested 48 h samples, indicating a putative mutation event (Fig. 2a). Upon cloning and sequencing of the amplicon, we found that the CRISPR/Cas9 construct induces the expected 273 bp deletion in *ELR*, which derives from cuts within three bases next to the protospacer adjacent motifs (PAM), as expected. Therefore, the designed CRISPR/Cas9 construct was considered suitable to edit ELR and efficient for use in potato transformations.

Transformation of wild *Solanum* with the *ELR*-targeting CRISPR/Cas9

To study the function of *ELR* in its native background, the designed CRISPR/Cas9 construct was initially transformed in *S. microdontum* genotypes MCD360-1 and GIG712-5 in order to generate a stable *ELR* knock-out. However after several attempts, we were unable to obtain *S. microdontum* transformants using our standard *Agrobacterium*-mediated transformation protocols. After co-cultivation of explants with *Agrobacterium* (300 explants per genotype), calli were formed indicating initial transformation of cells, however there was no shoot formation. Since functional *ELR* homologs exist in several *Solanum* spp., four different wild potato species which were previously shown to carry a functional *ELR* (Du, 2014) were selected; i.e. *S. edinense* (EDN151-1), *S. phureja* (PHU371-7), *S. papita* (PTA369-1) and *S. chacoense* (CHC543-5). Prior to transformation, those genotypes were confirmed to respond to INF1 using PVX-agroinfection (Fig. 3a, b, c). A total of 19 EDN151-1, 12 PHU371-7, seven PTA369-1and one CHC543-5 plants were regenerated on selective media after *Agrobacterium*-mediated introduction of the construct (Fig. 3b).

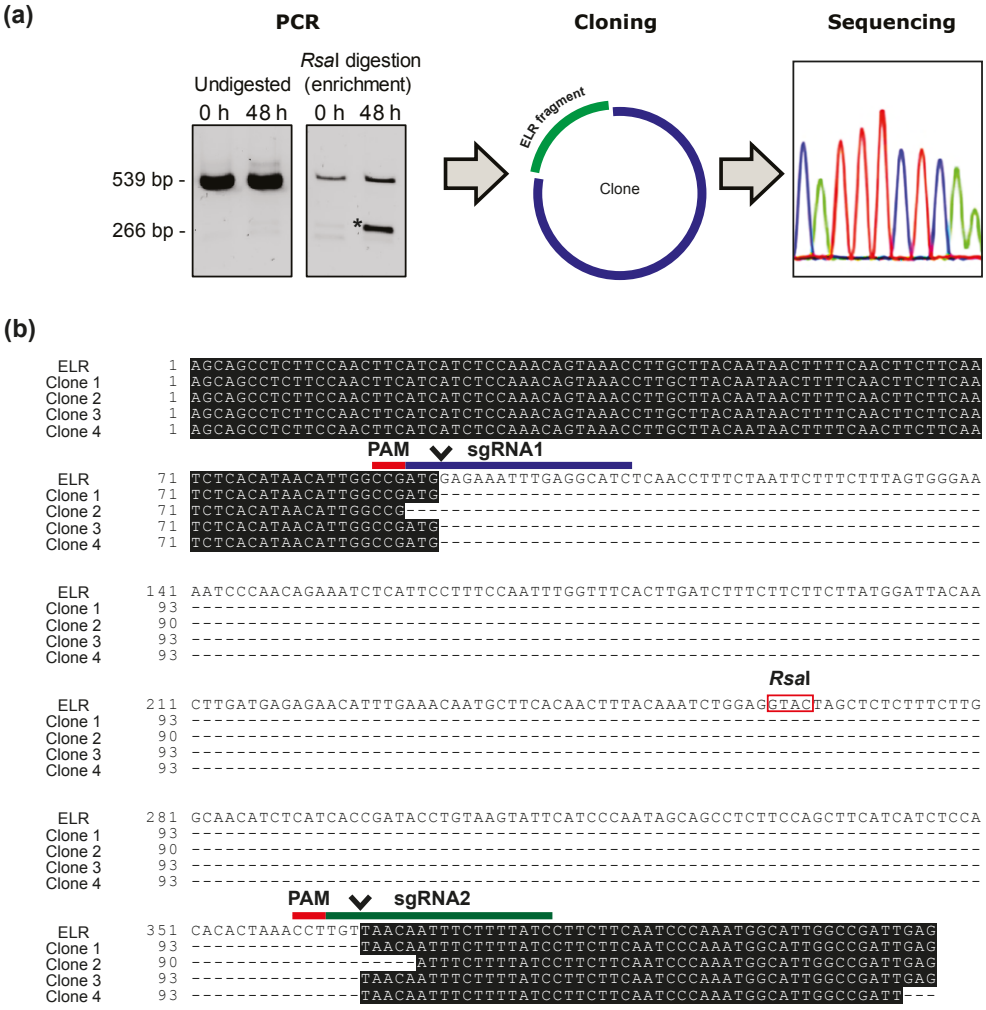
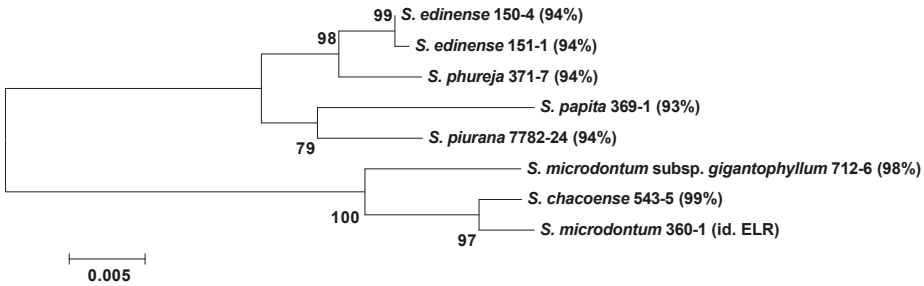


FIGURE 2 | Trial testing in *Nicotiana benthamiana* shows that *ELR*-targeting CRISPR/Cas9 constructs are functional. *N. benthamiana* leaves were co-infiltrated with a construct carrying *ELR* and the CRISPR/Cas9-*ELR* construct with two sgRNAs designed in this study. Genomic DNA was isolated from *N. benthamiana* at 0 and 48 h post-co-agroinfiltration. (a) PCR amplification of a partial *ELR* sequence was performed with or without *Rsa*I digestion, the divergent band (indicated with asterisk, *) was cloned and sequenced. (b) Sanger sequencing results of four independent clones showing the presence of a 273 bp deletion in *ELR* after CRISPR/Cas9 treatment.

(a)



(b)

Solanum sp.	Ploidy	Accession	# explants	# regenerants	# PCR positive transformants
S. edinense (EDN)	5n	151-1	300	>>20 (19 tested)	16
S. phureja (PHU)	2n	371-7	300	12	10
S. papita (PTA)	4n	369-1	300	7	7
S. microdontum subsp. *gigantophyllum* (GIG)	2n	712-6	300	0	0
S. chacoense (CHC)	2n	543-5	300	1	1
S. microdontum (MCD)	2n	360-1	300	0	0

(c)

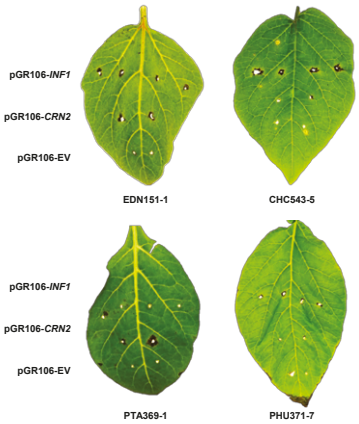


FIGURE 3 | Selection of *Solanum* carrying a functional ELR for stable transformation using CRISPR/Cas9. (a) Phylogenetic relationship of ELR homologs cloned from eight different *Solanum* sp. Amino acid sequences were aligned using MUSCLE and a maximum likelihood phylogenetic tree was created (1000 bootstrap) using MEGA5 software. Numbers in nodes indicate bootstrap values >50%. (b) Summary of obtained transformants from *Agrobacterium tumefaciens*-mediated transformation of potato internodes. (c) PVX-agroinfection with INF1, CRN2 (positive control) or empty vector (EV) on leaves of wild *Solanum* genotypes. Photos were taken at 14 days post inoculation and representative pictures are shown.

5

PCR confirmation of the integration of CRISPR/Cas9 into wild potato species

To analyse the identity of the 19 EDN151-1, 10 PHU371-7, seven PTA369-1 and the CHC543-5 regenerants (39 in total) obtained from the transformation, genomic DNA was isolated for PCR confirmation of transgene integration. To do so, a PCR was performed to detect part of the CRISPR/Cas9 transgene using primers Cas9-6f and RB-f1 (Table 2) compared to the respective untransformed genotypes (wild types, WT). Those primers amplify a fragment from the Cas9 to the edge of the right border of the binary vector (Fig. 4a). As internal controls for the DNA quality, the presence of *ELR* (primers *ELR* 190 Fwd and *ELR* 1206 Rev), as well as *StEF1a* (primers *St/NbEF1*α qPCR Fwd and *St/NbEF1*α qPCR Rev) was tested (Fig. 4b). We found that 34 out of 39 regenerants were carrying the CRISPR/Cas9 *ELR*-targeting

cassette and were considered actual transformants (Fig. 3b). However, we cannot exclude the possibility that some plants that were negative for the transgene could be derived from incomplete introgression of the right border since they grow on PPT selection media. So we included some non-PCR positive plants in our assays (i.e. EDN151-1 T6, T8 and T10). Overall, about 87% of the putative transformants of four genotypes were found to carry the CRISPR/Cas9 cassette. From the first experiments, eight of the transformants failed to grow sufficiently (EDN151-1 T15, PTA369-1 T4 and PHU371-7 T5, T6, T9, T10, T11, T12) and were excluded from further experiments, while others exhibited some degree of chlorosis or stunting phenotypes (Fig. 5).

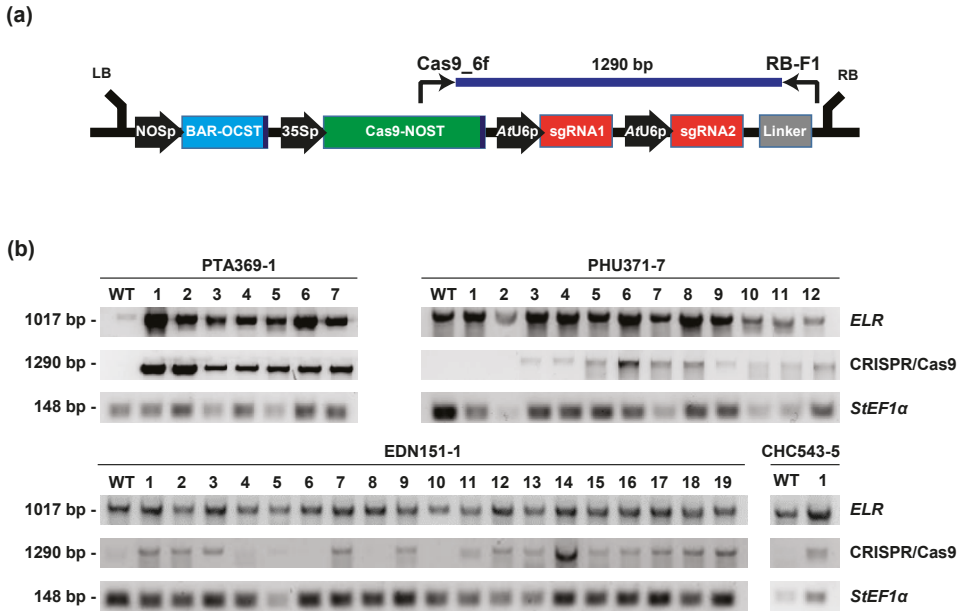
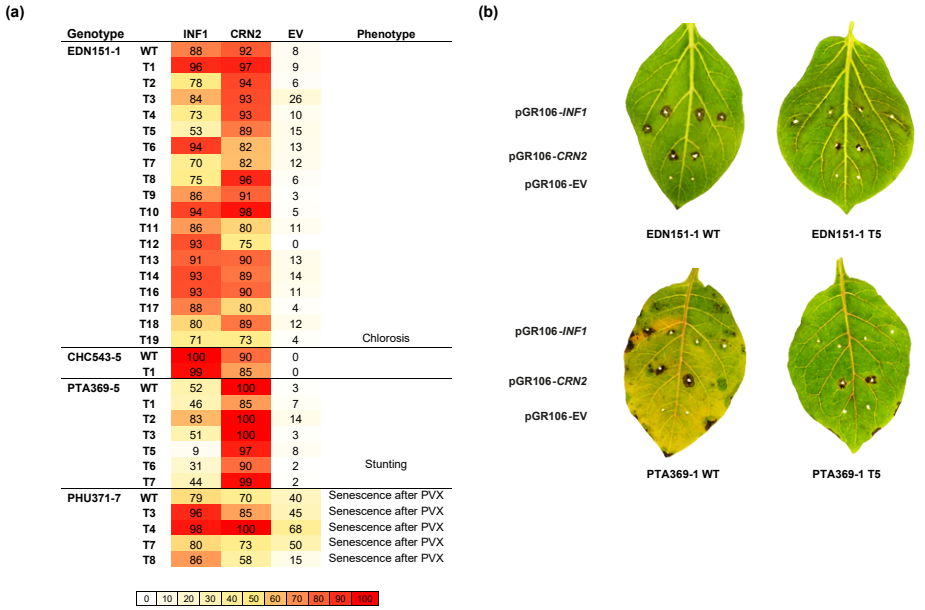


FIGURE 4 | PCR confirmation of CRISPR/Cas9 putative transformants. (a) CRISPR/Cas9 cassette used for *Solanum* transformation. The PCR fragment amplified for checking the transgene introgression is shown as blue line. (b) PCR confirmation of putative transformants obtained for *S. papita* PTA369-1, *S. phureja* PHU371-7, *S. edinense* EDN 151-1 and *S. chacoense* CHC543-5 with primers amplifying a fragment of the CRISPR/Cas9 construct as shown in (a). To check for DNA quality, fragments of *ELR* and *StEF1*α have been amplified as well.



Genotype		INF1	CRN2	EV	Phenotype
EDN151-1	WT	88	92	8	
	T1	96	97	9	
	T2	78	94	6	
	T3	84	93	26	
	T4	73	93	10	
	T5	53	89	15	
	T6	94	82	13	
	T7	70	82	12	
	T8	75	96	6	
	T9	86	91	3	
	T10	94	98	5	
	T11	86	80	11	
	T12	93	75	0	
	T13	91	90	13	
	T14	93	89	14	
	T16	93	90	11	
	T17	88	80	4	
	T18	80	89	12	
	T19	71	73	4	Chlorosis
CHC543-5	WT	100	90	0	
	T1	99	85	0	
PTA369-5	WT	52	100	3	
	T1	46	85	7	
	T2	83	100	14	
	T3	51	100	3	
	T5	9	97	8	
	T6	31	90	2	Stunting
	T7	44	99	2	
PHU371-7	WT	79	70	40	Senescence after PVX
	T3	96	85	45	Senescence after PVX
	T4	98	100	68	Senescence after PVX
	T7	80	73	50	Senescence after PVX
	T8	86	58	15	Senescence after PVX

FIGURE 5 | Characterization of CRISPR/Cas9 transformants for response to INF1. CRISPR/Cas9 transformants were subjected to PVX-agroinfection with pGR106-*INF1*, pGR106-*CRN2* or pGR106-EV. (a) Presence-absence of cell death was scored at 14 days post inoculation (dpi) and was expressed as the percentage of inoculation spots showing cell death relative to the total amount of inoculation spots. Data are representative of two biological repeats (22 ≤ n ≤ 400). (b) Photos of representative leaves for EDN151-1 WT, T5 and PTA369-1 WT, T5 treated with the same PVX constructs at 14 dpi.

TABLE 2 | Oligonucleotides used in this study.

Primer name	Sequence (5′ → 3′)	Reference
Cas9_6f	ATCTCCCGAAGATAATGAGCAGAAG	(Belhaj et al., 2013)
RB-F1	GGATAAACCTTTTCACGCCC	Universal
*ELR*sgRNA1 *Bsa*I Fwd	TGTGGTCTCAATTGATGCCTCAAATTTCTCCATGTTT	
*ELR*sgRNA2 *Bsa*I Fwd	TGTGGTCTCAATTGATAAAAGAAATTGTTAACAGTTT	
sgRNA *Bsa*I Rev	TGTGGTCTCAAGCGTAATGCCAACTTTGTAC	(Belhaj et al., 2013)
ELR 190 Fwd	AAGTCTTGGAATGAGAGTAGGGA	
ELR 728 Rev	CTCAATCGGCCAATGCC	
ELR 1206 Rev	AGAGAATTGGCATCCTGCAA	
*StEF1*α qPCR Fwd	TGACCAAGATTGACAGGCGT	This thesis, **Chapter 3**
*StEF1*α qPCR Rev	GCAAAACGACCCAATGGTGG	This thesis, **Chapter 3**

PVX agroinfection reveals differential cell death response to INF1 in some CRISPR transformants

The 26 positive CRISPR/Cas9 transformants plus the three EDN151-1 PCR-negative transformants were transferred to the greenhouse for evaluating their response to INF1 compared to their WT backgrounds. We hypothesized that putative *ELR* mutants could show a decreased response to INF1, however since some species are polyploids (EDN151-1, PTA369-4), they could be heterozygous for potential mutations and quantitative differences could be expected. PVX-agroinfection with pGR106-*INF1* was performed in each transformant. As controls, we used pGR106-*CRN2* which encoded a necrosis-inducing protein (Torto et al., 2003; Vleeshouwers et al., 2006) or pGR206-EV (empty vector). Cell death response was scored qualitatively at 14 days post inoculation (dpi) to determine the frequency of cell death (Fig. 5). After comparing the cell death scores of the transformants to their respective WT backgrounds, we found that transformants EDN151-1 T5 and PTA369-1 T5 showed 35% and 43% reduction in cell death response to INF1 compared to their respective WT background. Especially for PTA369-1 T5, the response to INF1 was as low as background (EV) (Fig. 5a, b). Interestingly, we observed that PTA369-1 T2 showed an increased cell death response to INF1 (by 31%) compared to its WT background, which was unexpected and could likely be due to biological variation. For PHU371-7 and CHC543-5 we did not observe major differences between the transformants and WT backgrounds in terms of INF1 response. In conclusion, two CRISPR/Cas9 transformants showed a consistent reduction in INF1-triggered cell death.

PCR amplification and sequencing identifies putative *ELR* mutations

To identify CRISPR/Cas9-induced mutations, a selection of transformants was done based on their responses to INF1. The transformants EDN151-1 T5 and PTA369-1 T5 were selected based on the consistently decreased response to INF1, the transformant PTA369-1 T2 based on its higher INF1 response and EDN151-1 T6, T7, T14, T17, PTA369-1 T3, T6 and T7 were selected based on their similar to WT response (background). To identify potential CRISPR/Cas9-induced mutations and to test whether all functional *ELR* homologs or only a portion have been mutated, we used a restriction enzyme enrichment method prior to PCR amplification (Belhaj et al., 2013; Nekrasov et al., 2013; Zischewski et al., 2017). We hypothesized that any mutation at the sgRNA2 site would cause loss of a chosen restriction site and therefore would enable PCR product amplification following digestion with the restriction endonuclease. On the other hand, we did not expect any mutations at sgRNA1 site, since there are mismatches with our MCD360-1-*ELR*-targetting CRISPR/Cas9 construct. Enzyme *Hpa*I was selected as its cutting site is located in

the sgRNA2 targeting position, exactly on the expected CRISPR/Cas9 cutting site (Fig. 1). A 1017 bp region of ELR was amplified with primers *ELR* 190 Fwd and *ELR* 1206 Rev (Table 2). PCR amplicons were obtained with proofreading polymerase from samples prior and after *Hpa*I digestion. Without *Hpa*I digestion PCR products were obtained for all samples, except for PTA369-1 T5 functional ELR allele. To search for mutations, enrichment with *Hpa*I was performed by digesting gDNA prior to PCR amplification and repeating on the obtained PCR products to ensure complete digestion. Agarose gel electrophoresis showed that the enrichment was successful, as bands in WT samples had disappeared. On the other hand, transformants of PTA369-1 T2, T6 as well as in EDN151-1 T5 and T17 showed *Hpa*I resistant bands corresponding to the expected ELR PCR product size. Those *Hpa*I-resistant bands as well as the WT bands were excised from gel and DNA was isolated for sequencing (Fig. 6a). Sequencing results have shown that PTA369-1 T2 has a SNP change in a likely functional ELR allele, which causes an amino acid substitution (V→A) while PTA369-1 T6 had a mutation in a likely non-functional ELR allele. For EDN151-1 two *Hpa*I-resistant bands were found. Since we were not able to obtain good sequencing results for the upper band, this PCR product was first cloned. After sequencing three clones, two variant sequences with one carrying a frameshift deletion were found (Fig. 6b). Interestingly, all EDN151-1 T5 *Hpa*I resistant bands had among other changes, an insertion of one L amino acid. The lower band of EDN151-1 T5 was found to contain a mutation leading to an amino acid substitution (K→T), however we were not able to detect the apparent deletion in the sequence, as observed by the smaller band. For EDN151-1 T17, similar sequences were obtained with T5. It is important to note that most variations were found in sgRNA2 position, as expected while the sequences we obtained were different from the previously cloned *ELR* homologs in these species (Du, 2014). Overall, following an enrichment strategy, we have identified variations in ELR-like sequences, for four CRISPR/Cas9 transformants which might explain the observed INF1 phenotype. From our data, EDN151-1 T5 potentially contains one deletion which we were not able to sequence fully. However in all cases, cloning of full length genes is required before making any definitive conclusions.

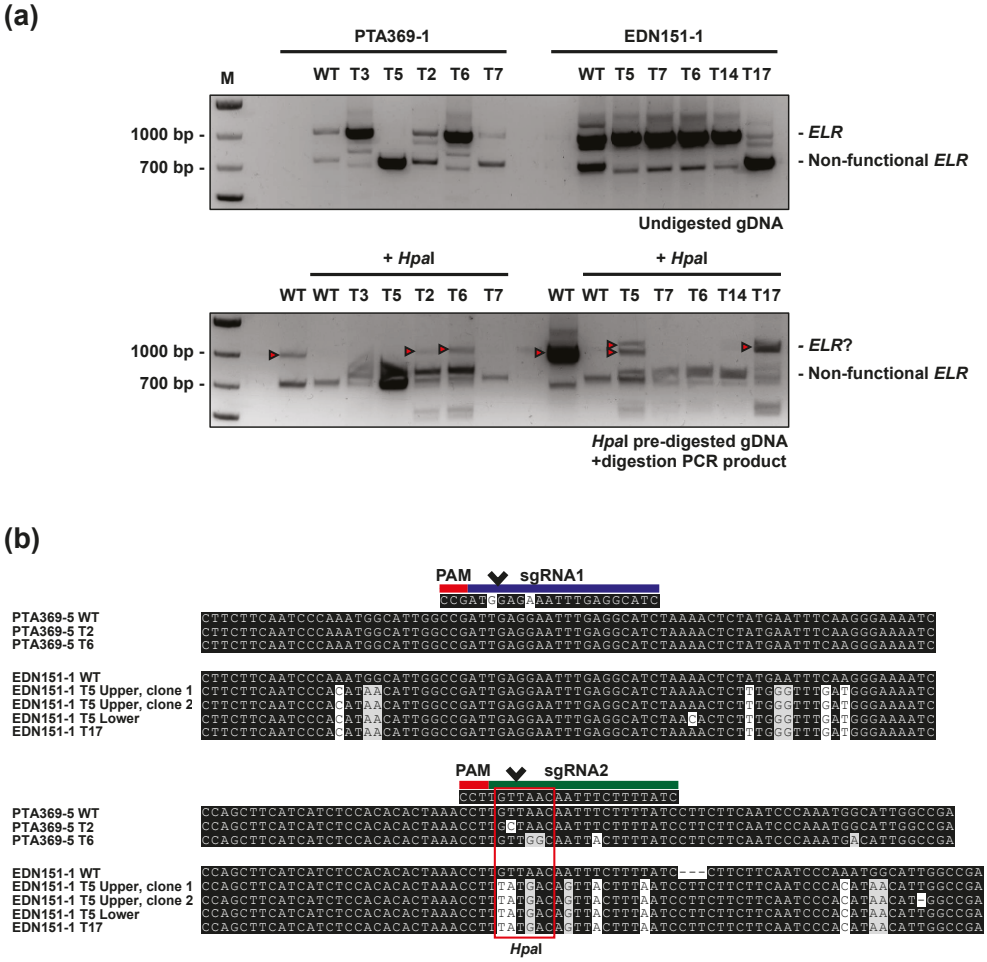


FIGURE 6 | Identification of CRISPR/Cas9-induced *ELR* mutations in stable potato transformants of *S. papita* PTA369-1 and *S. edinense* EDN151-1. PCR using proofreading polymerase was performed on a selection of transformants of *S. edinense* EDN151-1 and *S. papita* PTA369-1. (a) PCR before and after double digestion with *Hpa*I. Bands indicated with a red arrow were excised from gel and DNA was isolated and sequenced directly. In the case of EDN151-1 T5, two bands were obtained and the upper one was cloned prior to sequencing. (b) Sequencing results obtained from the indicated bands. Highlighted in red box is the *Hpa*I restriction site. Alignments were performed using Clustal Omega and BOXSHADE was used for visualization.

Discussion

ELR is the PRR responsible for elicitin recognition in *Solanum* (**Chapter 4**). ELR was initially identified in *S. microdontum* and its overexpression in *S. tuberosum* was shown to enhance resistance to *P. infestans* (Du et al., 2015). However the effect of ELR in late blight resistance was never explored in its native genetic background. In this study we attempted to knock-out *ELR* in INF1-responding wild *Solanum* sp. using CRISPR/Cas9-mediated genome editing. We first designed an *ELR*-targeting CRISPR/Cas9 construct which targets the gene at two positions. This construct was found to have a small number of predicted off targets based on Cas-OFFinder (Bae et al., 2014), all having at least one nucleotide difference. However, based on the study from Cho et al. (2013), the majority of potential off-targets identified could not be targeted by the CRISPR/Cas9 construct since the nucleotide differences are in non-tolerated positions, within the region of the gRNA adjacent to the PAM motif (Carroll, 2013; Cho et al., 2013; Fu et al., 2013; Hsu et al., 2013; Bae et al., 2014; Bortesi and Fischer, 2015). Therefore, we conclude that the construct used is highly specific to *ELR* and its close homologs with only one putative off-target, namely PGSC0003DMG400002910 that encodes for a Cf-like protein (60.4% amino acid identity to ELR).

To enable efficiency testing of the CRISPR/Cas9 construct, we developed an *in planta* screening assay in *N. benthamiana* using *Agrobacterium* co-infiltration. We found that indeed, the construct was able to induce double strand breaks (DSBs) in *ELR*, at both targeted sites, at the expected positions, creating a ~273 bp deletion in the gene. Since the construct was efficient in generating the expected mutation, we attempted to transform wild *Solanum* sp. which were known to have a functional *ELR* homolog (Du, 2014). We found that it was not possible to obtain transformants in the *S. microdontum* genotypes tested, likely due to the inability of the calli to regenerate shoots. Similar findings with regard to transformation attempts in *S. microdontum* have been described earlier (Rakosy-Tican L, 2004), which suggests that for *A. tumefaciens*-mediated transformation-recalcitrant species like *S. microdontum,* other potato transformation protocols or methods need to be explored, e.g. using protoplasts (Andersson et al., 2017). Fortunately, *ELR* homologs have been cloned from seven additional *Solanum* sp. (Du, 2014) and we were able to obtain CRISPR/Cas9 transformants for some of these species (for *S. papita*, *S. phureja*, *S. edinense*, *S. chacoense*). Among those, *S. edinense* gave the most transformants in numbers surpassing the routinely transformed *S. tuberosum* cv. Désirée, indicating that this wild species is highly amenable to *Agrobacterium* transformation.

Obtained transformants were transferred to the greenhouse where they were evaluated for their response to INF1. While most transformants did grow similar to their respective wild type background, some showed impaired growth or phenotype. Especially for *S. phureja*, from the 12 regenerants obtained, five showed severe stunting and four were lost before getting to the greenhouse for screening. This species also seems to suffer from PVX-induced senescence.

To identify CRISPR/Cas9 induced mutations in the transformants, we utilized mutation enrichment with a restriction enzyme cutting at the expected DNA modifying site of sgRNA2 (Zischewski et al., 2017). We found two *S. papita* and two *S. edinense* transformants which carry mutations in *ELR* or its close homologs. Based on the INF1 cell death screens, the majority of those mutations are likely either heterozygous or in a non-functional allele. For example, EDN151-1 T5 was shown to contain a mutation and the phenotype was a 35% decreased INF1 cell death. In another plant, PTA369-1 T5, a deletion mutation could be detected in *ELR* and response to INF1 was found to be not higher than the negative control. However, the previously identified *ELR* orthologue in this species had two mismatches present in the 3′ end of the sgRNA1 target site (Du, 2014). CRISPR-Cas9 has been reported to tolerate mismatches in the 5′ site but not the 3′ site of sgRNAs (Fu et al. 2013, Hsu et al. 2013, Carroll, 2013). Interestingly another transformant of *S. papita* (PTA369-5 T2), showed around 31% increased INF1 cell death response and was found to carry a mutation which leads to an amino acid substitution in the functional ELR protein. This could hint to the potential importance of this amino acid for response to INF1, but it requires further experimental confirmation.

So far, it was very hard to perform reverse genetics in potato due to its autotetraploid nature and the difficulty of obtaining mutants with similar genetic background to a reference (Uitdewilligen et al., 2013). In this chapter we attempted to create homozygous *ELR* mutants in wild *Solanum* sp. using CRISPR/Cas9-mediated genome editing. The functionality of reagents was assessed prior to transforming the wild potato genotypes in a transient co-expression setup. Obtained transformants showed differential responses to INF1 effector and we were able to pinpoint putative CRISPR/Cas9-induced alterations in *ELR* for a few of them. Our results show the potential of genome editing using CRISPR in potato and highlight that a high number of transformants needs to be rigorously screened for finding desired mutations, which is a challenge for multicopy genes or polyploid plants. The plants PTA369-5 T5 (likely to carry *ELR* mutation) and EDN151-1 T5 (heterozygous *ELR* mutations) that we obtained could be used for testing their response to *P. infestans* infection and complement the existing results on the ELR involvement in resistance using overexpression (Du et al., 2015). However, to increase chances of finding full *ELR* knock-outs in these species, a new CRISPR/Cas9 has to be designed, with more sgRNAs, to increase mutation efficiency.

Author contributions

ED, VGAAV and DO designed the research, ED, CAG, DW and MB performed experiments, JD provided sequence information. ED, RV and VGAAV supervised the research and wrote the manuscript.

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Chapter 6

General discussion

The potato crop suffers from many pests and pathogens, among which the late blight pathogen *Phytophthora infestans* is the most devastating (Haverkort et al., 2009). Breeding for resistance is of major importance for the global food security, yet, to achieve resistance, basic studies on the molecular interaction between plant and pathogen are essential. Plants are able to sense invading microbes and subsequently trigger immune responses by surface-localized pattern recognition receptors (PRRs) or nucleotide-binding leucine-rich repeat (NLR) cytoplasmic receptors. NLR-triggered immunity against *P. infestans* has been well studied in potato. Several NLRs have been cloned and bred into potato cultivars with proven effectivity. However, the lifespan of any introduced NLR has been relatively short due to the rapidly evolving nature of the pathogen (Haas et al., 2009; Haverkort et al., 2016). Contrary to the wealth of information on NLR-triggered resistance, cell surface-triggered immunity mediated by PRRs against oomycetes, and particularly *Phytophthora* has been understudied.

PRRs have been reported as the first line of defense that is mediated by apoplastic self- or microbe-derived molecules, which trigger a defense response that in many cases results in quantitative resistance against pathogens (Jones and Dangl, 2006). *Phytophthora* (and *Pythium*) pathogens are known to secrete elicitins, conserved apoplastic effectors that function in sterol nutrient uptake from the host (Stong et al., 2013; Derevnina et al., 2016). Since *P. infestans* cannot synthesize sterols, elicitins are indispensable proteins for its survival. Therefore, targeting plant receptors of elicitins was proposed as a promising approach to obtain a more durable resistance against this devastating pathogen (Du et al., 2015). Despite the fact that *Nicotiana benthamiana* and *N. tabacum* have been the model plants for elicitin perception since decades (Ricci et al., 1989; Kamoun et al., 1993; Panabieres et al., 1995), the first putative PRR responding to elicitins -ELR- was cloned from a wild potato species, *Solanum microdontum*.

At the start of this project it was known that ELR is able to respond to various elicitins by triggering cell death. This was shown by both transient co-expression in the genetic background of a non-responding *Solanum* genotype and with stable overexpression in *Solanum tuberosum* cv. Désireé (Du et al., 2015). However, it was not known whether ELR actually binds elicitins or is just part of a recognition complex. In addition, there was a need for producing recombinant apoplastic effectors for biochemical studies or for improving effectoromics screens in wild *Solanum* spp. to complement *Agrobacterium*-based screening assays. Therefore, our research questions were: is ELR a true PRR that binds elicitins? What are the first molecular players downstream of ELR? What would be the outcome of knocking out ELR in its native genetic background, and would these plants be less responsive to elicitins? And finally, can we optimize a heterologous system for efficient recombinant apoplastic effector production?

All these questions come together in the research objectives of my work, i.e. 1) biochemical characterization of elicitin-perception by ELR, 2) investigation of modern genome editing techniques for use in reverse genetic studies in wild potato, and 3) optimization of producing recombinant effectors for biochemical studies and effector screens.

With the work presented here I have unraveled the mechanism underlying elicitin perception by the ELR receptor in potato. I have shown that ELR is a true PRR that binds elicitins and that it associates with co-receptors in order to trigger cell death. With these studies, I am revealing the first players in the elicitin perception pathway. In addition, I explored the possibilities of using CRISPR/Cas9-mediated genome editing for mutating ELR in wild *Solanum*. I have also optimized the heterologous apoplastic effector protein production in yeast for, among others, functional screens by infiltration in wild potatoes. This work is complementing the routine *Agrobacterium*-based screens and enables screening of *Agrobacterium* or PVX-recalcitrant species. Overall, my work has significantly expanded our knowledge on how PRRs function against oomycetes in a crop plant; potato. In addition, it highlights similarities to the perception of fungi and bacteria, while revealing aspects of this complex immune system.

Recognition of the elicitin domain in *Solanum* is mediated by elicitin binding to ELR

ELR, the first PRR that specifically responds to MAMPs of *Phytophthora* has been cloned from a wild potato species, *Solanum microdontum* (Du et al., 2015). ELR is a RLP lacking a cytoplasmic kinase domain that recognizes several elicitins ranging from 45-65% amino acid similarity (Du et al., 2015). This spectrum includes *Phytophthora* elicitins belonging to class ELI-1 (e.g. INF1, RAMA1, ParA1, CRY2, β-CRY), ELI-2 (e.g. INF2A) and ELI-4 elicitins (e.g. INF5, INF6) (Jiang et al., 2006; Du et al., 2015)(**Chapter 4**). In this work we have shown that ELR physically binds INF1. This was evident from various experiments, including *in planta* and *in vitro* biochemical assays as well as binding studies with an INF1 cysteine mutant. Moreover, we have shown that ELR binds ParA1 and β-CRY elicitins (**Chapter 4**). The ELR-elicitin interaction is likely happening in the plant apoplast, since in all cases, the interaction was found to occur with the processed forms of elicitins (**Chapter 4**). We consider these findings as a milestone, as binding of MAMPs/effectors to their PRRs has only been reported for very few PRR-MAMP pairs, even after many years since the identification of some of these (see Fig. 2, **Chapter 1**).

We noted that elicitins that are recognized by ELR do not seem to share a conserved stretch of amino acids, as it is found in bacterial flagellin, EF-Tu or NLPs (Felix et al., 1999; Kunze et al., 2004; Bohm et al., 2014). Rather, the structure of elicitins is conserved and is likely recognized by ELR (Derevnina et al., 2016). Some elicitins form homo-oligomers, leaving their ω-loop region exposed, yet they are able to bind ELR. Therefore, we can hypothesize that this region might be important for their recognition by the receptor (**Chapter 4**).

Searching of ELR homologs in other species of *Solanaceae* using BLASTp revealed some potential homologs in potato, tomato and pepper (e.g. ≈80% similar amino acid sequences found in *S. tuberosum*, *S. pennellii* and *S. lycopersicum*, and ≈70% in *Capsicum annuum*), in *N. tabacum* (≈70% similar amino acid sequence found), while no close homologs exist in *N. benthamiana*. It is known that the sequenced potato DM1-3 516 R44 as well as most *S. tuberosum* cultivars tested so far are not able to respond to elicitins (Du et al., 2015). The specificity of the elicitin response seemed to be linked to the absence of some LRRs (LRR3-6) in the sequence of those ELR homologs (Du, 2014). For the rest of the *Solanaceae*, in particular *Nicotiana*, future studies will reveal whether the response to elicitins is always due to ELR homologs or whether completely different PRRs recognize elicitins as well. However, based on our findings that the co-receptor SOBIR1 is required (**Chapter 3**), we expect that those receptors should be RLPs (Liebrand et al., 2014).

ELR is the first oomycete-specific RLP ever cloned with a known ligand (**Chapter 4**). The only other known PRRs that can recognize oomycetes are RLP23 from *Arabidopsis* and the very recently-identified RXEG1 from *N. benthamiana*. For these PRRs, binding has been demonstrated with their respective MAMPs, i.e. the nlp20 and GH12 (Albert et al., 2015; Boutrot and Zipfel, 2017; Wang et al., 2018). Nlp20 can be found in *P. infestans*, however GH12 can only be found in the related oomycetes *P. sojae* and *P. parasitica* and *P. capsici*. Just like ELR, these RLPs were shown to increase resistance to pathogens producing those MAMPs.

Other studies on elicitin perception by *N. benthamiana*, *N. glutinosa*, and *C. annuum* have revealed proteins with an unclear role. The B-type LecRKs *Nb*LRK1 and *Ng*RLK1 have been reported to interact with INF1 and a homolog of *Phytophthora cactorum* (*Pc*INF1) *in vitro* (Kanzaki et al., 2008; Kim et al., 2010). For *Nb*LRK1, interaction with INF1 occurred through its cytoplasmic kinase domain, which was unexpected, since ELI-1 elicitins are proteins secreted in the apoplast (Kanzaki et al., 2008). *Pc*INF1 was also found to interact with the C2 domain of the calcium-binding protein SRC2-1 both *in planta* and *in vitro* (Liu et al., 2015). SRC2-1 is required for pepper responses to host and non-host pathogens and is also required for PcINF1-triggered cell death (Kim et al., 2008). Except for SRC2-1,

evidence of *in planta* interaction between *Nb*LRK1/*Ng*RLK1 and INF1/*Pc*INF1 are missing and currently it is not apparent how these studies link to the perception of elicitins by ELR. It is important to mention that our results on ELR are in line with other studies on elicitin/PRR signaling and fit our general model of surface receptor function (Boutrot and Zipfel, 2017).

SOBIR1 and SERK3 are both required for ELR function

As a RLP, ELR is lacking a cytoplasmic kinase domain and is thus incapable of downstream signaling. RLPs are known to associate with co-regulatory LRR-RLKs that are signaling competent (Chinchilla et al., 2009; Gust and Felix, 2014; Liebrand et al., 2014; Bi et al., 2016). SERK3 has previously been linked to cell death response caused by INF1 recognition in *N. benthamiana* (Chaparro-Garcia et al., 2011). Based on our studies, SOBIR1 was found to be required for INF1-triggered cell death and for basal resistance to *P. infestans*, similar to SERK3 and to what has been reported for other pathogens (Chaparro-Garcia et al., 2011; Liebrand et al., 2014; Peng et al., 2015; Domazakis et al., 2018).

We have shown that ELR constitutively associates with SOBIR1 in a ligand-independent manner, and associates with SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3) upon induction with INF1 (Gust and Felix, 2014; Liebrand et al., 2014; Bi et al., 2016; Postma et al., 2016; Domazakis et al., 2018). In other words, ELR is found in a preformed complex with SOBIR1 which recruits SERK3 upon ligand perception (Fig. 1).

From our experiments it was also evident that both SERK3 and SOBIR1 co-receptors are timely required for a robust response to elicitins. Artificial elicitins INF1-3HA, which likely forms homohexamers, and β-CRY chemically crosslinked dimer β-CRY^{DIM} (which is also found *in planta*) are binding to ELR (indirect proof for β-CRY; only the monomer was shown to bind) but were found to not allow co-receptors to form a complex with the RLP, probably due to steric hindrance (**Chapter 4**) (Fig. 1). With INF1-3HA, both SOBIR1 and SERK3 are inhibited for associating with ELR, while β-CRY^{DIM} affects association only with SOBIR1. In either case, elicitin-triggered cell death did not occur, but with β-CRY^{DIM} *PR* gene expression was found, likely due to SERK3 still being in the complex (**Chapter 4**). To our knowledge, this is the first report of co-receptor inhibition by a ligand and we propose that such a setup could find application in RLP signaling studies.

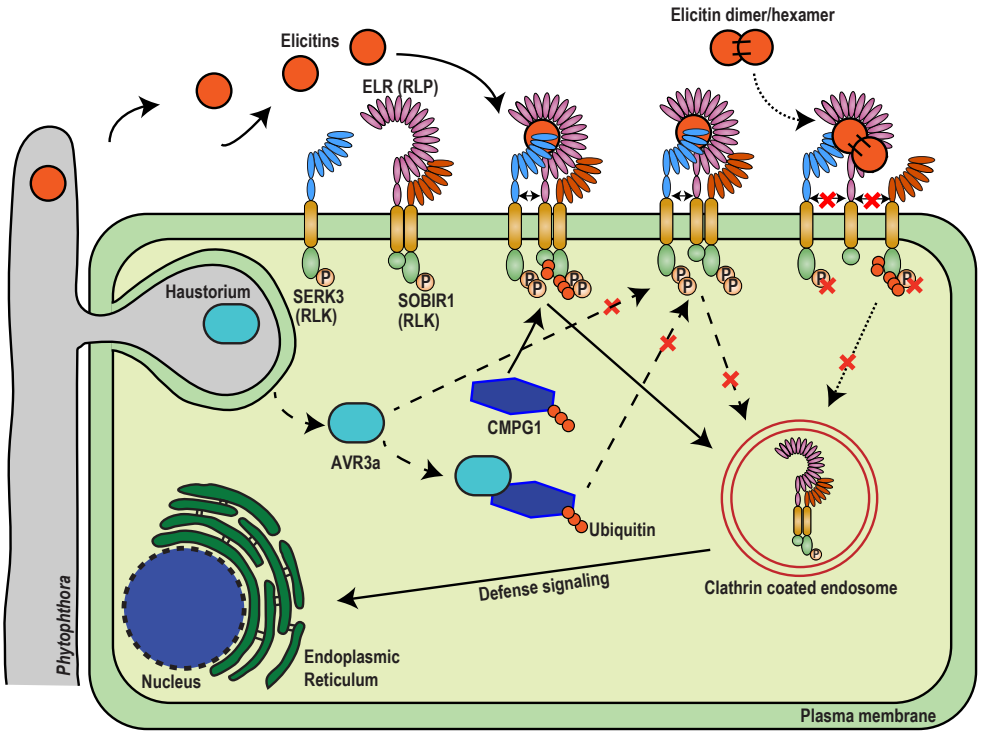

FIGURE 1 | A schematic overview of the first players in elicitin perception by ELR in *Solanum* sp. and different aspects affecting downstream responses. ELR is localized at the plasma membrane, where it is found in a preformed complex with the co-regulatory RLK SOBIR1 (Domazakis et al., 2018). Upon elicitin perception by ELR, the co-regulatory RLK SERK3 is recruited to the complex (dual arrow). After a series of phosphorylation (P) and ubiquitination reactions, the complex seems to undergo endocytosis and triggers downstream signaling (normal arrows). We propose that only ELR and SOBIR1 are endocytosed and SERK3 is released from the complex shortly after elicitin binding by ELR (Chapter 4). AVR3a is secreted from *Phytophthora infestans* and suppresses responses to INF1 (dashed arrows). This is done by stabilizing CMPG1 and inhibiting it from ubiquitinating the ELR-SOBIR1 complex, or by AVR3a acting as a general ubiquitination inhibitor (Chapter 4). In either case, the receptor complex fails to be internalized and as a consequence, the downstream responses are blocked. In this thesis, we have also discovered another way of blocking elicitin signaling (Chapter 4). Two structurally altered elicitins, i.e. an affinity-tagged INF1 or an *in planta* dimerized β-CRY are both binding to ELR, however these elicitins fail to trigger a robust cell death response. We have shown that this is because of inhibition of the ELR-SOBIR1-SERK3 complex formation (Chapter 4).

But what is the function of SOBIR1 in the complex? We found that the kinase of SOBIR1 is required for elicitin-triggered cell death and is continuously phosphorylated, similar to SERK3 (**Chapter 4**). Preliminary experiments have shown that a short elicitation with purified INF1 protein leads to massive changes in ELR-SOBIR1 complex accumulation (**Chapter 4**). Without INF1 elicitation, SOBIR1 has a stabilizing effect on ELR, but as soon as INF1 is introduced, the amounts of detected ELR and SOBIR1 are severely reduced (**Chapters 3, 4**). This

was not the case for SERK3, indicating that it may not stay in the complex after signal transduction occurs (Fig. 1). We hypothesize that this rapid decrease in ELR/SOBIR1 accumulation may happen due to the formation of insoluble endocytic vesicles rather than actual degradation of the proteins (Fig. 1). In line with this hypothesis, the elicitin ParA1 from *P. parasitica* was shown to induce SOBIR1 endocytosis (Peng et al., 2015). Moreover, the elicitin β-CRY from *P. cryptogea* has been shown to induce clathrin-mediated endocytosis in BY2 tobacco cells, which could be initiated by the tobacco elicitin receptor (Leborgne-Castel et al., 2008). For RLKs such as FLS2, PEPR1/2 and EFR it has been demonstrated that ligand-activated receptor complexes are internalized into clathrin-coated endosomes in a BRI1-ASSOCIATED KINASE 1 (BAK1)/SERK3-dependent manner (Mbengue et al., 2016). In a more related receptor to ELR, the RLP Cf-4, it was found that Avr4 elicitation leads to Cf-4 endocytosis together with SOBIR1, which is dependent on SERK3 (Postma et al., 2016). We propose that the activated ELR-SOBIR1 complex undergoes similar SERK3-dependent endocytosis, however this awaits experimental validation. This could be done with microscopy experiments similar to what has been performed for Cf-4 and the aforementioned RLKs (Postma et al., 2016).

Phytophthora infestans AVR3a could suppress INF1 recognition by being a potent ubiquitination inhibitor

P. infestans is able to suppress INF1-triggered defenses in two ways; by employing cytoplasmic effectors or by downregulating *INF1* expression (Kamoun et al., 1997; Derevnina et al., 2016). Known cytoplasmic effectors with such a function are PexRD8 and PexRD36$_{45\text{-}1}$, PexRD27 and AVR3a, with the latter being the most studied (Armstrong et al., 2005; Bos et al., 2006; Bos et al., 2009; Oh et al., 2009; Bos et al., 2010; Zheng et al., 2014). Besides INF1-triggered cell death, AVR3a has been also shown to suppress Cf-4-, Cf-9- and Pto-mediated cell death, indicating that it targets a shared signaling component of those PRRs (Gilroy et al., 2011). Moreover, AVR3a is able to inhibit the endocytosis of FLS2, a PRR not related with elicitin perception, but does not affect BRASSINOSTEROID INSENSITIVE 1 (BRI1) endocytosis (Chaparro-Garcia et al., 2015).

It has been reported that AVR3a is able to suppress MAMP-triggered responses in at least two ways, i.e. 1) by associating and inhibiting the E3 ubiquitin ligase CYS, MET, PRO, and GLY PROTEIN 1 (CMPG1) and 2) by blocking receptor endocytosis via association with DYNAMIN-RELATED PROTEIN 2 (DRP2), a protein involved in cellular trafficking (Bos et al., 2010; Chaparro-Garcia et al., 2015). CMPG1 and

DRP2 are both linked to the ubiquitination pathway, which plays a major role in immune receptor endocytosis function and is a common target for many effectors (Duplan and Rivas, 2014; Li et al., 2014; Banfield, 2015). During ubiquitination, a various number of ubiquitin moieties are covalently attached to a given substrate. This is done in a three step process involving ubiquitin ligases E1, E2 and E3, with the latter providing the substrate specificity (Vierstra, 2009; Duplan and Rivas, 2014). It is evident that many ubiquitin ligases are phosphorylated by interacting with RLKs indicating that they are involved in processes related to signaling (Furlan et al., 2012).

Preliminary data with two untagged AVR3a variants ($AVR3a^{KI}$ and $AVR3a^{EM}$) have shown that their expression *in planta* has a broad effect on inhibiting ubiquitination of proteins (**Chapter 4**). This was shown by the reduction in proteins carrying linked ubiquitin after total protein extraction in *N. benthamiana* (**Chapter 4**). Interestingly, similar findings have been reported previously, where $AVR3a^{KI}$ was shown to decrease smearing in the observed bands of FLS2, EFR and BAK1 (Fig. A2.3, (Chaparro-Garcia, 2012)). We hypothesize that AVR3a may be a potent general ubiquitination inhibitor and it would be interesting to test what is its effect on SOBIR1, which seems to be heavily modified (Fig. 1, **Chapter 3**). This may explain why AVR3a is able to inhibit the endocytosis of a range of activated immune receptors, even unrelated to elicitin perception and interact with different proteins such as CMPG1 and DRP2. It is conceivable that such an effector feature will be very beneficial for parasitic microbes such as *Phytophthora*.

Using purified effector proteins for effectoromics screens

Effector-assisted breeding was shown to accelerate the identification and cloning of R genes, speed up functional characterization of breeding material, and effectors can also be exploited for predicting the durability of introduced NLR genes (Vleeshouwers et al., 2011a; Vleeshouwers and Oliver, 2014). The successful cloning of ELR, which was possible thanks to accurate phenotyping based on effector responses, indicated that this approach could be extended to cell surface PRRs. However there are some difficulties which are often associated with high throughput effector screens in such diverse material as wild tuber-bearing *Solanum* species. In some cases, PVX agroinfection screens may be hindered by extreme resistance (ER) which is occurring at the single cell level (de Ronde et al., 2014) and leads to the absence of macroscopic cell death symptoms which are required for scoring. In other cases, agroinfiltration is hindered by unspecific cell death responses to *Agrobacterium* itself (Du et al., 2014).

In **Chapter 2**, we describe simplified protocols for effectoromics screens in wild potato for the identification of PRRs. In this chapter, we also describe the design and production of affinity-tagged recombinant *P. infestans* apoplastic effectors in the yeast *Pichia pastoris*. Previously, this system was used for the production of *P. infestans* elicitins (Ponchet et al., 1999; Vleeshouwers et al., 2006) and other fungal effector proteins (Kombrink, 2012; Sánchez-Vallet et al., 2013), however with our design we were focused on introducing affinity tags for proteomic assays (discussed below), accelerating the identification of high protein producing yeast clones and optimizing yield in small culture setups (without a fermenter).

With the protocols described in **Chapter 2**, we were able to produce six His-HA tagged effector proteins (INF1, SCR74-A10, SCR74-G1, SCR74-B3b, EPI1, EPIC2B) at reasonable yields and purity. The functionality of those effectors was confirmed as they gave expected responses when infiltrated in responding genotypes (**Chapters 2, 4**). Moreover, we were able to perform binding assays with His-HA-INF1 and ELR and obtained expected interactions (**Chapter 4**). No unspecific responses were observed when any of those effectors were infiltrated at concentrations up to 10 μM in non-responding wild potato (data not shown), indicating that potential contaminants from *P. pastoris* are unlikely to trigger macroscopic responses. For *Solanum* this is very important as proteins derived from *Escherichia coli* systems often cause unspecific cell death (data not shown).

We propose that His-HA effectors produced with the *P. pastoris* system are functional proteins that are suitable for effector screens and can be used to complement responses obtained by other methods (i.e. PVX agroinfection or agroinfiltration) or to enable screening of *Solanum* germplasm which less amenable to *Agrobacterium* assays. The protein infiltration assays can also be performed in greenhouse compartments without biological containment. Moreover, they can be used for biochemical assays to characterize receptor function, identify new PRRs or other *in planta* interactors (**Chapter 4**).

Proteomics as a tool for PRR identification

To identify plant PRRs, several strategies have been used with the most robust being forward and reverse genetics (Boutrot and Zipfel, 2017). Forward genetics takes advantage of the natural variation or uses mutagenized material in order to identify variation in resistance to pathogens, or in the response to a said MAMP/effector (**Chapter 2**, (Boutrot and Zipfel, 2017)). Using specific MAMPs/effectors for screenings instead of disease assays or crude protein extracts leads to a better understanding of the exact function of the PRRs cloned (**Chapter 2**, (Boutrot and

Zipfel, 2017)). Reverse genetic approaches often include mutant libraries of plants with mutations in genes that are expected to be involved in disease resistance (e.g. RLPs, RLKs). The increasing knowledge on PRR structure and sequence has significantly increased the speed and efficiency of such methods, however, these have so far been restricted to model plants such as *Arabidopsis thaliana*.

However, there are cases that data from forward genetics do align with expected Mendelian segregation or the obtained resistance proteins are not PRRs, but rather participate indirectly in the defense pathway. Biochemical approaches have shown potential in identifying PRRs, though their application is still not very wide because of technical difficulties (Boutrot and Zipfel, 2017). Several PRRs have been cloned with methods such as ligand affinity (*Os*CEBiP, LYM2, PEPR1) or co-receptor binding (*Nb*CSPR) (Kaku et al., 2006; Yamaguchi et al., 2006; Petutschnig et al., 2010; Saur et al., 2016).

In this thesis we provide evidence that ELR can be isolated from diluted total protein extracts suspension by using beads with immobilized INF1 (**Chapter 4**). Similar findings were obtained by infiltrating INF1 into the plant apoplast in *N. benthamiana* leaves expressing ELR (data not shown). These data suggested that ELR could potentially be identified from its native background using a similar setup. Alternatively, since it was known that INF1 response requires SERK3, an approach could be to transform *S. microdontum* potato plants with an affinity-tagged version of SERK3, but since this is difficult with current *Agrobacterium* protocols (**Chapter 5**), different species that carry a functional ELR receptor could be used (Du, 2014). It is anticipated, that overexpression of SERK3 may cause aberrant phenotypes in *Solanum* due to its involvement in several signaling processes, but if this is the case, inducible promoters could be considered (Chinchilla et al., 2009; Liebrand et al., 2014). Alternatively, the experiment could be performed in *N. benthamiana* which also carries an INF1 receptor and is more easy to transiently transform (Saur et al., 2016). Then, after performing a short elicitation by infiltrating INF1 protein, immunoprecipitation of SERK3 should also immunoprecipitate ELR. Such an approach was described in Saur et al. (2016), which led to the identification of *Nb*CSPR. In Fig. 2 we schematically describe a general model for PRR identification using proteomics. It is important to mention that the use of SERK3 as a bait for capturing a PRR requires the confirmation that response to a said MAMP/effector requires the specific co-receptor. RNAi or virus-induced gene silencing (VIGS) could be used for this purpose. Moreover, proteomic databases are required for analyzing the data.

With the increased basic knowledge on immune receptor properties, it is anticipated that proteomics will be increasingly employed in the future in order to understand plant immunity. Provided that these experiments are performed with

the right negative controls, the information derived from such methods can be valuable not only for PRR identification, but also for the identification of proteins such as effector targets and downstream signaling interactors (Fig. 2). Effector targets hold great promise for modification as they are often found to function as susceptibility (*S*-) genes (van Schie and Takken, 2014).

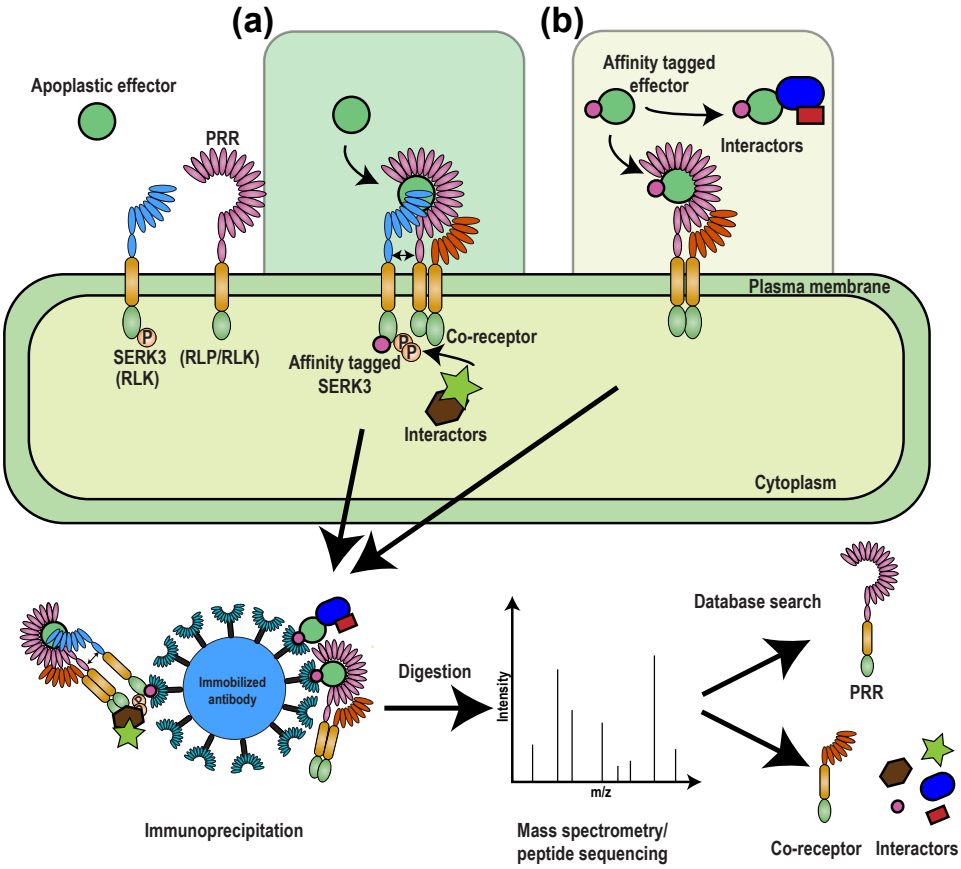

FIGURE 2 | Proposed proteomic approaches for accelerating the identification of PRRs, based on affinity-tagged co-receptors (a) or effectors (b). a) After confirming that a co-receptor (e.g. SERK3) is involved in a response to a said apoplastic effector, transgenic or transiently transformed plants expressing an affinity-tagged co-receptor can be generated. After a short elicitation with purified effector protein, the co-receptor will associate with the matching PRR. b) An alternative way is the use of affinity-tagged recombinant apoplastic effector. The protein is infiltrated into the plant apoplast where it will bind with PRRs or other interacting proteins (e.g. effector targets), or it may be first immobilized in a matrix and plant extract is applied (Chapter 4). In both cases (a and b), several negative controls must be included; e.g. for a); tagged control protein which localizes to plasma membrane, carrying the same tag as SERK3. Samples obtained from both elicited and non-elicited plants. For b); a different unrelated apoplastic effector carrying the same tag can be used. In both a) and b) cases, plant material, is collected for protein extraction and immunoprecipitation. Subsequently, samples are digested and peptides are sequenced using mass spectrometry. The identity of the PRRs and other proteins found in the mix is determined by database searches.

Genome editing for the functional characterization of PRRs in *Solanum*

In recent years, genome editing has accelerated the targeted generation of desired mutations in model and crop plants (Belhaj et al., 2013; Bortesi and Fischer, 2015). The technology known as clustered regular interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) was relatively recently shown to function in plants and gained immediate attention from the research community. Since potato is not a model species, assays to assess gene function were difficult due to the high ploidy and self-incompatibility. Those problems have been largely overcome with the CRISPR/Cas9, and several reports emerged with the application of the method to alter agronomically important genes, with the main limitation being the transformation efficiency (Wang et al., 2015; Andersson et al., 2017). For ELR, we hypothesized that its activity could be more effective in its own genetic background *S. microdontum*, compared to *S. tuberosum* cv. Désireé (Du et al., 2015).

To understand the contribution of ELR in resistance to *P. infestans* in its native background, we explored the use of CRISPR/Cas9 in wild *Solanum* for generating an *ELR* knock-out (**Chapter 5**). Two *ELR*-targeting single guide RNA sequences (sgRNAs) were designed for this purpose. Their efficiency was successfully determined with a transient assay that we developed in *N. benthamiana*. We found that mutation enrichment was necessary for identifying mutations (Nekrasov et al., 2013). The constructs were then used for transforming *S. microdontum*. However, we were unable to obtain transformants using our routine *Agrobacterium*-mediated transformation protocols, similar to what has been reported for this wild species (Rakosy-Tican L, 2004). To overcome this, we initiated transformations in various other *Solanum* sp. (*S. edinense*, *S. papita*, *S. phureja*, *S. microdontum* subsp. *gigantophyllum* and *S. chacoense*), which are known to carry functional *ELR* homologs (**Chapter 5**). Positive transformants were found in *S. edinense*, *S. papita*, *S. phureja* and *S. chacoense,* which we then tested for altered responses to INF1. One transformant of *S. edinense* and one of *S. phureja* showed a decreased cell death frequency upon treatment with INF1, and those plants were studied further. Following a similar mutation enrichment strategy, we found evidence that some *S. edinense* and *S. papita* may contain heterozygous mutations. No full *ELR*-knock-outs were obtained. The reasons for this could be that these species are polyploids (4n – 5n) that are more challenging for this approach, and we have used only one sgRNA sequence, which limits the targeting efficiency (**Chapter 5**). However increasing the sgRNA targets is known to increase mutation efficiency in polyploids and is something to be investigated (Wang et al., 2014). Interestingly,

most mutations we have identified were not deletions. We hypothesize that this could be due to the high repeat content in *ELR* itself as well as the genomic locus in general, which is full of *ELR*-like sequences. We propose that such sequences have potentially been used by the plant DNA repair machinery in order to repair the CRISPR-induced double strand breaks.

From our experiments it was evident that performing CRISPR/Cas9-mediated mutagenesis in wild potato is possible, provided that the genotype used is amenable to transformation. Moreover, for polyploid genotypes, it is preferable to use CRISPR/Cas9 designs with more sgRNAs, which is easy to do based on current cloning methods and available plasmids (Weber et al., 2011). We anticipate that CRISPR/Cas9 will be increasingly used for characterizing functionally characterizing genes such as PRRs. However developments in homologous directed repair could enable us to actually modify those receptors at will and e.g. enhance their recognition spectrum, though this is still not possible for plants (Belhaj et al., 2013).

Final remarks

Crop plants are continuously threatened by diseases that limit their yields. To feed the ever-increasing world population, such crop losses should be minimized. One way to do this is breeding for disease resistant cultivars. My work has contributed to understanding how cell surface-triggered immunity works in potato against the devastating pathogen *P. infestans*. I have shown that ELR is a true PRR and revealed the first interactors downstream of elicitin binding. I have also provided evidence that PRRs can potentially be identified using proteomics. Moreover, I explored the applicability of genome editing in wild *Solanum* for reverse genetic studies with the ultimate goal to enable characterization of PRR genes in their native background. My data show that immunity that is triggered by PRRs has potential for disease resistance against potato late blight (**Chapter 3**), however it is often suppressed by cytoplasmic effectors. In my opinion, no single strategy for obtaining durable disease resistance exists. However, by carefully selecting and combining several layers of defense, including PRRs, NLRs and *S*-genes in bred cultivars, and combining with pathogen monitoring can maximize our chances to outcompete rapidly evolving pathogens like *P. infestans*.

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Summary

Samenvatting

Acknowledgements

About the author

List of publications

Education statement

Summary

Potato (*Solanum tuberosum*) is the most important non-cereal crop for human consumption and its starch and fibers are also used to produce several industrial products. Potato suffers from many pests and diseases. The most threatening and re-emerging disease of potato worldwide is late blight, which is caused by the notorious oomycete *Phytophthora infestans* (**Chapter 1**). This pathogen is a hemi-biotrophic organism that secretes a huge arsenal of apoplastic and host-translocated cytoplasmic effectors in order to colonize the host. Interestingly, wild potato plants have evolved receptors that recognize some of those effectors and trigger defense responses. These wild plants are a major source of resistance genes that can be transferred to the cultivated potato. Resistant cultivars obtained by breeding are highly desirable because the costs for chemical control of the disease are very high and there is also a need to reduce their use in order to preserve the environment. Moreover, the pathogen has developed resistance to some of those chemicals.

Receptors that recognize cytoplasmic effectors often belong to the nucleotide-binding leucine-rich repeat (NLR) family of resistance genes, (*R* genes), and they have been used for several years in resistance breeding. Despite the effectivity of NLRs in providing resistance, so far, most single NLR genes introduced into cultivated potato have been defeated by *P. infestans* rather quickly. To effectively control the pathogen in the long term, resistance gene stacking approaches in combination with new layers of defense have to be considered.

Like NLRs, cell surface-residing receptors, or pattern recognition receptors (PRRs) trigger defense responses and contribute to basal or non-host resistance against pathogens. They do so by recognizing apoplastic effectors or microbe-associated molecular patterns (MAMPs). Usually PRR-triggered resistance is quantitative and not as robust as NLR-based resistance, however it is believed to be more durable. In potato, PRR-based immunity that is triggered by the recognition of MAMPs/effectors has remained unexplored. The first receptor against oomycetes was recently identified from a wild potato species, *S. microdontum*. The receptor, named ELICITIN RESPONSE (ELR), was found to recognize elicitins, a family of conserved apoplastic effectors with MAMP features, found specifically in *Phytophthora* and *Pythium* (**Chapter 1**). The research described in this thesis focused on studying PRR-triggered immunity in potato against the devastating pathogen *P. infestans*, by using ELR as a model (**Chapter 1**).

Effector-assisted breeding has proven to be a great tool for identifying resistances against pathogens and was pioneered from research on potato late blight. In a process also known as effector genomics (effectoromics), candidate effectors are predicted from the genome of pathogens such as *P. infestans*. Candidate effectors

are cloned in plant expression vectors and are screened in a wild resistant germplasm for occurrence of specific responses. In **Chapter 2** we show how effectoromics can be used to identify PRRs in *Solanum* spp. Simplified protocols are described for performing the effector screens, selecting plants for crosses and genetically mapping the responses. For performing effector screens, besides the routinely used potato virus X (PVX) agroinfection and agroinfiltration, we describe the use of recombinant apoplastic effector proteins. This strategy can complement the results obtained by the other routinely applied *Agrobacterium*-based methods or enable screening of *Agrobacterium*- or PVX-recalcitrant plants. We provide protocols for heterologous apoplastic effector expression in the yeast *Pichia pastoris*. This includes recombinant effector design, cloning, high throughput *P. pastoris* clone selection and small scale protein production. We also provide an example with the production of six different *P. infestans* effectors using this system.

ELR is a receptor-like protein (RLP) and as such, it lacks a cytoplasmic signaling domain that is required for triggering defense responses. It was known that SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3) is a receptor-like kinase (RLK) required for cell death triggered by the elicitin INF1 and that it also biochemically associates with ELR. In **Chapter 3** we investigated the association of ELR with another interactor, which has been proposed to be specific for RLPs; the RLK SUPPRESSOR OF BIR1-1 (SOBIR1). Virus-induced gene silencing (VIGS) assays showed that *SOBIR1* is required for cell death response triggered by INF1 and for basal resistance to *P. infestans* in *Nicotiana benthamiana*. Genetic complementation assays demonstrated that the kinase of SOBIR1 is required for INF1-triggered cell death. Protein co-immunoprecipitation studies showed that ELR is associating with *S. microdontum* SOBIR1 and its close homolog SOBIR1-like. From our findings it seems that ELR is found in a constitutive complex with SOBIR1, which recruits SERK3 upon INF1 elicitation.

True PRRs are known to physically interact with their ligands, however, this was not explored yet since the identification of ELR. In **Chapter 4** we studied whether ELR is able to interact with various elicitins by using *in planta* and *in vitro* co-immunoprecipitation assays. We showed that ELR is able to physically bind INF1 in both setups, indicating that the interaction is very specific. Moreover, we found that ELR binds with the elicitins ParA1 (*P. parasitica*) and β-CRY (*P. cryptogea*). We also found that ELR is able to trigger cell death with additional elicitins, but likely binds them with lower affinity that remained under the detection limit. Interestingly, we observed that one C-terminally tagged version of INF1 was binding to ELR but failed to trigger cell death, in contrast to N-terminally tagged INF1. Similarly, a β-CRY dimer failed to trigger cell death when infiltrated in leaves expressing ELR. We hypothesized that the cause of these phenotypes could be due to altered interaction

of ELR with the RLKs SOBIR1 or SERK3. Indeed, with the C-terminally tagged INF1, we found that both SOBIR1 and SERK3 were not in complex with ELR, while the β-CRY dimer was not allowing SOBIR1 to associate. To our knowledge, this is the first time that such a co-receptor inhibition is reported. Our data, therefore, highlight the necessity of performing functional control experiments for tagged proteins and further strengthen our earlier finding that both SOBIR1 and SERK3 are required for INF1-triggered cell death by ELR. These discoveries could prove useful for enabling detailed studies on RLP signaling.

When transformed into cultivated potato, ELR was known to enhance resistance against *P. infestans*, however, it remained unknown what the resistance contribution of this PRR in its native genetic background (i.e. *S. microdontum*) is. We hypothesized that recent developments of genome editing technologies can be used to perform such studies in non-model plants like (wild) potato. In **Chapter 5**, we used clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9)-mediated genome editing to target *ELR* in wild *Solanum*. We generated a CRISPR/Cas9 construct that was found to be effective in inducing targeted mutations in *ELR* when tested in a transient setup *in planta*. Since it was impossible to transform the wild *S. microdontum* with the construct, we attempted the transformation of five different species that were known to carry *ELR* homologs. We obtained transformants from *S. edinense*, *S. papita*, *S. phureja* and *S. chacoense*, which were then screened for altered responses to INF1. Promising transformants with altered responses to INF1 were obtained in the pentaploid *S. edinense* and tetraploid *S. papita*. These transformants were subsequently searched for mutations using a mutation enrichment approach, followed by PCR and sequencing. No full *ELR* knock-outs were obtained, however, partly mutated and partly wildtype alleles could be found in these transformants. Altogether, these findings show that CRISPR/Cas9 approaches are effective for functional characterization of genes in wild *Solanum* spp. However, they are limited by the transformation efficiency and ploidy level of a said genotype.

Finally, in **Chapter 6**, the findings of this thesis are discussed and placed in a broader perspective. A schematic overview of the perception of elicitins by ELR in wild potato is provided including all of our findings on elicitin binding, interaction with co-receptors SERK3 and SOBIR1, as well as some preliminary findings on AVR3a, an effector that suppresses INF1-triggered cell death as well as receptor endocytosis. In addition we propose two proteomic approaches for PRR identification, taking advantage of the obtained knowledge on effector/PRR or PRR/co-receptor interactions. Overall my research has contributed to the characterization of the first line of induced defense against *Phytophthora* in potato and could be instrumental for achieving durable resistance against late blight.

Samenvatting

Aardappel (*Solanum tuberosum*) is een belangrijk gewas voor de wereld-voedselvoorziening, en voor de zetmeel- en vezelindustrie. De aardappelplant is echter vatbaar voor een groot aantal ziekten. De belangrijkste, de zogenaamde 'aardappelziekte' wordt veroorzaakt door de oomyceet *Phytophthora infestans* (**Chapter 1**). Deze hemi-biotrofe ziekteverwekker scheidt een arsenaal aan apoplastische (buiten de cel) en cytoplasmatische (in de cel) effectoren uit, om de plant te kunnen koloniseren. In wilde aardappelplanten zijn tijdens de evolutie receptoren ontstaan die sommige effectoren kunnen herkennen, en dit kan vervolgens leiden tot een afweerreactie van de plant. De wilde aardappelsoorten vormen een belangrijke bron van resistentiegenen, die gebruikt kunnen worden voor de veredeling van rassen. Rassen die resistent zijn tegen *Phytophthora* zijn zeer gewild aangezien de kosten voor chemische bestrijding hoog zijn, en bovendien een bedreiging voor het milieu. Helaas heeft *Phytophthora* resistentie ontwikkeld tegen een aantal bestrijdingsmiddelen.

Receptoren die de effectoren ín de cel herkennen behoren over het algemeen tot de familie van de nucleotide-binding leucine-rich repeat (NLR) resistentiegenen (*R* genen). Deze *R* genen worden al jarenlang gebruikt in de resistentieveredeling. Ondanks dat ze in eerste instantie een goede resistentie geven, worden de individueel ingebrachte NLR tot dusver snel doorbroken door *P. infestans*. Voor een effectievere ziektebeheersing op de langere termijn, moeten we overwegen of we NLR kunnen combineren met nieuwe vormen van afweer.

Receptoren aan het celoppervlak, oftewel de 'pattern recognition receptors' (PRRs) kunnen ook afweerreacties aanschakelen, net als NLR. Deze PRR dragen bij aan de basale resistentie, en de niet-waardresistentie tegen ziekteverwekkers. Dit doen ze door middel van de herkenning in de plant van apoplastische effectoren of 'microbe-associated molecular patterns' (MAMPs). De PRR-gebaseerde resistentie is over het algemeen kwantitatief van aard en minder robuust dan NLR-gebaseerde resistentie, maar wordt wel gezien als potentieel meer duurzaam. PRR-gebaseerde resistentie is in aardappel tot dusver nog niet bestudeerd. Een aantal jaren geleden is in een wilde aardappelsoort, *Solanum microdontum,* de eerste PRR receptor tegen oomyceten geïdentificeerd. Deze receptor, genaamd ELICITIN RESPONSE (ELR), herkent elicitinen, een familie van geconserveerde apoplastische effectoren / MAMP van *Phytophthora* en *Pythium* soorten (**Chapter 1**). Het onderzoek dat is beschreven in dit proefschrift is gericht op het bestuderen van PRR-gebaseerde resistentie tegen *Phytophthora infestans* in aardappel, door gebruik te maken van ELR als modelsysteem (**Chapter 1**).

Effector-gestuurde veredeling is een moderne, beproefde manier om nieuwe ziekteresistenties te identificeren. Pionierend onderzoek naar *Phytophthora* resistentie in aardappel heeft geleid tot de ontwikkeling van 'Effectoromics'. Uit de genoom sequentie van *Phytophthora* worden kandidaatgenen van effectoren voorspeld, die in expressievectoren worden gekloneerd en gescreend op het aanschakelen van specifieke afweerreacties in resistente wilde *Solanum* soorten. Optreden van een reactie wijst op de potentiële aanwezigheid van *R* genen. In **Chapter 2** laten we zien dat effectoromics ook kan worden toegepast om PRRs te identificeren in de wilde *Solanum* soorten. Vereenvoudigde protocollen zijn omschreven voor effectortoetsen, kruisingen, en voor genetische kartering van onderliggende genen. Naast de routinematige experimenten die gebaseerd zijn op het gebruik van *Agrobacterium tumefaciens* (agro-infiltratie), en/of aardappel virus X (PVX) (agroinfectie) beschrijven we het gebruik van recombinante apoplastische effectoren op eiwit niveau. De eiwitinfiltratie screening is een uitbreiding van de beschikbare functionele effectorscreening voor aardappel, en o.a. goed bruikbaar voor planten die niet geschikt zijn voor *Agrobacterium*- en/of PVX-gebaseerde experimenten. We ontwikkelden protocollen voor heterologe expressie van apoplastische effectoren in de gistsoort *Pichia pastoris*. Dit omvat het ontwerp van de recombinante effector, klonering, *P. pastoris* kloon selectie met een geïnserteerde effector en eiwit productie op kleine schaal. Ook laten we een voorbeeld zien van de productie van zes verschillende apoplastische effectoren van *P. infestans,* gebruikmakend van dit systeem.

ELR is een 'receptor-like' proteïne (RLP) en als zodanig mist het een cytoplasmatisch signaleringsdomein dat nodig is om afweerreacties aan te schakelen. Het is bekend dat het 'receptor-like kinase' (RLK) SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3) nodig is om de celdoodreactie tegen INF1 elicitine aan te schakelen, en dat SERK3 associeert met ELR. In **Chapter 3** onderzoeken we de associatie van ELR en een andere component die gedacht wordt specifiek te zijn voor RLPs, namelijk de RLK SUPPRESSOR OF BIR1-1 (SOBIR1). 'Virus-induced gene silencing' (VIGS) experimenten lieten zien dat SOBIR1 nodig is voor de INF1-geinitieerde celdood reactie en voor basale resistentie tegen *P. infestans* in *Nicotiana benthamiana*. Tevens bleek het kinase domein van SOBIR1 nodig voor de celdoodreactie tegen INF1. Eiwit co-immunoprecipitatie (CoIP) studies tonen aan dat ELR is geassocieerd met SOBIR1 (en SOBIR1-like) van *S. microdontum*. Uit onze resultaten blijkt dat ELR constitutief een complex vormt met SOBIR1, waar SERK3 zich bij aansluit na elicitatie met INF1.

Authentieke PRRs kunnen fysiek binden met hun ligand, echter, dit was nog niet onderzocht voor ELR. In **Chapter 4** bestuderen we of ELR kan binden met verschillende elicitinen, door middel van *in planta* en *in vitro* CoIP experimenten.

We laten op beide manieren zien dat de interactie zeer specifiek is en dat ELR fysiek bindt met INF1. We vonden tevens dat ELR ook kan binden met de elicitinen ParA1 (*P. parasitica*) en β-CRY (*P. cryptogea*). Daarnaast bleek dat ELR celdood induceert met een aantal andere elicitinen, maar deze blijkbaar met een latere affiniteit bindt, die onder de detectiegrens van de gebruikte methoden blijft. We zagen dat C-terminaal gelabelde INF1 wel ELR kon binden maar geen celdood kon induceren, terwijl N-terminaal gelabeld INF1 dit wel kon. Ook een β-CRY elicitine dimeer kon geen celdood induceren na infiltratie in bladeren die ELR tot expressie brengen. We stelden dat deze fenotypen veroorzaakt kunnen worden door een veranderde interactie van ELR met SOBIR1 of SERK3. Inderdaad, met C-terminaal gelabeld INF1 vonden we zowel SOBIR1 als SERK3 niet terug in het complex met ELR, en dat het β-CRY dimeer de associatie met SOBIR1 verhinderde. Zover we weten is dit de eerste keer dat zo'n remming door een co-receptor wordt gerapporteerd. Onze resultaten geven daarmee het belang aan van functionele controle experimenten met gelabelde eiwitten, en versterken verder onze eerdere vinding dat zowel SOBIR1 als SERK3 nodig zijn voor de INF1-geinitieerde celdoodreactie met ELR. Deze ontdekkingen kunnen bruikbaar zijn voor meer gedetailleerde studies aan RLP signalering.

Stabiele transformatie van ELR naar aardappelplanten leidt tot een verhoogde resistentie tegen *P. infestans*. Echter, het was nog niet bekend wat de precieze resistentiebijdrage van dit specifieke PRR is in zijn oorspronkelijke achtergrond (*S. microdontum*). We stelden vast dat we de recente ontwikkelingen in 'genome editing' technologieën ook kunnen gebruiken in niet-modelsystemen als wilde aardappel. In **Chapter 5**, gebruiken we 'clustered regularly interspaced short palindromic repeat' (CRISPR)/CRISPR-associated protein 9 (Cas9)-mediated genome editing' om *ELR* uit te schakelen in wilde *Solanum* soorten. We hebben een CRISPR/Cas9 construct gemaakt dat gerichte mutaties kon aanbrengen in ELR, in een proef met transiënte expressie. Aangezien het niet mogelijk bleek om de wilde *S. microdontum* stabiel te transformeren, hebben we geprobeerd om het construct te transformeren naar verschillende wilde *Solanum* soorten waarvan bekend was dat ze *ELR* homologen bevatten. We verkregen transformanten van *S. edinense*, *S. papita*, *S. phureja* en *S. chacoense*. Deze hebben we getest op de INF1 reactie. Veelbelovende transformanten die een veranderde reactie op INF1 lieten zien, werden verkregen van de pentaploïde *S. edinense* en de tetraploïde *S. papita*. Deze transformanten hebben we vervolgens onderzocht op mutaties door middel van een mutatieverrijkingsbenadering, gevolgd door PCR en sequencing. Volledige *ELR* knock-outs met alleen gemuteerde allelen werden niet verkregen, echter, planten met gedeeltelijk wildtype en gedeeltelijk gemuteerde allelen wel. Onze resultaten geven aan dat de CRISPR/Cas9 methode effectief is voor

functionele karakterisering van genen in wilde *Solanum* soorten, maar er zijn grenzen aan de transformatie efficiëntie van de soort en aan het ploïdie niveau van gegeven genotypen.

Uiteindelijk, in **Chapter 6**, worden de resultaten van die proefschrift bediscussieerd en in een breder perspectief geplaatst. Een schematisch overzicht van de herkenning van elicitinen door ELR in wilde aardappel geeft een samenvatting van onze resultaten over de binding van ELR met elictinen, de interactie met de co-receptoren SERK3 en SOBIR1, evenals voorlopige bevindingen met AVR3a, een effector die INF1-geinitieerde celdood en receptorendocytose onderdrukt. Daarnaast stellen we twee nieuwe proteomics benaderingen voor om PRRs te identificeren, waarbij we gebruik maken van de verkregen kennis rond effector/PRR en PRR/co-receptor interacties. Over het geheel genomen heeft mijn onderzoek bijgedragen aan de karakterisering van een vroege kwantitatieve afweerreactie tegen *Phytophthora* in aardappel die van nut kan zijn voor het verkrijgen van een meer duurzame resistentie.

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About the author

Emmanouil (Manos) Domazakis was born on the 10th of January, 1987 in Rethymno, Crete, Greece. He grew up at a (very) small village called Melissourgaki. After finishing his primary and secondary education, in 2004, he moved to Athens for a 5-year BSc program in Plant Sciences at the Agricultural University of Athens. At the mid of his BSc studies, he became fascinated by the field of Phytopathology and graduated with the specialization plant protection and environment. His BSc thesis was about biocontrol of *Fusarium* vascular wilt in melon, while both his BSc internships (Benaki Phytopahological Institute, Laboratory of Phytopathology) were at phytopathology-related subjects including diagnostics and disease trials. In 2010, for his MSc studies, he came to the Netherlands, at Wageningen University & Research. There, he gained more knowledge on molecular plant-microbe interactions. He did his MSc thesis on *Verticillium* vascular wilt and *Cladosporium* leaf mold of tomato at the Laboratory of Phytopathology and his internship on disease quantification at Monsanto Vegetable Seeds. For his PhD, he moved to Plant breeding at Wageningen University & Research where he worked with the major pathogen affecting potato production, *Phytophthora infestans*, under the supervision of Dr Vivianne G. A. A. Vleeshouwers and Prof. Dr Richard G. F. Visser. The outcome of this research is described in this thesis. Soon after finalizing his experimental work, in November 2017, he was employed as molecular phytopathologist at Enza Zaden B.V.

List of publications

Domazakis, E., Wouters, D., Visser, R.G.F., Kamoun, S., Joosten, M.H.A.J., and Vleeshouwers, V.G.A.A. 2018. The ELR-SOBIR1 complex functions as a two-component RLK to mount defense against *Phytophthora infestans* Mol Plant Microbe Interact (Accepted).

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Tzima, A.K., Paplomatas, E.J., Schoina, C., **Domazakis, E.**, Kang, S., and Goodwin, P.H. 2014. Successful Agrobacterium mediated transformation of *Thielaviopsis basicola* by optimizing multiple conditions. Fungal Biology 118:675-682.

Charalambous, A., Tjamos, S.E., **Domazakis, E.**, and Paplomatas, E.J. 2013. Incorporation into the transplant soil plug of the plant protective agent *Paenibacillus alvei* strain K165 confers protection to melon against *Fusarium* wilt. BioControl 58:685-692.

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: Emmanouil Domazakis
Date: 1 June 2018
Group: Plant Breeding
University: Wageningen University & Research

1) Start-up phase	date
▶ **First presentation of your project**	
Title: Apoplastic immunity against Phytophthora infestans in potato	26 Nov 2013
▶ **Writing or rewriting a project proposal**	
Title: Identification and characterization of novel cell surface immune receptors against potato late blight	Jan 2014
▶ **Writing a review or book chapter**	
Effectoromics-based identification of cell surface receptors in potato, IN: Plant Pattern Recognition receptors: Methods and Protocols, Methods in Molecular Biology, Vol.1578, DOI 10.1007/978-1-4939-6859-6_29, Springer (2017)	Jul 2016
▶ **MSc courses**	
Laboratory use of isotopes	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	date
▶ **EPS PhD student days**	
EPS PhD student day, Leiden, NL	29 Nov 2013
EPS PhD student day 'Get2Gether', Soest, NL	29-30 Jan 2015
EPS PhD student day 'Get2Gether', Soest, NL	28-29 Jan 2016
▶ **EPS theme symposia**	
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten Day, Amsterdam, NL	25 Feb 2014
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten Day, Utrecht, NL	20 Feb 2015
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten Day, Leiden, NL	22 Jan 2016
▶ **National meetings (e.g. Lunteren days) and other National Platforms**	
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	22-23 Apr 2013
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	14-15 Apr 2014
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	13-14 Apr 2015
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	11-12 Apr 2016
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	10-11 Apr 2017

► **Seminars (series), workshops and symposia**	
Mini-symposium: How to write a world-class paper	17 Oct 2013
Crop Pathology and Plant-Microbes Interaction symposium	08 May 2015
EPS flying seminar Plant intracellular immunity: evolutionary and molecular underpinnings	21 Jan 2016
EPS flying seminar How do plants read their own shape	16 Mar 2016
Invited seminar 'Rewriting our genes', Jennifer Doudna and Edze Westra	30 Sep 2016
EPS flying seminar dr. Rossana Henriques: Circadian regulation of long non-coding RNAs	16 Nov 2015
Invited seminar 'Reprogramming cells for defense in plant innate immunity', Jane Parker	09 Apr 2014
Invited seminar: 'Dissecting the interactions between Phytophthora sojae and soy bean: making sense of signaling and effectors', Yuanchao Wang	16 Jul 2014
EPS Symposium 'Omics Advances for Academia and Industry - Towards True Molecular Plant Breeding', Wageningen, NL	11 Dec 2014
► **Seminar plus**	
► **International symposia and congresses**	
2014 XVI International Congress on Molecular Plant-Microbe Interactions (MPMI), Rhode Island, Greece	06-10 Jul 2014
2016 XVII International Congress on Molecular Plant-Microbe Interactions (MPMI), Portland, Oregon, USA	17-21 Jul 2016
► **Presentations**	
Talk: COST SUSTAIN 2nd Annual Conference, Poland	14-15 Oct 2014
Poster: MPMI (Greece)	09 Jul 2016
Poster: MPMI (USA)	19-20 Jul 2016
Talk: IACGS (International Academic Conference for Graduate Students), China	16-19 Oct 2016
Talk: Understanding how the ELicitin Response receptor (ELR) functions against Phytophthora infestans, Lunteren, NL	11 Apr 2017
► **IAB interview**	
► **Excursions**	
Excursion to Rijk Zwaan, De Lier, NL	27 Sep 2013
Subtotal Scientific Exposure	*14.9 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Advanced course: 'Bioinformatics: A user's approach'	26-30 Aug 2013
Advanced course: 'The power of RNA-seq'	16-18 Dec 2013
► **Journal club**	
Member of literature dicussion at Plant Breeding	2015-2017
► **Individual research training**	
TALEN & CRISPR training school (COST Action), Halle-Wittenberg, Germnay	24-28 Mar 2014
Subtotal In-Depth Studies	*5.9 credits**

4) Personal development	date
► **Skill training courses**	
Social Dutch for employees	May-Jul 2013
Scientific artwork with Photoshop and Illustrator	01-02 Mar 2016
WGS course 'Career Perspectives'	10, 17, 24 Nov-01, 08 Dec 2016
► **Organisation of PhD students day, course or conference**	
Monday Seminars organizer for Plant Breeding	Jan 2015-Jan 2017
Organization of EPS 'Get2Gether' event	Sep 2015-Jan 2016
Organization Lab trip Plant Breeding	17 Sep 2015
► **Membership of Board, Committee or PhD council**	
Member of EPS PhD council	Feb 2015-Mar 2017
Subtotal Personal Development	*8.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**42.9**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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