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Chapter 1

General introduction

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The importance of potato

Potato originates from the Andes in South America and is part of the tuber-bearing *Solanum* species that belong to the section *Petota*. The cultivated potato (*Solanum tuberosum* L.) is an important food crop and ranks fourth in the world, after maize, wheat and rice. Potato was first introduced from the New World into Europe by the Spanish Conquistadors around 1570. However, the exact origin of European potato is still unclear, since Chile and Peru are both competing for this honour. Subsequently, the potato was introduced to the rest of the world and it is assumed to have reached China in the late 16^{th} century.

The ability of potato plants to grow at fast rates allows poor families to cultivate it on small plots and break the circle of poverty. Hundred millions of people around the world depend on potato to survive. Potato is grown in more than 150 countries, under temperate, subtropical and tropical conditions. China is now the largest potato producer followed by Russian Federation, India and Ukraine (http://faostat.fao.org/). The year 2008 was set as the International Year of the potato by the United Nations, which aimed to raise awareness on the importance of the "humble tuber" as a staple food.

Besides for human consumption, potatoes can also be used for industrial purposes, such as the starch industry. Potato starch can be processed in different products, e.g. textile, paper, glue, coating, sizing, flocculating agents and building materials. More uses can be anticipated for the future such as biopharmaceuticals for encapsulation, controlled release of functional ingredients and biofuel (Bradshaw et al. 2006, Li et al. 2009).

The devastation of late blight

Potato late blight is one of the most devastating diseases in the world. The notorious late blight disease led for example to the Irish famines between 1845 and 1852. Ever since, it has remained the most destructive disease in the world, resulting in annual losses of potatoes that would be sufficient to feed hundreds of millions of people (Fisher et al. 2012). Late blight is caused by *Phytophthora infestans* (Mont.) de Bary, which can infect the entire crop, including the leaf, stem and tuber (Figure 1). Infected tissues develop characteristic black necrotic lesions, appearing 'blighted'. *P. infestans* can also infect more *Solanaceous* species e.g tomato and *Nicotiana benthamiana*.

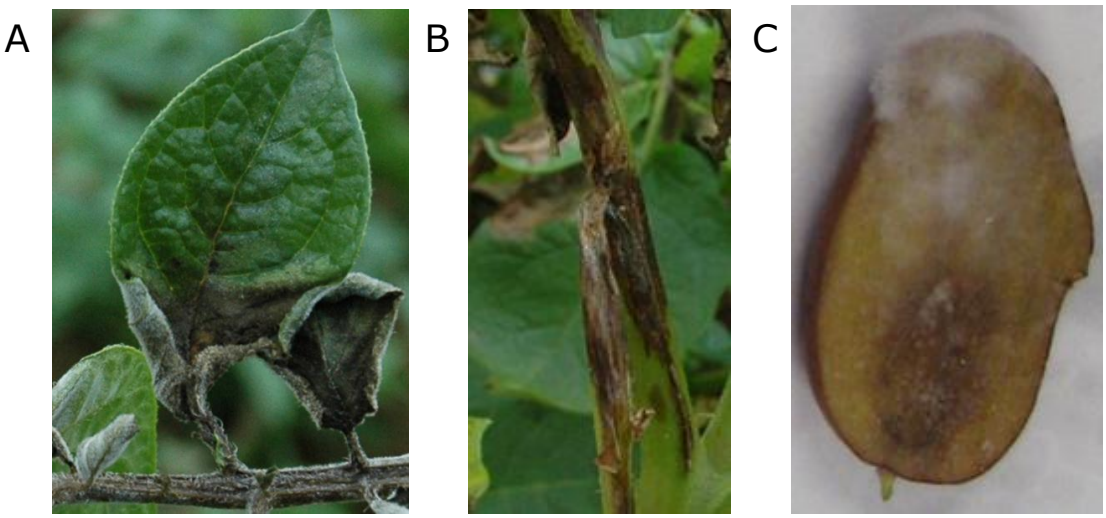

Figure 1. Late blight infection on potato A) leaf, B) stem and C) tuber.

Because of its filamentous growth habit, *P. infestans* was previously incorrectly referred to as a fungus. However, modern biochemical analyses and phylogenetic analyses of ribosomal and mitochondrial genes have shown that *P. infestans* belongs to the oomycetes. Oomycetes share little taxonomic affinity to filamentous fungi in the Kingdom Unikonts, but are more closely related to brown algae in the Kingdom Chromalveolates (Kumar and Rzhetsky 1996, van de Peer and de Wachter 1997) (Figure 2). Obviously, oomycetes have many different aspects compared to true fungi. For example, like plants and algae, the oomycete cell wall is composed of β-glucans, whereas fungal cell walls mainly consist of chitin (Bartnicky-Garcia and Wang 1983). Moreover, some oomycetes are not able to synthesize sterols, but obtain them from their environment (Hendrix and Guttman 1970).

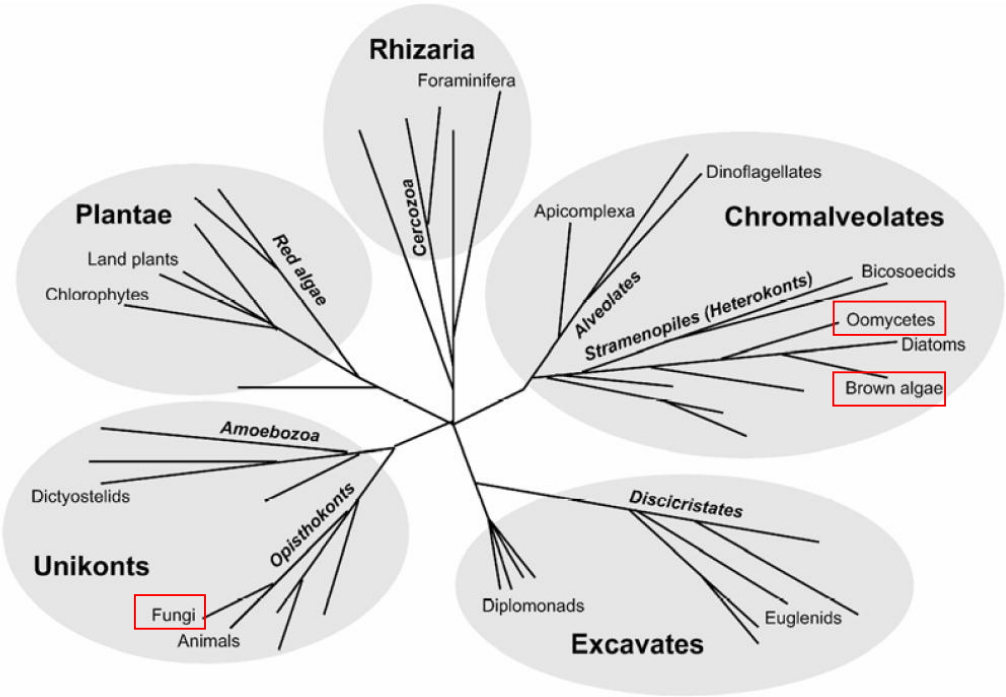

Figure 2. A schematic phylogeny of eukaryotes.

The five proposed eukaryotic super-groups are shaded and labelled in bold font. For simplicity, other labels correspond to selected examples; major sub-branches are shown in italics, and representative groups of organisms are provided at the branch termini. Plantae, Rhizaria, Chromalveolates, and Excavates represent the bikont lineages (published in (Govers and Gijzen 2006).

The genus *Phytophthora* comprises over 65 phytopathogenic species that cause many economically important diseases and can have devastating effects on natural habitats (Erwin and Ribeiro 1996). *P. infestans* is notorious for the plasticity and highly peculiar architecture of its genome (Haas et al. 2009). As will be described later, this enables *P. infestans* to thrive as a rapidly-adapting pathogen. The genome of *P. infestans* is the largest (240 Mb) among other plant-pathogenic *Phytophthora* species (Haas et al. 2009). The *P. infestans* genome also contains many transposons, rendering it to be highly dynamic (Haas et al. 2009). Combined with the mixed reproduction system, this accounts for the rapid increase in genetic diversity of the *P. infestans* population witnessed in recent years (Goodwin and Drenth 1997). Indeed, the 'old' (A1) population was rapidly replaced by new (A1/A2) populations (Goodwin and Drenth 1997).

Two layers of plant immunity

Pathogens can secret microbial and pest molecules that alter host-cell processes or structures that generally promote the microbe lifestyle. These molecules are called effectors. Effector functions are as diverse as suppressing immune responses or enhancing access to nutrients (Win et al. 2012). Effectors can be occur outside or inside plant cells, and are designated apoplastic or cytoplasmic effector, respectively.

Apoplastic effectors are secreted into the host extracellular space. The apoplastic effectors interact with host extracellular proteins and can be recognized by pattern recognition receptors (PRRs). Among apoplastic effectors, pathogen associated molecular patterns (PAMPs) are conserved in whole classes of microbes (nonself) for which they have an important function (Medzhitov and Janeway 1997, Brunner et al. 2002, Gomez-Gomez and Boller 2002, Nurnberger et al. 2004, Zipfel and Robatzek 2010). For example flagellin, the main building block of the flagellum, can bind to FLS2, a LRR-receptor-like kinase from *Arabidopsis* (Felix et al. 1999, Chinchilla et al. 2006, Boller and Felix 2009). FLS2 is highly conserved in plant species across the Brassicaceae (Robatzek et al. 2007, Boller and Felix 2009). Studies on flagellin and some other bacterial PAMPs stand model for apoplastic immunity (Kunze et al. 2004, Zipfel et al. 2006, Zipfel and Robatzek 2010).

Cytoplasmic effectors are translocated inside the host cell through specialized microbial structures such as the type-III secretion system apparatus of bacteria or haustoria in the case of oomycetes. Inside the host cell, the cytoplasmic effectors traffic to different subcellular compartments where they exert their

functions. Cytoplasmic effectors are generally much less conserved than PAMPs. Many known *avirulence (Avr)* genes are cytoplasmic effectors, such as *Avr1* (Tyler 2009), *Avr2* (Saunders et al. 2012), *Avr3a* (Armstrong et al. 2005), *Avr3b* (Li et al. 2011), *Avr4* (van Poppel et al. 2008), and *Avrblb1* (Vleeshouwers et al. 2008), *Avrblb2* (Oh et al. 2009) and *Avrvnt1* (Pel 2010), which can interact with the corresponding *R* genes, conferring a gene-for-gene resistance.

For inducing defense responses that can lead to plant immunity, the pathogen recognition is essential. Perception of pathogen molecules can occur at the surface (the first line of defense) or inside the cell, and accordingly induce apoplastic immunity and intracellular immunity (Dodds and Rathjen 2010) (Figure 3). Apoplastic immunity can be induced when extracellular effectors or PAMPs of pathogens are perceived by PRRs. The binding activates PRRs and triggers profound physiological changes in plant cells, including bursts of calcium and reactive oxygen species (ROS), as well as the activation of mitogen-associated and calcium-dependent protein kinases (MAPKs and CDPKs), leading to massive transcriptional reprogramming (Nicaise et al. 2009, Tena et al. 2011).

As PAMP-triggered immunity (PTI) is sufficient to halt infection of most microbes, loss of individual PRRs leads to enhanced disease susceptibility to both adapted and non-adapted pathogens (Boller and Felix 2009). Besides, adapted pathogens secrete numerous cytoplasmic effector proteins. In case of oomycetes, such effectors generally contain an RXLR motif, which is involved in translocation inside plant cells (Whisson et al. 2007, Dou et al. 2008, Kale et al. 2010). Some of these RXLR effectors have been reported to be able to suppress PTI (Dodds and Rathjen 2010). An example is *P. infestans* AVR3a, which can partially suppress the response to some oomycete PAMPs (Bos et al. 2006, Bos et al. 2010, Gilroy et al. 2011). Recognition of intracellular effectors by intracellular immune receptors containing nucleotide-binding (NB) and leucine-rich repeat (LRR) domains induces the second layer of immunity - intracellular immunity. This layer of immunity can activate robust defense programs often culminating in localized cell death (Dodds and Rathjen 2010).

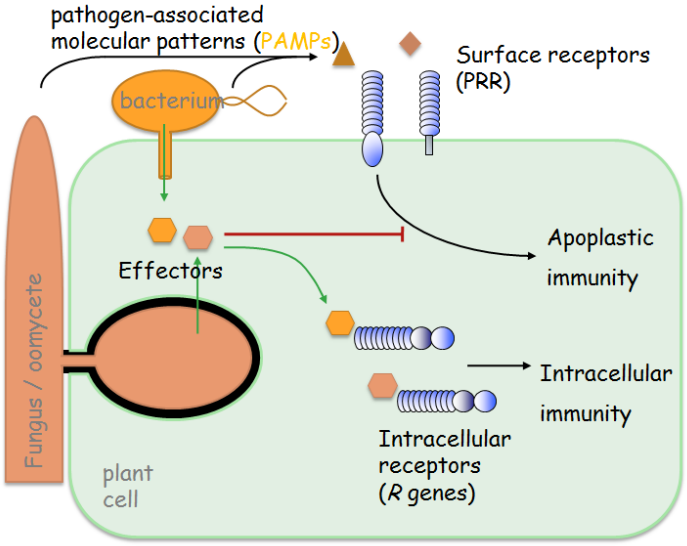

Figure 3. Two layers of immunity in plants

Apoplastic immunity and intracellular immunity are two layers of immunity in plants. Apoplastic immunity is induced when PAMPs of pathogens are perceived by PRR. Intracellular immunity is induced upon recognition of intracellular effectors by NB-LRR proteins, encoded by *R* genes (Dodds and Rathjen 2010, Kamoun 2013).

The challenges of *R*-gene based intracellular immunity in potato breeding

P. infestans remarkable ability of fast evolution is due to high mutation rates, large effective populations, a high gene/genotype flow, a mixed reproduction system and an efficient directional selection (McDonald and Linde 2002). Moreover, the revelation of *P. infestans* genome plasticity and its abundance of putative effectors, 563 genes predicted for the RXLR family (Haas et al. 2009), show that this oomycete remains a formidable foe.

The use of pathogen effectors in breeding and deployment has recently proven a successful strategy to understand and achieve resistance to late blight in potato (Vleeshouwers et al. 2008, Ellis et al. 2009, Hein et al. 2009, Vleeshouwers et al. 2011). Effectoromics strategies, i.e. high throughput functional screens with effectors on germplasm, has contributed to accelerate and improve the exploitation of *R* genes in contemporary potato resistance breeding (Vleeshouwers et al. 2008).

In the past years, potato breeders devoted to breeding resistant cultivars, as it is much more economic and environmental-friendly than using pesticides. Early breeding efforts were focused on the introgression of 11 resistance (*R*) genes from *Solanum demissum*, a wild relative of potato. The initial exploitation of new cultivars containing these *R* genes was successful (Black et al. 1953, Malcolms and Black 1966). However, rapidly changing populations of *P. infestans* overcame the *R1-R11* genes, even in pyramiding systems (Hein et al. 2009). Encouragingly, there are many more wild potato species that could be exploited besides *S. demissum*. The tuber-bearing *Solanum* section *Petota* consists of 189 species including the cultivated species *S. tuberosum* (Spooner and Salas 2006).

Contemporary potato breeding for *P. infestans* resistance is exploring the wealth of *R* gene diversity in a broad range of *Petota* species to build up a collection of diverse *Rpi* (Resistance to *P. infestans*) genes (Vleeshouwers et al. 2011). Till now, *R1*, *R2*, *R3a* and *R3b* have been cloned from *S. demissum* (Ballvora et al. 2002, Huang et al. 2005, Lokossou et al. 2009, Li et al. 2011). Additional *Rpi* genes have been cloned from different wild species, e.g. *Rpi-blb1/RB* (Song et al. 2003, van der Vossen et al. 2003), *Rpi-blb2* (van der Vossen et al. 2005), and *Rpi-blb3* from *S. bulbocastanum* (Lokossou et al. 2009); *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* (Vleeshouwers et al. 2008); *Rpi-abpt*/*R2-like* from unknown species used in a pre-breeding program (Lokossou et al. 2009), and *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* from *S. venturii* (Foster et al. 2009, Pel et al. 2009). More *Rpi*-genes are in the pipeline and are expected to be cloned in the near future, which will develop more avenues to protect potatoes against late blight (Smilde et al. 2005, Rauscher et al. 2006, Tan et al. 2008, Hein et al. 2009, Jacobs et al. 2010).

However, despite the successes of effectoromics approaches, intracellular immunity based on *R* genes rarely confers a durable broad-spectrum immunity that remains effective against all races of the pathogen. Especially for late blight, there are many concerns about resistance breeding based on *R* genes

alone (Gebhardt and Valkonen 2001). We hypothesize that apoplastic immunity may provide another layer of defense that can be recruited to control the disease.

The availability of apoplastic immunity

Apoplastic immunity is triggered by apoplastic effectors. Among apoplastic immunity, PAMP-triggered immunity (PTI) is an emerging topic in the field of molecular microbe-plant interactions. PAMPs have been widely identified in various pathogens, e.g. bacteria, fungi and oomycetes. Various matching PRRs against bacteria and fungi are also being reported, but remain relatively unexplored in oomycetes till now (Table 1). Those identified PRRs consist of receptor-like proteins (RLPs) or receptor-like kinases (RLKs) and the latter contain an intracellular kinase domain (Boller and Felix 2009).

Apoplastic immunity against bacteria

Flagellin -triggered apoplastic immunity

The 22-amino-acid flagellin epitope flg22, which is present in the conserved N-terminus of flagellin, is recognized by most plant species (Felix et al. 1999). Flg22 binds directly to the leucine-rich repeat receptor kinase (LRR-RLK) FLAGELLIN SENSING2 (FLS2) (Chinchilla et al. 2006). Then FLS2 interacts with the adaptor protein *brassinosteroid-insensitive1 (BRI1)-associated receptor kinase 1* (BAK1) and activates the PTI responses against bacteria (Felix et al. 1999, Gomez-Gomez and Boller 2002, Chinchilla et al. 2007, Heese et al. 2007). The perception of flg22 by FLS2 is one of the most well studied examples of PTI. Pretreatment of *Arabidopsis* plants with flg22 restricts the growth of bacterial speck disease pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), and *fls2* mutant plants are more susceptible to this pathogen following spray inoculation (Zipfel et al. 2004). In addition, lack of flagellin recognition allows more growth of the nonadapted bacteria *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *tabaci* (Li et al. 2005, de Torres et al. 2006). Interestingly, there is a second epitope of flagellin, termed flgII-28 that is sufficient to trigger immunity in tomato (Cai et al. 2011). However, flgII-28 could not be perceived by FLS2 but may modulate indirectly flg22 perception by FLS2 (Cai et al. 2011).

Ef-Tu-triggered apoplastic immunity

The elf18 epitope of the elongation factor Tu (Ef-Tu) is another well-studied PAMP of bacteria. Ef-Tu of *Agrobacterium tumefaciens* is fully active as an elicitor in *Arabidopsis* (Kunze et al. 2004). Ef-Tu is detected by EF-Tu receptor (EFR), a *Brassicaceae* specific LRR-RLK (Kunze et al. 2004, Zipfel et al. 2006). *Efr* mutants show enhanced susceptibility to *Agrobacterium tumefaciens* (Zipfel et al. 2006).

PGN -triggered apoplastic immunity

Peptidoglycan (PGN) is a bacterial N-acetyl glucosamine oligomer that is structurally similar to chitin. PGN binds proteins LYM1 and LYM3 that contain LysM domain. LYM1 and LYM3 were recentlydemonstrated to mediate plant sensitivity to PGN and contribute to immunity to *Pst* DC3000 (Willmann et al. 2011).

AX21-triggered apoplastic immunity

Rice bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most serious rice diseases worldwide. The bacterial gene *AX21* does not encode one of the usual effectors, but instead seems to be involved in the production of a secreted, sulphated molecule that possibly acts as a quorum sensing signal (Han et al. 2011). Since AX21 sequence is conserved in all *Xanthomonas* species, this peptide is clearly a PAMP and hence redefines the cognate receptor XA21 as a true PRR (Han et al. 2011, Segonzac and Zipfel 2011). XA21 confers resistance to a number of races of *Xoo* (Song et al. 1995).

eMAX -triggered apoplastic immunity

The protein eMAX is recognized as a PAMP of *Xanthomonas.* AtRLP1 has recently been identified as the receptor for eMAX, and consequently AtRLP1 was named ReMAX (Jehle et al. 2013). Interfamily transfer of *ReMAX* to *Nicotiana benthamiana* was successful after using hybrid receptors with the C-terminal part of *ReMAX* replaced by that of a tomato RLP named *Eix2* (Jehle et al. 2013). A comprehensive survey revealed that knockout lines of *Remax* did not exhibit significant changes in susceptibility to *Xanthomonas campestris* pv. *campestris* (Wang et al. 2008).

Table 1. Representative PAMP-PRR pairs in plants.

Source	PAMP	Epitope	Reference	PRR	PRR Type	Reference
Bacteria	Flagellin	flg22& flgII-28	(Felix et al. 1999, Cai et al. 2011)	FLS2	LRR-RLK	(Gomez-Gomez and Boller 2000)
	Ef-Tu	Elf18	(Kunze et al. 2004)	EFR	LRR-RLK	(Zipfel et al. 2006)
	Peptidoglycan	PGN	(Gust et al. 2007)	LYM1 & LYM3	LysM-RLP	(Willmann et al. 2011)
	Ax21	*ni*	(Han et al. 2011)	Xa21	LRR-RLK	(Song et al. 1995)
	eMAX	*ni*	(Jehle et al. 2013)	ReMAX/ AtRLP1	LRR-RLP	(Jehle et al. 2013)
Fungi	Xylanase	TKLGE pentapeptide	(Fuchs et al. 1989, Rotblat et al. 2002)	LeEIX1 & LeEIX2	LRR-RLP	(Ron and Avni 2004)
	Chitin	chitin oligosaccharides	(Felix et al. 1993)	CEBiP & CERK1	LysM-RLK	(Kaku et al. 2006, Miya et al. 2007)
	Avr2	*ni*	*ni*	Cf-2	LRR-RLP	(Dixon et al. 1996)
	Avr4	*ni*	(Joosten et al. 1994)	Cf-4	LRR-RLP	(Joosten et al. 1994)
	Avr4E	*ni*	(Westerink et al. 2004)	Cf-4E	LRR-RLP	(Takken et al. 1999)
	Avr5	*ni*	*ni*	Cf-5	LRR-RLP	(Dixon et al. 1998)
	Avr9	*ni*	(van Kan et al. 1991, van Den Ackerveken et al. 1992)	Cf-9 & 9DC	LRR-RLP	(Jones et al. 1994, van der Hoorn et al. 2001, Kruijt et al. 2004)
	Ave1	*ni*	(de Jonge et al. 2012)	Ve1	LRR-RLP	(Kawchuk et al. 2001)
Oomycetes	*ni*	*ni*	*ni*	LecRK-I.9	legume-like lectin RLK	(Bouwmeester et al. 2011)
	ni	*ni*	*ni*	IOS1	LRR-RLK	(Hok et al. 2011)
	Elicitin	*ni*	(Ricci et al. 1989, Vleeshouwers et al. 2006, Chaparro-Garcia et al. 2011)	ELR	LRR-RLP	This thesis

ni means not identified.

Apoplastic immunity against Fungi

Chitin -triggered apoplastic immunity against various fungal pathogens

LysM domain-containing RLP CEBiP was identified as a chitin-binding protein required for chitin recognition in rice (Kaku et al. 2006). CEBiP forms a chitin-enhanced heteromeric complex with the LysM-RLK CERK1 (Shimizu et al. 2010). Although it is unclear whether CERK1 also binds chitin, it is required for chitin responsiveness in rice (Shimizu et al. 2010). In *Arabidopsis*, however, CERK1 is the major chitin binding protein (Iizasa et al. 2010, Petutschnig et al. 2010), and is required for chitin-induced responses (Miya et al. 2007, Wan et al. 2008) and resistance against *Alternaria brassicicola* (Miya et al. 2007).

Avr protein-triggered apoplastic immunity against Cladosporium fulvum

Tomato resistance to the biotrophic leaf mold fungus *Cladosporium fulvum* is fully based on apoplastic immunity. The first *RLP* gene discovered involved in pathogen defense was *Cf-9* in tomato (*Solanum lycopersicum*). Cf9 can recognize the Avr9 effector secreted by *C. fulvum* and trigger the resistance (van Kan et al. 1991, Jones et al. 1994). By now, all cloned tomato *Cf* genes are known to encode RLPs and can be grouped into two large gene families. The *Cf-4*, *Cf-4E*, *Cf-9*, and *9DC* genes that mediate recognition of the cognate *Avr4*, *Avr4E*, and *Avr9* (both *Cf-9* and *9DC*), respectively, are highly homologous and belong to the *Hcr9* (homologs of *C. fulvum* resistance gene *Cf-9*) gene family (Jones et al. 1994, Joosten et al. 1994, Takken et al. 1999, van der Hoorn et al. 2001). Similarly, the *Cf-2* and *Cf-5* genes that mediate recognition of the cognate *Avr2* and *Avr5*, respectively, belong to the *Hcr2* (homologs of the *C. fulvum* resistance gene *Cf-2*) gene family (Dixon et al. 1996, Dixon et al. 1998, Luderer et al. 2002). Recognition leads to defense responses including the hypersensitive cell death response (HR), which results in a complete inhibition of the pathogen at the site of penetration (Joosten and de Wit 1999, Wulff et al. 2009). Although *Cf* genes and their matching *Avr* genes fit the gene-for-gene resistance (Keen 1990), the distinction between resistance proteins and PAMP receptors cannot strictly be maintained (Thomma et al. 2011).

In addition to Avr proteins that match Cf receptors, *C. fulvum* also abundantly secretes other extracellular proteins (Ecp). A well-known example is Ecp6 (for extracellular protein 6), which contains LysM domains like the plant chitin receptors CEBiP and CERK1 (Bolton et al. 2008). Although Ecp6 can bind chitin fragments to suppress chitin-triggered immune responses, Ecp6 is not qualified as an avirulence gene but a PAMP, since LysM effectors are widely conserved in the fungal kingdom (Bolton et al. 2008, de Jonge and Thomma 2009). Tomato genotypes that have evolved to recognize Ecp6 develop an HR upon Ecp6 infiltration and presumably carry a cell surface receptor for this molecule. This surface receptor is tentatively called Cf-Ecp6, for *C. fulvum* resistance to Ecp6 (Thomma et al. 2011).

Ave1-triggered apoplastic immunity against Verticillium species

Verticillium spp. can cause wilt disease, resulting in severe losses of yield and quality in many crop species such as tomato, potato, eggplant and strawberry. Two closely linked inverted genes, *Ve1* and *Ve2*, have been cloned from tomato (Kawchuk et al. 2001). Both *Ve1* and *Ve2* encode RLPs, containing 38 imperfect extracellular LRRs and sharing 84% amino acid identity (Kawchuk et al. 2001). Subsequent analysis has demonstrated that Ve1, but not Ve2, acts as a true resistance protein against race 1 strains of *V. dahliae* and *V. albo-atrum* in tomato (Fradin et al. 2009). Ave1 was later identified by high-throughput population genome sequencing, and confirmed to activate *Ve1*-mediated resistance (de Jonge et al. 2012).

Xylanase-triggered apoplastic immunity against Trichoderma viride

The tomato RLP LeEix confers recognition of an ethylene inducing xylanase (EIX) of the biocontrol fungus *Trichoderma viride* (Ron and Avni 2004). Molecular cloning of the *LeEix* locus resulted in the identification of two functional genes, *LeEix1* and *LeEix2* (Ron and Avni 2004). Both genes bind the EIX elicitor, but only LeEix2 can induce an HR (Ron and Avni 2004). LeEix2 contains a short cytoplasmic domain with an endocytosis motif (YXXØ) that is required for HR induction (Ron and Avni 2004). Binding of the EIX elicitor to LeEix2 leads to ligand-induced endocytosis of the receptor (Bar and Avni 2009).

Apoplastic immunity against oomycetes

Studies with bacterial and fungal pathosystems have shown that plant receptors that target conserved PAMPs have great promise for engineering effective and durable disease resistance in crops (Dodds and Rathjen 2010). However, this PAMP-triggered immunity (PTI) has not yet been widely exploited against oomycete pathogens (Monaghan and Zipfel 2012). For oomycetes, in particular *Phytophthora* species, a few extracellular proteins have been identified as PAMPs (Table 1). Among the best-characterized oomycete PAMPs are elicitins.

Elicitins are oomycete PAMPs

Elicitins represent a superfamily of structurally conserved extracellular proteins that share a 98-amino-acid elicitin domain (Ponchet et al. 1999, Qutob et al. 2003, Jiang et al. 2006). Elicitins induce cell death and other responses associated with defense in a range of plant species, such as *Nicotiana*, *Solanum* (*Solanaceae*) and *Brassicaceae* (Kamoun et al. 1993, Pernollet et al. 1993, Kamoun et al. 1997). Since elicitins occur only in oomycetes, specifically in *Phytophthora* and *Pythium* species, they represent non-self-molecules for plants. For the oomycete species that cannot synthesize sterols, elicitins fulfill an important function as sterol scavengers (Mikes et al. 1997, Yousef et al. 2009, Stong et al. 2013). For these reasons, elicitins are hypothesized to act as oomycete PAMPs (Vleeshouwers et al. 2006, Chaparro-Garcia et al. 2011).

Elicitin-triggered defense responses

Most *Phytophthora* species secrete elicitins. Elicitins of *P. infestans* (*Inf*) are expressed during the interaction with the host (Kamoun et al. 1997, Huitema et al. 2005). INF1 is a 10-kDa protein produced in almost all *P. infestans* isolates (Kamoun et al. 1998), which suggests that immune responses to INF1 can target a broad spectrum of isolates. In addition to INF1, a complex family of INFs and INF-likes occurs in *P. infestans* (Jiang et al. 2006). INF1 was demonstrated to function as an avirulence factor in the interaction between *N. benthamiana* and *P. infestans*. *P. infestans* strains that produce INF1 induce hypersensitive cell death responses in *N. benthamiana*, whereas INF1-silenced strains showed less hypersensitive cell death and a gain of virulence on these plants (Kamoun et al. 1998). Furthermore, INF1 can also elicit cell death responses in various species of *Solanum* section *Petota* (Vleeshouwers et al. 2006, Rietman 2011). In tomato, INF1 activates jasmonic acid- and ethylene-mediated signalling pathways but does not induce a cell death response (Kawamura et al. 2009). Elicitins also cause hypersensitive cell death in some cultivars of *Brassica rapa* (rape) and *Raphanus sativus* (radish) (Kamoun et al. 1993, Keizer et al. 1998, Takemoto et al. 2005). Moreover, two elicitins named cryptogein and capsicein were reported to promote defense responses against *Phytophthora cinnamomi* (Medeira et al. 2012), which is cause of the decline of cork oak (*Quercus suber*) in Iberian Peninsula.

Other oomycete PAMPs-triggered defense responses

In addition to elicitins, more oomycete PAMPs have been identified. β-glucan, a fragment of the mycelial cell walls, was one of the first elicitors discovered in *Phytophthora megasperma* and recognized to be actively involved in plant-pathogen interactions (Ayers et al. 1976). As a typical oomycete PAMP, β-glucan elicits a variety of defense reactions in tobacco, conferring resistance to the soft rot disease caused by bacterium agent *E. carotovora* (Klarzynski et al. 2000). The extracellular beta-glucan-binding protein (GBP) in legumes was reported to bind beta-glucan (Fliegmann et al. 2004), but how it accomplishes intracellular signaling is not clear.

Pep-13, first described as a peptide elicitor of defense responses in parsley, constitutes a surface-exposed fragment within a calcium-dependent cell wall transglutaminase (TGase) from *Phytophthora sojae* (Nürnberger et al. 1994). Later, Pep-13 was found to be conserved among *Phytophthora* transglutaminases and also activates defense in potato (Brunner et al. 2002). In the case of the non-host plant, parsley, receptor-mediated recognition of Pep-13 may trigger defense reactions that contribute to, or are sufficient for, resistance against *Phytophthora* infection (Nürnberger et al. 1994). However, in the potato-*P. infestans* interaction, pathogen recognition through the Pep-13 motif is clearly insufficient to provide resistance (Brunner et al. 2002).

Arachidonic acid, a fatty acid characteristic of oomycetes but absent from plants, acts as a potent elicitor in potato and elicits phytoalexin accumulation in potato tuber tissue (Bostock et al. 1981). Arachidonic

acid is still sometimes used as an elicitor, and would fit perfectly in the definition of a PAMP, but it has never been further explored (Boller and Felix 2009).

Cellulose binding elicitor lectin (CBEL), a cell wall glycoprotein, was first cloned from *Phytophthora parasitica var nicotianae*, the causal agent of the black shank disease of tobacco (*Nicotiana tabacum*) (Mateos et al. 1997). CBEL is widespread in *Phytophthora* and induces immune responses in several plant species, including tobacco and *Arabidopsis thaliana* (Khatib et al. 2004). CBEL contains two cellulose binding domains (CBDs), which is a novel class of molecular patterns in oomycetes that are targeted by the innate immune system of plants and might act through interaction with the cell wall (Gaulin et al. 2006). In tobacco, CBEL induces local hypersensitive response (HR)-like lesions, defense responses, and protection against subsequent infection with the oomycete (Mateos et al. 1997). In Arabidopsis, it has been shown that three signaling pathways that involve salicylic acid, jasmonic acid, or ethylene are triggered by CBEL and that its necrosis-inducing activity depends on ethylene and jasmonic acid (Khatib et al. 2004). A more recent study reported that CBEL-triggered immunity is essential to trigger immunity and resistance to the root pathogen *Phytophthora parasitica* in *Arabidopsis* (Larroque et al. 2013).

More interestingly, some PAMPs exist beyond pathogen kingdoms, not only in oomycetes. A typical example is a family of necrosis-inducing proteins that belongs to the superfamily of Nep1-like proteins (NLPs) present in bacteria, fungi, and oomycetes (Bailey 1995, Veit et al. 2001, Fellbrich et al. 2002, Qutob et al. 2002, Mattinen et al. 2004, Kanneganti et al. 2006). As mentioned above, in 1995, the 24-KDa protein Nep1 was first purified from *F. oxysporum* (Bailey 1995), and later more Nep1-like protein family members have been identified. These proteins induce ethylene biosynthesis and necrosis in a wide variety of Dicotyledoneae, but not likely in Monocotyledoneae (Veit et al. 2001).

There are many oomycete PAMPs known, but not many matching PRR. In this thesis we characterize ELR (elicitin response), the first RLP that matches a known oomycete PAMP, namely elicitin. A few other PRR against oomycetes are known such as LecRK-I.9 and IOS1. The *Arabidopsis* legume-like lectin receptor kinase LecRK-I.9 is known to bind to the *Phytophthora infestans* RXLR-dEER effector IPI-O in vitro via a RGD cell attachment motif present in IPIO (Gouget et al. 2006). LecRK-I.9 is associated with the plasma membrane. Two T-DNA insertions lines deficient in LecRK-I.9 show a 'gain-of-susceptibility' phenotype specifically towards the oomycete *Phytophthora brassicae* (Bouwmeester et al. 2011). Accordingly, overexpression of LecRK-I.9 leads to enhanced resistance to *P. brassicae* (Bouwmeester et al. 2011). Another oomycete LRR-RLK was denominated Impaired Oomycete Susceptibility 1 (IOS1) (Hok et al. 2011). A knockout mutant of IOS1 showed reduced downy mildew infection, but susceptibility was fully restored through complementation of the mutation, suggesting that IOS1 contributes to the disease (Hok et al. 2011).

Scope of the thesis

The aim of research described in this thesis is to study apoplastic immunity against potato late blight caused by the oomycete pathogen *P. infestans*. Our studies are based on an apoplastic immune receptor ELR, which was recently cloned from the wild potato *Solanum microdontum* using effectoromics strategies (Verzaux 2010). In this thesis we first describe effectoromics approaches and two most-commonly-used transient assays, namely, agroinfiltration and PVX agroinfection. Then we further study apoplastic immunity conferred by ELR in potato.

Potato resistance breeding, based on *R* genes (intracellular immunity), has been used widely but has been unsuccessful for more than a century. A further drawback is that traditional resistance breeding is slow and inefficient. Encouragingly, the use of pathogen effectors in breeding has recently been proven a successful strategy to understand and achieve resistance to late blight in potato (Vleeshouwers et al. 2008, Ellis et al. 2009, Hein et al. 2009, Vleeshouwers et al. 2011). In **Chapter 2**, we analyzed the application aspects of effectoromics in the forms of do's and don'ts.

As two routinely high-throughput functional assays for effectoromics, agroinfiltration and PVX agroinfection, have been shown to be successful on *Phytophthora* effectors (Qutob et al. 2002, Torto et al. 2003, Huitema et al. 2004, Vleeshouwers et al. 2011), there is a clear desire to use these approaches by many different researchers. As they are rather difficult to implement from scratch we provided in **Chapter 3** detailed protocols and discuss about the advantages and disadvantages of both assays.

Effectoromics has initially been focused on RXLR effectors that interact with intracellular *R* genes, but is also effective for identifying apoplastic immune receptors such as ELR (Verzaux 2010). In **Chapter 4**, we characterize ELR. ELR is predicted to encode an RLP. To study whether ELR confers resistance to *P. infestans*, we transformed *ELR* to a susceptible potato cultivar (Désirée) and analyzed the resistance of *ELR* transformants to *P. infestans* isolates. Indeed, the *ELR* transformants were more resistant than the susceptible Désirée controls. Furthermore, we tested the recognition spectrum of *ELR* transformants and found that ELR could recognize a broad spectrum of *Phytophthora* elicitins and induce defense responses.

In **Chapter 5**, we analyze the genetic variation of *ELR*. First, we studied the geographical and phylogenetic location of those INF1-recognizing species. Results indicate that phylogenetically diverse *Solanum* section *Petota* species containing *ELR* homologs are distributed in both Central and South America. The amino acid sequences of 7 ELR orthologs that recognize elicitins are highly identical, ranging from 93% to 100% sequence identity. In addition, we found a significant loss of about 300 bp in the coding sequence of most non-functional homologs. To further analyze the variation of functional *ELR* orthologs, we tested their recognition spectrum by agro-co-infiltration with diverse elicitins. Results show that elicitin response patterns are similar with the elicitin response patterns on *N.*

benthamiana. Moreover, we also tested if elicitin responses can be suppressed in potato like they can be in *N. benthamiana*. And indeed, elicitin responses can also be suppressed in potato. This provided additional evidence that elicitin response patterns are conserved between *Solanum* and *Nicotiana*.

In **Chapter 6**, we discuss the possibilities of several strategies for durable resistance to potato late blight, e.g. the availability of apoplastic immunity, the application of effectoromics, the exploitation of defense-responsive genes and stacking multiple defense layers.

Chapter 2

The *Do's* and *Don'ts* of effectoromics

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The *Do's* and *Don'ts* of effectoromics

Abstract

Effectoromics, a high throughput, functional genomics approach that uses effectors to probe plant germplasm to detect *R* genes has proven a potent contribution to modern resistance breeding. Advantages of effectoromics are summarized in four aspects, 1) accelerating *R* gene identification; 2) distinguishing functional redundancy; 3) detecting recognition specificity and 4) assisting in *R* gene deployment. In this manuscript, we provide suggestions as well as some reminders for applying effectoromics in the breeding process. The two routine functional assays that are widely used, agroinfiltration and agroinfection, are presented. We briefly explain their advantages and disadvantages, and provide protocols for applying them in the model system *Nicotiana benthamiana* as well as in potato (*Solanum tuberosum*).

Introduction

Effectoromics has recently emerged as a powerful tool to identify resistance (*R*) genes in crop plants and matching avirulence (*Avr*) genes of pathogens (Vleeshouwers et al. 2008, Ellis et al. 2009, Oh et al. 2009, Vleeshouwers et al. 2011). This high-throughput functional genomics approach uses effectors to probe plant germplasm for specific recognition of R proteins (Figure 1). The required inflow of predicted effectors from plant pathogen genome sequences is becoming increasingly easy with the rapid development of sequencing technology. Still, after analysing the huge amount of sequences by bioinformatics for putative functional genes, efficient biological assays are essential. Effectoromics perfectly bridges this gap, as we have shown for the late blight pathogen *Phytophthora infestans* and its interactions with wild potato species. Currently, researchers and breeders from various fields are adopting this approach for their own specific pathosystem.

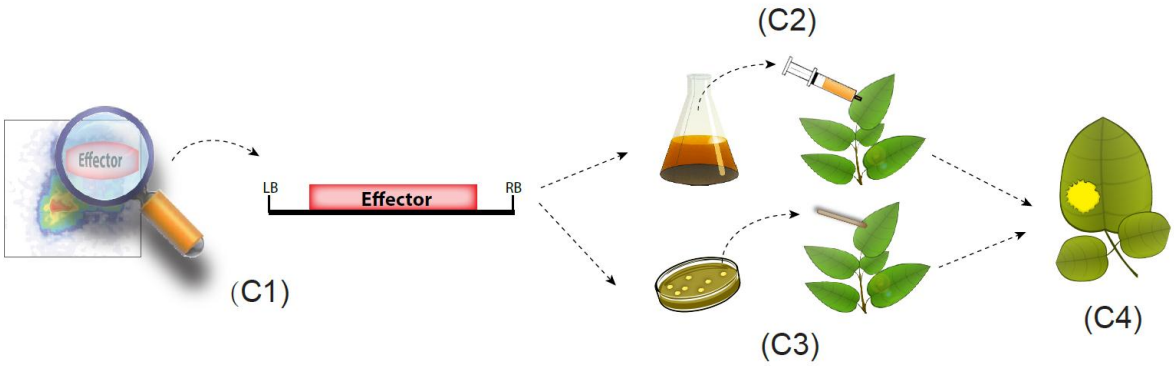

Figure 1. A scheme for effectoromics.

The overall goal of effectoromics assay is to test whether effectors of pathogens (C1) are recognized by resistance proteins in plants. This is achieved by first infecting plant leaves with a liquid culture of Agrobacterium (C2), or through PVX agroinfection (C3), which will transiently express effectors in the plant tissue. Then the plants are allowed to incubate and the plants are scored for cell death. Results are obtained that show cell death responses based on recognition of transiently expressed effectors by resistance proteins in plants (C4).

For effectoromics in *Solanaceous* plants, two *Agrobacterium*-based functional assays, namely, agroinfiltration (Figure 2) and PVX (*Potato Virus X*) agroinfection (Figure 3), are routinely used to transiently express effectors in plant cells (Kanneganti et al. 2007, Bhaskar et al. 2009, Vleeshouwers and Rietman 2009). Agroinfiltration is performed by infiltrating the suspensions of recombinant *Agrobacterium* into a plant leaf, which will transfer the desired gene into plant cells. PVX agroinfection is also based on *Agrobacterium,* in this case as a binary PVX expression system. Agroinfiltration and PVX agroinfection both have advantages and disadvantages (Table 1). Briefly, the agroinfiltration assay (Box 1) is very suitable for functional analysis of single genes, such as resistance (*R*) or avirulence (*Avr*) genes, as well as for reconstructing the R-AVR relationships by delivering two transgenes into the same cell. However, nonspecific defense to *Agrobacterium* is regularly observed, especially in some potato genotypes. PVX agroinfection (Box 2) is more sensitive, more high-throughput and less sensitive to nonspecific defense responses to *Agrobacterium*. However, in this case, nonspecific defense to PVX can occur and there is a risk to miss responses due to virus-induced extreme resistance (ER). Generally, agroinfiltration experiments are more laborious in preparing the inoculation culture of *Agrobacterium*, while PVX agroinfection experiments take longer until symptoms can be scored.

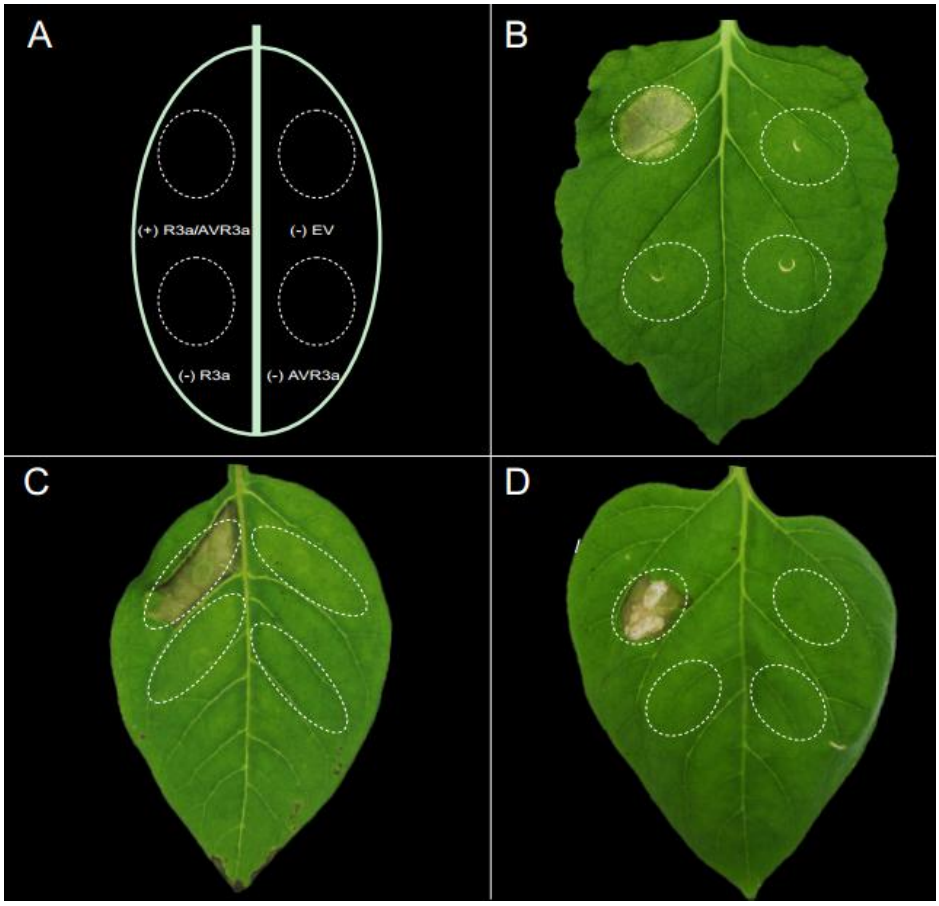

Figure 2. Agroinfiltration in *N. benthamiana* and potato

Agrobacterium clones expressing *R3a* and *AVR3a* were co-infiltrated in leaves of *N. benthamiana* (B) and two potato species *S. hjertingii* 349-3 (C) and Désirée (D). The empty pK7WG2 vector, *R3a* and *AVR3a* were included as negative controls, individually. Three days after inoculation, confluent cell death is visible in the leaf panel co-infiltrated with pBINplus-R3a and pK7WG2-AVR3a. No cell death occurs in the leaf panel infiltrated with pK7WG2-empty, pBINplus-R3a or pK7WG2-AVR3a.

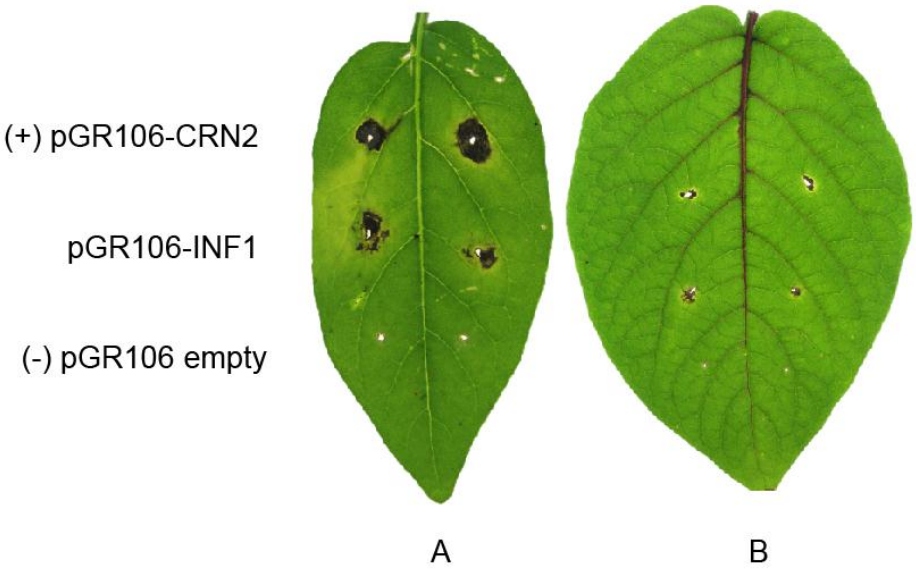

Figure 3. PVX agroinfection in potato

Agrobacterium clones expressing *Inf1* elicitin of *P. infestans* were tooth-pick inoculated in leaves of two potato species *S. huancabambense* 354-1 (A) and *S. microdontum* 360-1 (B). Crn2, a general cell death inducing gene from *P. infestans*, was included as a positive control, the empty pGR106 vector as a negative control. Two weeks after inoculation, expanding cell death is visible at the sites inoculated with pGR106-Crn2 and pGR106-Inf1. No expanding cell death is observed at the sites inoculated with pGR106-empty.

Table 1. Comparison of characteristics of agroinfiltration and PVX agroinfection

	Agroinfiltration	**PVX agroinfection**
Sensitivity	sensitive	very sensitive
Efficiency	medium throughput	high throughput
Nonspecific responses	sometimes suffers from plant responses to *Agrobacterium*	sometimes suffers from plant responses to PVX
Reliability	reliable	sometimes risky to miss responses due to extreme resistance
Preparation of inoculation cultures	relatively laborious	quick
Phenotyping results	short incubation time (3-5 days)	long incubation time (up to 2 weeks)

Box 1. Agroinfiltration protocol

1. Around 4-5-week-old seed-grown *N. benthamiana* or potato from *in vitro* tissue culture can be used for agroinfiltration. Choose young, healthy and fully developed leaves for infiltrations.
2. Inoculate 20 µl glycerol stock of the desired *Agrobacterium* strains into 10 ml YEB medium supplemented with 1 µl acetosyringone (3'-5' dimethoxy-4'-hydroxy acetophenone, 200 mM stock, 39,3 mg/ml DSMO), 100 µl MES buffer (2-(N-morpholino)-ethane sulfonic acid, 1 M stock, 195 g/L) and the appropriate antibiotics. Incubate cells for 1-2 days at 28 ℃ at 200 rpm to an OD_{600} of approximately 1.0.
3. Harvest cells by centrifugation at 4 000 rpm for 10 min, pour off the supernatant and resuspend the pellet in freshly made MMA medium (20 g sucrose, 5 g MS salts, 1.95 g MES, pH adjusted to 5.6 with NaOH, and 1 ml acetosyringone/L) to an OD_{600} of 0.3. For co-infiltration, mix the culture in a 1:1 ratio. Then incubate cells at room temperature for 1-6 h.
4. Place *Agrobacterium* suspensions into a 1 ml needleless syringe. Carefully inject the suspension from the syringe to the leaf.
5. Response can be macroscopically scored about 3 days after infiltrations. Results can be quantified by assessing cell death percentages.

Box 2. PVX agroinfection protocol

1. Around 2-3-week-old seed-grown *N. benthamiana* or potato from *in vitro* can be used for PVX agroinfection. For large-scale tests, slightly older (4-5 weeks) plants can be used.
2. Inoculate 20 µl glycerol stock of the desired *Agrobacterium* strains into 3 ml YEB medium supplemented with the appropriate antibiotics. Incubate cells for 1-2 days at 28 ℃ and 200 rpm to an OD_{600} of approximately 1.0.
3. Pipet about 300 µl of each *Agrobacterium* strain and spread them onto LB solid agar medium plates supplemented with the appropriate antibiotics. Incubate cells at 28 ℃ for 1-2 days.
4. Dip a wooden toothpick in the culture of the recombinant *Agrobacterium* strain and pierce the leaves.
5. To make a quantitative scoring possible, make multiple inoculations sites for each strain. Use three leaves per plant to serve as triplicates. Inoculate at least three plants for each strain.
6. Symptoms can be scored about two weeks after inoculation. For high-throughput screens, summarize the qualitative responses (yes/no) for each inoculation spot. Then calculate the percentage of responsive sites and compare them with controls.

As indicated above, the main issue for agroinfiltration and PVX agroinfection is the chance that nonspecific defense responses are raised against the infection agents, *Agrobacterium* or PVX, respectively, in diverse plant materials (Figure 4A). To solve this issue, we usually search for related plant genotypes that carry the same resistance gene but do not suffer from this unpractical response to the infection agents (Figure 4B). One way to do this is to identify resistant offspring plants that are amenable to the expression assays, and subject those genotypes to subsequent effector studies (Figure 4C and D).

So far, effectoromics has shown to contribute to resistance breeding in four aspects, i.e. accelerating *R* gene identification, distinguishing functional redundancy, detecting recognition specificity, and assisting in *R* gene deployment (Vleeshouwers et al. 2011). Distributed over these four aspects, we list 7 "Do's" that may be helpful to design a resistance breeding program involving effectoromics in potato or other crops. In addition, we discuss three "Don'ts" that are important to consider when assessing the results of effectoromics experiments.

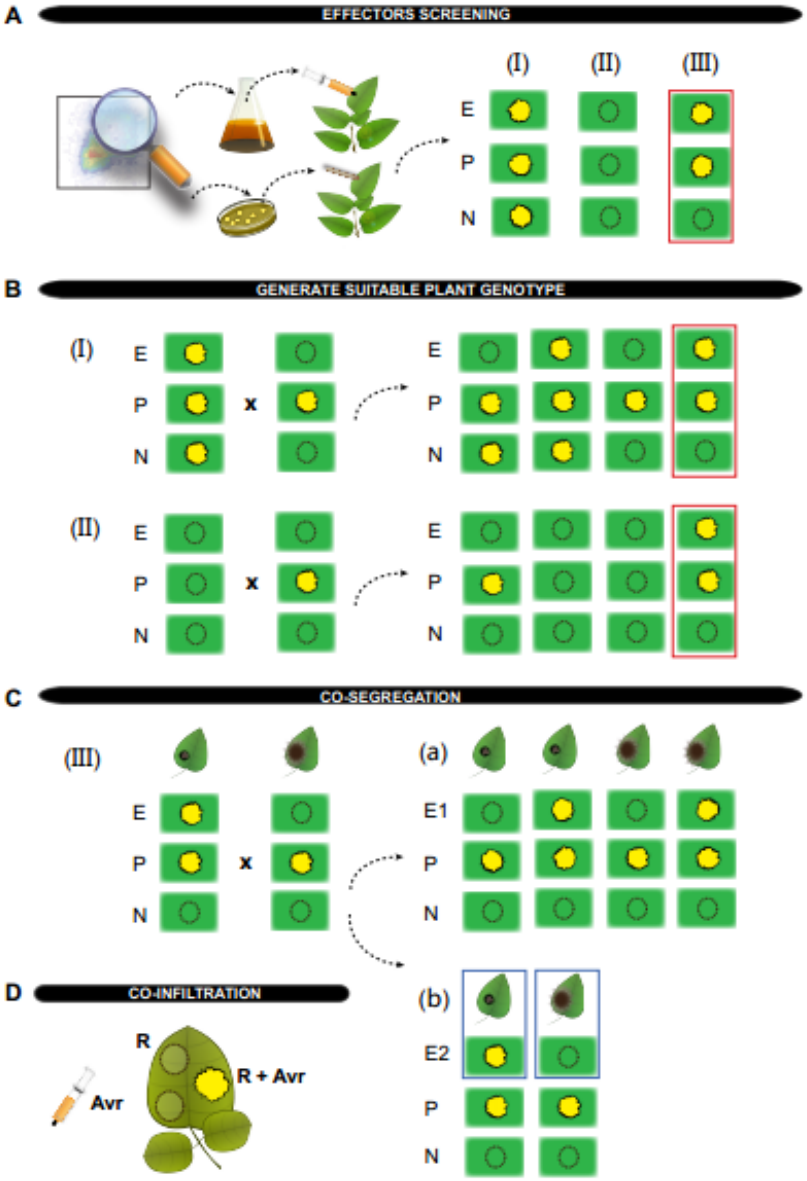

Figure 4. Identification of *R* and *Avr* genes by effectoromics.

A) RXLR effectors are retrieved from the *Phytophthora infestans* genome sequences and cloned into expression vectors. Constructs are then introduced in *Agrobacterium tumefaciens* for functional screening by agroinfiltration or *Potato Virus X* (PVX) agroinfection. Plant genotypes that are suitable for effectoromics screens (III) show response to the positive control (P) but not the negative control (N), and response to effectors (E) can be studied (in red box). However, some potato genotypes (I) show nonspecific responses to negative controls, other genotypes (II) fail to show response to positive controls, and such genotypes are not suitable. **B)** To generate more suitable genotypes, genetic crosses are made. Among the progeny, potato genotypes that show response to the effector as well as the positive control but not the negative control, are selected (in red boxes). **C)** Genetic studies for co-segregation of response to effectors with resistance to *Phytophthora infestans*. In (a), response to effector E1 does not co-segregate with resistance. In (b), response to one effector E2 co-segregates with resistance (in blue boxes), and E2 is a candidate *Avr* gene that matches the *R* gene in the tested plant. **D)** Further validation by agro-co-infiltration of the candidate *Avr* gene with the matching *R* gene. Specific cell death occurs in leaf panels where *R* and *Avr* gene are simultaneously expressed.

Do's

Acceleration

1) Exploit effectors to accelerate *R* gene cloning

Distinguishing functional redundancy

2) Exploit effectors for circumventing genetic crossing barriers
3) Exploit effectors for dissecting resistance specificities

Detecting specificity

4) Exploit effectors for detecting weak resistance phenotypes
5) Expand effector recognition specificity of *R* genes; target for broad-spectrum resistance

Deployment

6) Exploit effectors that are important for *P. infestans*; target for durable resistance
7) Deploy effectors for *R* gene application in agriculture

In the following paragraphs, these points will be elaborated on:

1) Exploit effectors to accelerate *R* gene cloning

During *R* gene cloning processes, various candidate genes are often obtained. Complementation studies are needed to select the true functional *R* gene. In a classic complementation test e.g. for potato, it takes up to six months to generate stable transgenic plants that can be inoculated with *P. infestans* and assessed for resistance. In contrast, if the matching *Avr* gene is available, the test can be limited to several days; one needs simply to agro-co-infiltrate the candidate *R* gene with the matching *Avr* gene in leaves of *Nicotiana benthamiana* to test for *R*-gene-specific cell death responses. In this way, the identification and characterization of *R* genes in potato can greatly be accelerated (Vleeshouwers et al. 2008, Li et al. 2011, Kim et al. 2012, Rietman et al. 2012).

2) Exploit effectors for circumventing genetic crossing barriers

Since most cloned *R* genes have been defeated by the fast evolving pathogen *P. infestans*, potato breeders explore wild *Solanum* germplasm for new *R* genes. However, genetic crossing barriers together with linkage drag remain a problem in potato resistance breeding and can drastically delay introgression of *R* genes. By effectoromics approaches, this problem can be solved. Functional allele mining with *Avr* genes in large collections of germplasm can quickly lead to identification of functional *R* gene homologs in various species. Sexually, more compatible species with particular resistance specificity can then be selected for introgression. A good example is the well-known broad-spectrum potato resistance gene *RB*/*Rpi-blb1* (Song et al. 2003, van der Vossen et al. 2003), which originates from *Solanum bulbocastanum* that is not directly sexually compatible with cultivated potatoes (Figure 5). In the past,

difficult somatic hybridisation experiments (Song et al. 2003) and time-consuming bridge-crossing experiments (Hermsen and Ramanna 1973) were used to introgress the *Rpi-blb1/RB* gene into *S. tuberosum*. Functional screens with *Avrblb1* in *Solanum* germplasm quickly led to identification of specific cell death responses in *Solanum stoloniferum*, which is directly crossable with cultivated potatoes. Based on *Rpi-blb1* homology and functional equivalence, the two *Rpi-blb1* homologs *Rpi-sto1* and *Rpi-pta1* were rapidly cloned from *S. stoloniferum* (Vleeshouwers et al. 2008). In addition to trans- or cisgenic approaches, traditional breeding strategies can now be more quickly used to introgress the *Rpi-blb1* specificity into potato.

Figure 5. A wild *Solanum bulbocastanum* plant

Solanum bulbocastanum belongs to the tuber-bearing *Solanum* species of section *Petota*, but is rather distantly related to cultivated potato. *S. bulbocastanum* is a Mexican diploid *Solanum* spp. that is not directly crossable with *S. tuberosum* (Hermsen and Ramanna 1973, Jacobs et al. 2008).

In addition to identifying sexually compatible resistant species, breeders can avoid redundant breeding or cloning efforts by classifying germplasm or *R* genes based on their responses to effectors (Vleeshouwers et al. 2008, Ellis et al. 2009, Oh et al. 2009). This is particularly important for *R* genes with broad-spectrum effects for which diagnostic pathogen races are not available. Therefore, effector-based resistance breeding also enables pyramiding of functionally complementary *R* genes.

3) Exploit effectors for dissecting resistance specificities

Although most cloned *R* genes have been defeated by local *P. infestans* populations, some cultivars and wild species still retain a certain resistance and plants remain healthy. In many cases, the resistance of those plants is based on pyramided *R* genes. Compared to traditional inoculations with *P. infestans*

isolates, the effectors can more easily and more accurately dissect the activities of otherwise indistinguishable *R* genes into discrete recognition specificities. For example, resistance specificities were dissected in a segregating population of potato cultivar 'Sarpo Mira', which has retained resistance in practice over many years. Responses to *P. infestans* RXLR effectors were matched with race-specific resistance responses to different *P. infestans* strains and 'Sarpo Mira' was shown to contain at least three known *R* genes *R3a, R3b* and *R4*, and two new genes *Rpi-Smira1* and *Rpi-Smira2* (Rietman et al. 2012). In the same way, the two potato *R* gene differentials MaR8 and MaR9 (Mastenbroek 1953) were dissected to have at least four (*R3a, R3b, R4, and R8*) and seven (*R1, Rpi-abpt1, R3a, R3b, R4, R8, R9*) *R* genes, respectively (Kim et al. 2012). Knowing which combinations of *R* genes are present in the resistant genotypes, breeders can introgress their favourite *R* genes into the current potato cultivars. The main prerequisite is obvious, namely that the matching *Avr* genes should be known.

4) Exploit effectors for detecting weak resistance phenotypes

For many years, potato breeding has been focussed on the introgression of field resistance, which has been claimed to be more durable in various studies. However, the genetic basis of field resistance has remained unclear for two reasons. One is that the weak phenotypes of field resistance are too difficult to follow in the genetically complex potato breeding. The other reason is that the avirulence profiles of infecting *P. infestans* strains cannot always be accurately determined in routine detached leaf assays for *R* genes with weaker phenotypes (Kim et al. 2012). In such cases, effectors can play an important role. For example, the so-called "field resistance gene" *Rpi-Smira2* in potato cultivar 'Sarpo Mira' could not be distinguished in detached leaf assays with *P. infestans*, but was detected by response to *Avr-Smira2* (Rietman et al. 2012). Thus, field resistance phenotypes, which are often too weak to be reliably detected under laboratory conditions on detached leaves, can be accurately detected by effectors. It shows that effectors can act as functional markers and contribute to more efficient resistance breeding in potato.

5) Expand effector recognition specificity of *R* genes; target for broad-spectrum resistance

Expanding the effector recognition specificity of a given *R* gene to new virulent alleles can further improve breeding for durable resistance breeding. As described above, *R* genes that can target all allelic forms of *Avr* genes could provide a full-spectrum resistance to pathogen isolates. A potential tool to accomplish this goal is the artificial evolution by random mutagenesis, as previously demonstrated for the PVX resistance gene *Rx* (Bendahmane et al. 1999, Farnham and Baulcombe 2006). Whenever the original *R* gene is present in the crop species, one can implement targeted mutagenesis (genome editing) by new technologies, such as zinc finger nuclease–based approaches (Shukla et al. 2009, Townsend et al. 2009) or transcription activator-like (TAL) effectors that can be fused to DNA nucleases to target a precise site in a genome to create genetic variation (Boch et al. 2009, Bogdanove and Voytas 2011). Genome editing could provide a non-transgenic resistant variety that does not carry extraneous pieces

of DNA (Marton et al. 2010). To design an efficient screening system to identify the mutated *R* gene candidates, basic knowledge of the pathogen effectors is a key requirement. Also, the right choice of the R-AVR pair will influence the new *R* gene durability in the field. For instance, as mentioned above, *R3a* but not *R4* would be a good target to manipulate and an engineered *R3a* that also recognizes *Avr3a*^{EM} could provide a broader spectrum resistance than the original *R3a*. Indeed, expanded recognition specificity by a new *R* gene variant could be due to a single aa change in the R protein, as recently discovered for *R3a* (Kamoun et al. 2013).

6) Exploit effectors that are important for *P. infestans*; target for durable resistance

Essential effectors of *P. infestans* are expected to be useful targets for potato resistance breeding. The genome and expression analysis of three *P. infestans* strains shows that only a small subset of 45 RXLR genes is consistently induced *in planta* during the biotrophic infection stage (Cooke et al. 2012). Among those "core effectors", most known *Avr* genes occur, for example the extensively studied *Avr3a*. In *P. infestans* populations, two alleles of *Avr3a* have been identified that encode secreted proteins AVR3a$^{K80/I103}$ (AVR3a^{KI}) and AVR3a$^{E80/M103}$ (AVR3a^{EM}), which differ in two amino acids (aa) in their effector domains (Armstrong et al. 2005). Only AVR3a^{KI} can induce potato resistance to *P. infestans* by activating the resistance protein R3a. Interestingly, AVR3a was recently shown to be essential for full virulence of *P. infestans* (Bos et al. 2010). These findings suggest that *R3a* can be an important target for durable resistance breeding when an *R3a* variant that targets both allelic forms of *Avr3a* can be identified from natural sources (see Do nr 2) or otherwise (see Do nr 5). In contrast, *R* genes such as *R4* are not considered as useful targets for durable resistance breeding because the matching *Avr4* gene encodes dispensable effectors (van Poppel et al. 2008).

In addition to breeding for resistance genes that recognize core RXLR effectors, another layer of resistance in the form of apoplastic receptors offers an alternative for durable resistance breeding. These apoplastic receptors can recognize apoplastic effectors that in some cases represent conserved pathogen associated molecular patterns (PAMPs). Among the best-characterized oomycete PAMPs are elicitins, a conserved family of extracellular proteins that share a 98-amino-acid elicitin domain (Ponchet et al. 1999, Qutob et al. 2003, Jiang et al. 2006, Vleeshouwers et al. 2006). For oomycete species that cannot synthesize sterols, elicitins fulfil an important function as sterol scavengers. Therefore, we argue that receptors of PAMPs like elicitins can be good targets for breeding durable resistant potato.

7) Assist in *R* gene deployment in agriculture

Monitoring effector allelic diversity in pathogen populations can improve the spatio-temporal deployment of *R* gene-based disease resistance. Functional profiling of *Avr* genes in local *P. infestans* populations can inform about the distribution of virulence alleles. This information can help breeders evaluate the potential of a given *R* gene. Moreover, it will help breeders to detect the emerging virulent races of *P. infestans* before they reach epidemic proportions. Normally, primary inoculum will increase

during the following season along with genetic adaptation and selection and thus accelerate the emergence of highly aggressive clones. Once detected, breeders can choose appropriate cultivars to buffer the occurrence of the clone to manage the epidemics, or apply instant chemical control measures. With *P. infestans* genome sequences, next-generation sequencing technologies and sensitive tools based on e.g. real-time PCR, it is now possible to rapidly profile the effector repertoires of emerging *P. infestans* genotypes. This effector profiling can assist in decision making for *R* gene deployment and chemical control measures in current and subsequent potato growing seasons.

Don'ts

1) Don't give up too fast
2) Don't conclude too fast on AVR activity
3) Don't conclude too fast on *R* gene identity

1) Don't give up too fast

As described above, some plant genotypes can give nonspecific responses to agents like *Agrobacterium*, which is in fact also a pathogen on a wide range of plants (Figure 4A). Also, some germplasm may not be amenable to *Agrobacterium*-mediated transient transformation. Especially in plant breeding, when working with a wide diversity of plant germplasm, such issues can occur at a certain frequency. Don't give up. One can easily generate alternative plant genotypes that do not suffer from these matters and respond to controls as they should. This procedure to generate and select suitable plant genotypes is presented in Figure 4B.

2) Don't conclude too fast on AVR activity

Proteins that show cell death in transient assays do not necessarily have avirulence activity in the natural potato-*P. infestans* interaction. Other characteristics such as level and timing of expression or localization to different sites in the host also influence whether the proteins act as avirulence determinants (Schornack et al. 2009). For example, effector gene *PEX147-3* is a close relative of *Avr3a* that induces cell death with *R3a* by agro-co-infiltration (Bos 2007), but is not expressed during infection (Armstrong et al. 2005) and not likely to represent a true *Avr* gene. Also, for example some *crinklers* (necrosis-inducing proteins) induce cell death in a broad range of plants (and *Crn2* is therefore often used as a positive control) (Torto et al. 2003). Some of these proteins target the host nucleus where the outcome of the plant-microbe interaction is determined, but avirulence activity has not been detected (Torto et al. 2003, Huitema et al. 2004, Schornack et al. 2009). To draw conclusions about presumed AVR activity, one should perform follow-up experiments with independent assays. We often use genetic studies for co-segregation of response to the effector with resistance to *P. infestans* in a segregating population (Figure 4C). In addition, co-infiltration of *R* genes and candidate *Avr* genes can

provide complementary information (Figure 4D). Complementation studies in *P. infestans* will provide the ultimate answer on avirulence activity (Gilroy et al. 2011).

3) Don't conclude too fast on *R* gene identity

Once specific responses to an AVR protein are detected (Figure 4A) and have been confirmed with independent assays (Figure 4C and D), we can carefully conclude that the tested plant contains an *R* gene that matches the inoculated *Avr* gene. In functional allele mining studies, when an *Avr* gene is screened on diverse plant germplasm (see Do nr2), identified specific resistance responses are most likely due to homologs of the known *R* gene (Vleeshouwers et al. 2008). However, it cannot be excluded that one effector interacts with different families of *R* genes, similarly as one *R* gene can interact with different effectors (Vos et al. 1998, Lozano-Torres et al. 2012, Angel and Schoelz 2013). Therefore, independent experiments are required to confirm the identity of detected *R* genes.

Conclusions

Effectoromics provides innovative advantages that contribute to resistance breeding and *R* gene deployment. We summarize seven advantages in the *Do's*, and basically we classify them in four themes, i.e. acceleration, distinguishing functional redundancy, detecting specificity and deployment. *Don'ts* are less frequent than *Do's*. Apart from the prerequisite that sequence libraries should be available to apply effectoromics, the three presented *Don'ts* are just some practical notes to draw scientifically sound conclusions from phenotyping exercises. Ironically, these *Don'ts* mainly advocate patience during the experiments, whereas the effectoromics strategy in general focusses on enhancing the speed of *R* and *AVR* gene identity.

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Chapter 3

Agroinfiltration and PVX agroinfection in potato and *Nicotiana benthamiana*

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Agroinfiltration and PVX agroinfection in potato and *Nicotiana benthamiana*

Abstract

Agroinfiltration and PVX agroinfection are two efficient transient expression assays for functional analysis of candidate genes in plants. The most commonly used agent for agroinfiltration is *Agrobacterium tumefaciens*, a pathogen of many dicot plant species. This implies that agroinfiltration can be applied to many plant species. Here, we present our protocols and expected results when applying these methods to the potato (*Solanum tuberosum*), its related wild tuber-bearing *Solanum* species (*Solanum* section *Petota*) and the model plant *Nicotiana benthamiana*. In addition to functional analysis of single genes, such as resistance (*R*) or avirulence (*Avr*) genes, the agroinfiltration assay is very suitable for recapitulating the R-AVR interactions associated with specific host pathogen interactions by simply delivering *R* and *Avr* transgenes into the same cell. However, some plant genotypes can raise nonspecific defense responses to *Agrobacterium*, as we observed for example for several potato genotypes. Compared to agroinfiltration, detection of AVR activity with PVX agroinfection is more sensitive, more high-throughput in functional screens and less sensitive to nonspecific defense responses to *Agrobacterium.* However, nonspecific defense to PVX can occur and there is a risk to miss responses due to virus-induced extreme resistance. Despite such limitations, in our experience, agroinfiltration and PVX agroinfection are both suitable and complementary assays that can be used simultaneously to confirm each other's results.

Introduction

Effectoromics, a high-throughput functional genomics approach has recently emerged as a powerful tool to identify resistance (*R*) genes in crop plants and matching avirulence (*Avr*) genes of pathogens (Vleeshouwers et al. 2008, Ellis et al. 2009, Oh et al. 2009, Vleeshouwers et al. 2011). In contrast to the more time-consuming stable transformation with *R* genes, the effectoromics strategy is based on transient assays of pathogen gene sequences.

Since the genomics era, genomes of plant pathogens have widely been explored. For example for oomycetes, which include the most devastating plant pathogens, large collections of sequences have been generated and analyzed for genes that play a role during the interaction with the plant (Tyler et al. 2006, Lamour et al. 2007, Haas et al. 2009, Levesque et al. 2010, Kemen et al. 2011, Stassen et al. 2012). One class of pathogen proteins represents effectors, which manipulate host cell structure and function either to facilitate infection (virulence factors) or to trigger defense responses (avirulence

factors) (Kamoun 2006, Hogenhout et al. 2009, Win et al. 2012). Expression of *Avr* genes in plant cells containing *R* genes usually results in the hypersensitive cell death response (HR) (Dangl and Jones 2001; van der Hoorn et al. 2000). *In planta* expression of *R* and *Avr* genes can be accomplished using transient expression systems such as *Agrobacterium tumefaciens*-based transient transformation (agroinfiltration) (Kapila et al. 1997). This transient transformation can also be applied in combination with viral expression systems (agroinfection) (Kanneganti et al. 2007, Vleeshouwers and Rietman 2009).

For agroinfiltration, the most commonly used agent is *A. tumefaciens*, a broad host range pathogen of dicot plants. *A. tumefaciens* contains a tumor-inducing (Ti) plasmid. Transfer DNA (T-DNA) from a Ti plasmid will translocate into the plant cells after the virulence machinery of the bacterium is activated. This can be triggered in wounded plant cells, by the released low-molecular-weight phenolic compounds and monosaccharaides in a slightly acidic environment (Peng et al. 1998). The virulence gene is activated after the infiltration of *Agrobacterium* suspensions into leaf panels defined by major veins. Then plant cells in the leaf panels will be transformed and express the transgene(s) contained in the T-DNA region.

Agroinfection is based on wound-inoculated *Agrobacterium*, which mediates translocation of a virus to plant cells. The virus than further spreads to adjacent plant tissues, in the absence of *Agrobacterium*. For agroinfection, several plant viruses can be used. RNA viruses are ideal vectors for gene expression because they can multiply to very high levels in infected plants. Among plant RNA viruses, *Potato Virus X* (PVX) is widely used for effectoromics screens. To facilitate functional tests for an inserted gene, binary vectors that contain the PVX genome flanked by the Cauliflower mosaic virus 35S promoter and the nopaline synthase terminator, were cloned into the T-DNA of *A. tumefaciens* (Lu et al. 2003). After the T-DNA is transferred into plant cells, the PVX genome contained in the T-DNA is transcribed from the 35S promoter. Then virus particles spread systemically in the infected plants, resulting in the expression of the inserted gene. This method based on both *Agrobacterium* and PVX is called PVX agroinfection.

Here we show examples for both the agroinfiltration and PVX agroinfection assays. As host plants we use potato germplasm (*Solanum* section *Petota*), for which effectoromics approaches have been pioneered and proven successful (Vleeshouwers et al. 2008, Oh et al. 2009). We also use *Nicotiana benthamiana*, which is renowned as a model plant in *Solanaceous* plants (van der Hoorn et al. 2000, Bhaskar et al. 2009, Ma et al. 2012).

Protocol

1. Plant growing and testing conditions

Grow and maintain plants in controlled greenhouses or climate chambers within the temperature range of 18-22 ℃ and under natural light regime or with a 16 hours/8 hours day/night regime. Remove axillary branches in order to make the plants more manageable.

For potato, maintain *in vitro* plantlets in sterile plastic jars containing *Murashige-Skoog* (MS) medium (20 g/L sucrose, 5 g/L MS salts with vitamins, 8 g/L agar, pH 5.8) under controlled conditions in climate chambers at 18 ℃ with a 16 hours/8 hours day/night regime for two weeks and then transfer them into pots of sterilized soil in regulated greenhouse compartments.

2. Agroinfiltration

A. Plant material

For *Nicotiana benthamiana*, use around 4-5-week-old seed-grown plants. For potato, use around 4-5-week-old transplants from *in vitro* shoots. Choose young, healthy and fully developed leaves for infiltrations.

B. *Agrobacterium* culture

1) Prepare the YEB medium in advance (Table 1). Fill the 50 ml tubes with 10 ml YEB medium supplemented with 1 µl acetosyringone (200 mM), 100 µl MES buffer (2-(N-morpholino)-ethane sulfonic acid, 1 M) and the appropriate antibiotics. Pipet 20 µl glycerol stock of the desired strains (Table 2) (containing the gene of interest) into the YEB. Initiate cultures for all the *Agrobacterium* strains in this assay at the same time. Incubate cells by shaking for 1-2 days at 28 ℃ and 200 rpm to an OD_{600} of approximately 1.0.
2) Harvest cells by centrifugation at 3 000 x g for 10 min. Pour off the supernatant and resuspend the pellet in freshly made MMA medium (Table 3) to an OD_{600} of 0.3. Cells should be gently vortexed to resuspend them. For co-infiltration of two bacterial strains, mix the culture in a 1:1 ratio.
3) Leave the culture on the bench at room temperature for 1-6 h before infiltrations. In the meantime, label the plants to be infiltrated and the date of the experiment.

C. Infiltrations

Use a 1 ml needleless syringe to infiltrate the *Agrobacterium* suspensions. Carefully and slowly inject leaf panels with the suspensions from the syringe (for health and safety reasons eye protection should be worn during this process). Infiltrate at least three plants for each strain. Use three leaves per plant to serve as triplicates. Note: Normally, 1 ml of *Agrobacterium* suspensions could be enough for 3 plants of *N. benthamiana*. For potato, more suspension is needed because that leaves of *Solanum* species are more difficult to infiltrate. Required volumes of suspensions depend on the

Solanum species. Avoid cross-contamination by changing gloves or sterilizing gloves with ethanol between infiltrations and by not watering the plants until the next day after the inoculation.

D. Scoring

Score macroscopic responses about 3 days after infiltration when the cell death phenotype is clear (Figure 1). If the cell death phenotype is quantitative, then use the given criteria (Figure 2). Briefly, the scales for macroscopic scoring of cell death mainly depend on the cell death percentages of the infiltrated area. Percentages of cell death are depicted on a scale from 0% (no symptoms) to 100% (confluent cell death). Intermediate responses range from weak responses such as chlorosis to increasing levels of cell death. If desired, the macroscopic scoring can also be quantitatively assessed using modern photo-imaging equipment.

3. PVX agroinfection

A. Plant material

For *N. benthamiana*, use around 2-3-week-old seed-grown plants. For potato, use around 2-3-week-old transplants from *in vitro*. For large-scale tests, use slightly older (4-5 weeks) plants, as these plants have more and larger leaves to accommodate higher numbers of *Agrobacterium* inoculation spots.

B. *Agrobacterium* culture

1) Prepare the YEB medium in advance. Fill the 10 ml tubes with 3 ml YEB supplemented with the appropriate antibiotics. Pipet 20 µl glycerol stock of the desired strains (Table 2) (containing the gene of interest) into the YEB. Initiate cultures for all the *Agrobacterium* strains in this assay at the same time. Incubate cells by shaking for 1-2 days at 28 ℃ and 200 rpm to an OD_{600} of approximately 1.0.
2) Pipet about 300 µl of each *Agrobacterium* strain and spread them onto LB solid agar medium plates supplemented with the appropriate antibiotics and incubate cells at 28 ℃ for 1-2 days.

C. Infections

Use a spatula to collect the *Agrobacterium* culture in the plate. Dip a wooden toothpick in the *Agrobacterium* culture on the spatula and pierce the leaves to inoculate large amount of bacteria. Inoculate at least three plants for each strain. Use three leaves per plant to serve as triplicates. In each leaf, make multiple inoculation sites for each strain.

D. Scoring

Score macroscopic responses about two weeks after inoculation (Figure 3). For high-throughput screens, record the qualitative responses (yes/no) for each inoculation spot. Then calculate the percentage of responding sites and compare them with controls.

Representative results

Figure 1 shows a representative experiment of agroinfiltration with 7 different effectors (E1-E7) in potato and *N. benthamiana*. Cell death appears in the infiltrated leaf panels about 3 days after infiltration. The extent of the cell death phenotype needs to be compared with the controls. A mixture of the *Agrobacterium* strain AGL1 (pVirG) (van der Fits et al. 2000) containing pBINplus-R3a and pK7WG2-AVR3a was used as positive control (Armstrong et al. 2005, Huang et al. 2005), while AGL1 (pVirG) was used as negative control. **Figure 1A** and **Figure 1B** show good examples of agroinfiltration in the potato genotype MaR8 (Mastenbroek R8) (Kim et al. 2012) and *N. benthamiana*, respectively, which are very amenable for agroinfiltration. There is confluent cell death in the leaf panel co-infiltrated with positive control, while no cell death response occurs with negative control or pBINplus-R3a. In MaR8, two effectors AVR3a (E2) and AVR4 (E3) induce a cell death, while the other effectors (E1 and E4-E7) do not. In *N. benthamiana*, none of the tested effectors induce a cell death response. **Figure 1C** and **Figure 1D** show examples of agroinfiltration in the wild potato *Solanum berthaultii* 483-1 and *Solanum rechei* 210-5, which are not well amenable for the agroinfiltration technique. In *S. berthaultii* 483-1, the leaf tissue shows a nonspecific necrosis to negative controls as well as to all tested effectors. In *S. rechei* 210-5, infiltrated leaf panels show very weak cell death response to positive control.

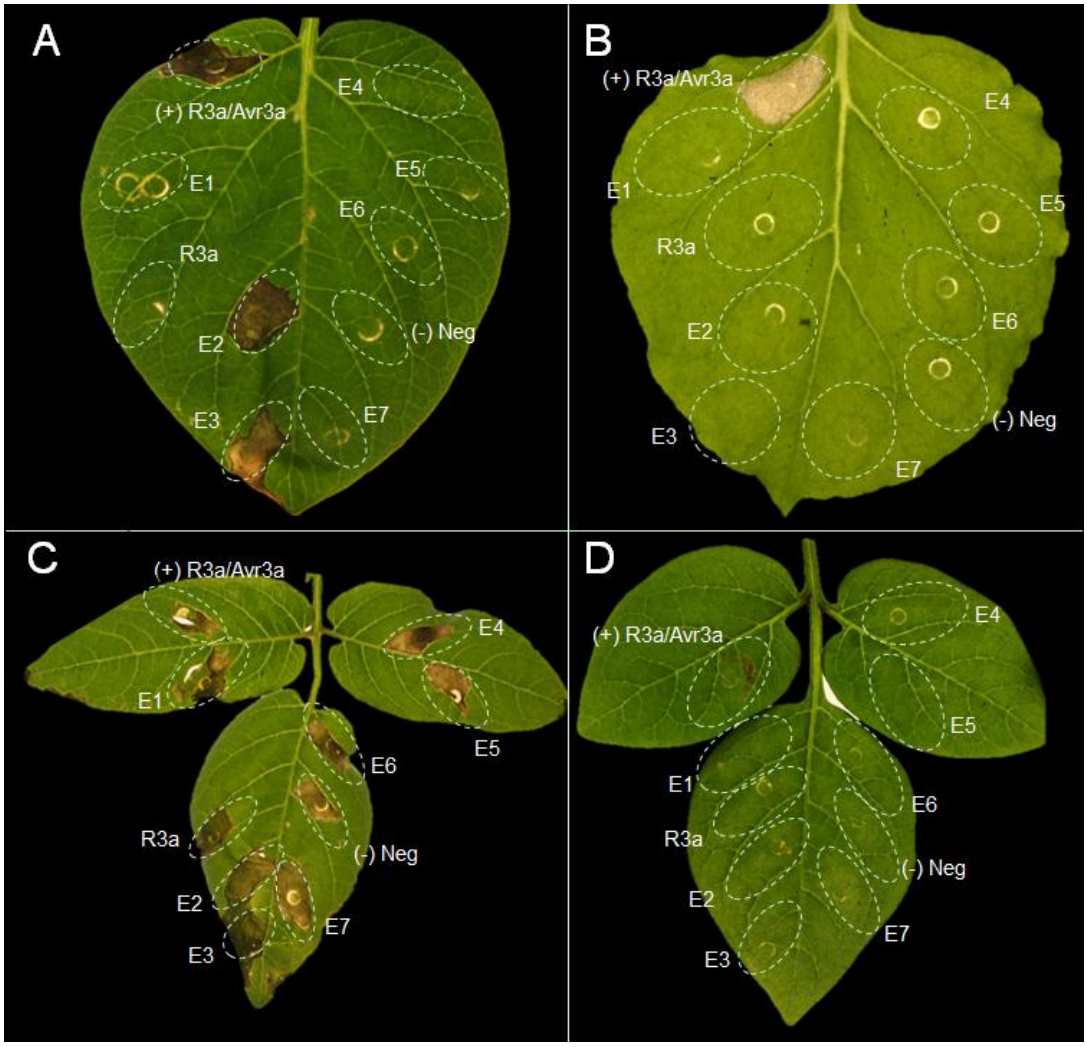

Figure 1. Examples of agroinfiltration in potato and *N. benthamiana*

Plants including **(A)** a potato genotype MaR8 (Mastenbroek R8), **(B)** *N. benthamiana*, **(C)** *Solanum berthaultii* 483-1 and **(D)** *Solanum rechei* 210-5 were infiltrated with a mixture of the *Agrobacterium* strain AGL1(pVirG) (van der Fits et al. 2000) containing pBINplus-R3a and pK7WG2-AVR3a (positive control), AGL1(pvirG) (negative control), pBINplus-R3a, and seven effectors (E1-E7).

Figure 2 shows a range of scoring scales that can be used to quantify the response to agroinfiltrated *Agrobacterium*. Percentages of cell death are depicted on a scale 0% – 100%. Observed phenotypes range from macroscopically not visible symptoms (0%), through a range of intermediate responses displaying chlorosis and increasing levels of cell death, up to confluent cell death (100%).

0 25% 50% 75% 100%

Figure 2. Quantification of agroinfiltration responses

The photograph shows representative scoring scales for cell death, ranging from 0% (no symptoms) to 100% (confluent cell death). Intermediate responses range from weak responses such as chlorosis to increasing levels of cell death.

Figure 3 shows a representative experiment after PVX agroinfection in potato. Normally, expanding cell death can be found at the sites about two weeks after tooth-pick inoculation. As shown in both Figure panels, expanding cell death is present at the sites that were tooth-pick inoculated with pGR106-CRN2 (positive control using the effector crinkler Crn2), which is a general cell death inducing gene from *P. infestans* (Torto et al. 2003). Apart from minor response to wounding, no expanding cell death is noted at the sites that were tooth-pick inoculated with the pGR106-empty (negative control). In **Figure 3A**, representing the potato genotype MaR3 (Mastenbroek R3), two pGR106-effectors (E1 and E2) did not induce cell death. In **Figure 3B**, a positive result of effector screening in the wild *Solanum huancabambense* 354-1 is presented; cell death response was observed to two pGR106-effectors (E1-E3, representing elicitins) (Vleeshouwers et al. 2006).

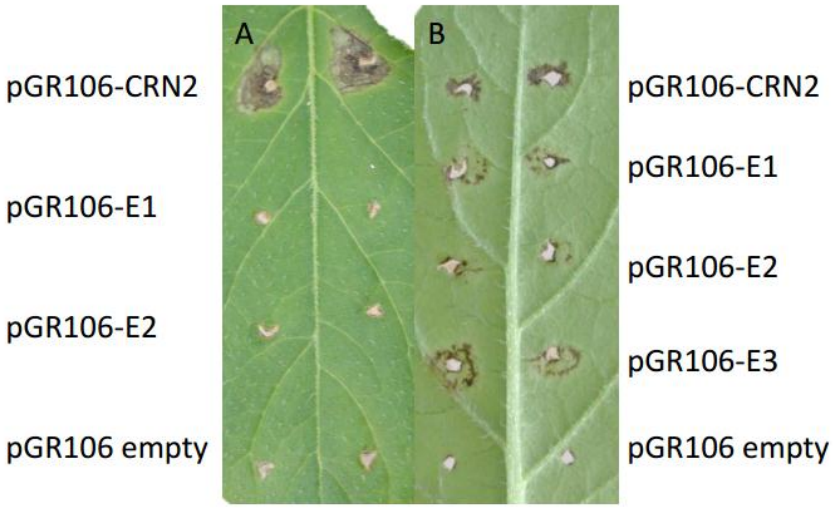

Figure 3. Examples of PVX agroinfection in potato

Potato genotype MaR3 (Mastenbroek R3) (A) and *Solanum huancabambense* 354-1 (B) tooth-pick inoculated with pGR106-CRN2 (positive control), pGR106-empty (negative control) and pGR106-E1-2 (effectors), or pGR106-E1-E3, respectively.

Table 1. YEB medium

1 L	distilled H2O
5 g	sucrose
5 g	beef extract
5 g	bacteriological peptone
1 g	yeast extract
2 ml	$MgSO_4$ (1M)

Table 2. Vectors and strains used for agroinfiltration and PVX agroinfection

Several binary vectors can be used. Vectors in the list below allow high expression of the candidate genes and have worked well in our hands. We prefer using the *A. tumefaciens strain* GV3101 (pMP90) (Koncz and Schell 1986) in *N. benthamiana* and find AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG (pBBR1MCS-5.virGN54D) (van der Fits et al. 2000) more suitable in potato (Rietman 2011). Additional strains have been analyzed in other groups on various model plants including *N. benthamiana* but not in potato (Wroblewski et al. 2005).

Assay	**Vector**	**Reference**	**Antibiotics for vector selection (50 µg/ml)**
Agroinfiltration	pBINplus (GW)	(van engelen et al. 1995)	Kanamycin
	pK7WG2 (GW)	(Karimi et al. 2002)	Spectinomycin
	pCB302-3 (GW)	(Xiang et al. 1999)	Kanamycin
	pMDC32 (GW)	(Curtis and Grossniklaus 2003)	Kanamycin
PVX agroinfection	pGR106	(Lu et al. 2003)	Kanamycin

GW: gateway version (Curtis and Grossniklaus 2003).

Table 3. MMA medium

1 L	distilled H2O
20 g	sucrose
5 g	MS salts (without vitamins)
1.95 g	MES
2 ml	NaOH (1M)
1 ml	Acetosyringone (200 mM)

Adjust the pH to 5.6.

Discussion

Transient assays like agroinfiltration and agroinfection are efficient methods that are vital to modern molecular plant pathology research. Despite some limitations, these methods meet the demand for efficient and robust high-throughput functional analysis in plants.

The agroinfiltration system is a widely used functional assay in a range of plant species. Agroinfiltration facilitates the delivery of several transgenes into the same cell with simultaneous expression of interacting proteins. This is advantageous for recapitulating R-AVR relationships, by co-infiltrating *Agrobacterium* strains that express *Avr* genes with strains that express the matching *R* genes. Also, for known R-AVR pairs, such co-infiltrations can be used as positive controls. Including such controls is important because in some plant genotypes, the transformation efficiency can be below the threshold to detect responses. Including negative controls, e.g. an *Agrobacterium* strain containing a vector without a gene insert, is also essential to determine whether a certain plant genotype raises nonspecific defense responses to the *Agrobacterium*. This feature occurs at a certain frequency in potato germplasm, and not all *Solanum* species are well suitable for this *Agrobacterium*-based expression system. Generally, the agroinfiltration assay works very well in *N. benthamiana* and most potato genotypes. In addition to effectoromics, there are various other potential applications for the agroinfiltration technique, such as production of proteins from transgenes and protein localization in plant cells by confocal microscopy.

PVX agroinfection is a highly sensitive screening system and typically more suitable for high-throughput screenings. Since the *Agrobacterium* is now only locally present, nonspecific responses to this bacterium are now not very disturbing, as the PVX virus takes over further spread of the transgene. However, plants may be resistant to PVX, or mount extreme resistance (ER) responses, and in that case the agroinfection method is not suitable. Another limitation of the PVX agroinfection method is the insert size of the gene of interest. Observed phenotypes of responses may vary from an intense black necrosis surrounding the wound to faint necrosis near the inoculation spot. In both *N. benthamiana* and *Solanum* species, PVX agroinfection is recognized as more sensitive than agroinfiltration.

Keeping into account that the genetic background of the diverse tested plant genotypes can have some restrictions (see above), we generally obtain similar conclusions by PVX agroinfection and agroinfiltration. These results are also comparable as obtained in other assays, such as protein infiltrations (Vleeshouwers et al. 2006) and ELISA (Vleeshouwers et al. 2008). Considering the advantages and limitations of both systems, we recommend using both methods to either complement each other or confirm independent results.

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Chapter 4

Elicitin recognition confers enhanced resistance to the Irish potato famine pathogen

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Elicitin recognition confers enhanced resistance to the Irish potato famine pathogen

Abstract

Potato late blight, caused by the destructive Irish famine pathogen *Phytophthora infestans*, is a major threat to global food security. All known late blight resistance genes identified to date belong to the coiled-coil, nucleotide-binding, leucine-rich repeat class of intracellular immune receptors. However, virulent races of the pathogen quickly evolved and evade recognition by these cytoplasmic immune receptors. Here we demonstrate that the receptor-like protein ELR from the wild potato *Solanum microdontum* mediates extracellular recognition of the elicitin domain, a molecular pattern that is conserved in *Phytophthora* species. ELR associates with the immune co-receptor BAK1/SERK3 and mediates broad-spectrum recognition of elicitin proteins from several *Phytophthora* species, including four diverse elicitins from *P. infestans*. Transfer of ELR into cultivated potato resulted in enhanced resistance to *P. infestans*. Pyramiding cell surface pattern recognition receptors with intracellular immune receptors maximizes the potential of generating a broader and potentially more durable resistance to this devastating plant pathogen.

One Sentence Summary

The cell surface receptor-like protein ELR recognizes the elicitin molecular pattern of oomycetes and enhances resistance to the Irish potato famine pathogen *Phytophthora infestans* in potato.

Potato (*Solanum tuberosum* L.) is the most important non-grain food crop and a major source of calories for the world's poor. Increasing potato production is critical to prevent global malnutrition and hunger in an era of expanding world population (Fisher et al. 2012). Unfortunately, potato suffers from the devastating late blight disease which is caused by the notorious oomycete pathogen *Phytophthora infestans* (Fry 2008). To limit losses to late blight, potato breeders rely on fungicide treatment and breeding of disease resistance (*R*) genes, all of which belong to the coiled-coil, nucleotide-binding, leucine-rich (CC-NB-LRR) class of immune receptors (Vleeshouwers et al. 2011). These intracellular proteins recognize pathogen avirulence (*Avr*) proteins of the RxLR class of effectors to mount defense responses. However, RxLR effectors display high evolutionary rates (Raffaele et al. 2010), and as a result, *P. infestans* can rapidly circumvent recognition by intracellular R immune receptors thereby limiting the development of sustainable and durable genetic resistance. Therefore, novel types of immune receptors that recognize a broader spectrum of pathogen molecules are needed.

To fend off pathogens, plants rely on two classes of immune receptors that either reside inside the plant cell (NB-LRRs) or on the cell surface. The first line of defense is initiated by surface receptors, also called pathogen recognition receptors (PRRs). PRRs are typically receptor-like proteins (RLPs) or receptor-like kinases (RLKs) that recognize conserved pathogen-associated molecular patterns (PAMPs) (Zipfel and Robatzek 2010). So far, only a relatively few cell surface receptors against agronomically important pathogens have been identified.

Elicitins are structurally conserved extracellular proteins in *Phytophthora* and *Pythium* pathogen species (Pfam PF00964) (Ponchet et al. 1999, Jiang et al. 2006, L évesque et al. 2010). *P. infestans* contains six elicitin genes that are conserved among different strains (Jiang et al. 2006, Cooke et al. 2012). Elicitins are recognized as oomycete PAMPs but their intrinsic function in oomycetes is to bind lipids. Some elicitins sequester sterols from plants thereby fulfilling an important biological function in *Phytophthora* and *Pythium* species that cannot synthesize sterols (Ponchet et al. 1999). Targeting such conserved "Achilles heel" proteins of pathogens is expected to lead to a more broad-spectrum resistance.

To identify novel types of immune receptors against the potato late blight pathogen, we initiated the cloning of *ELR*, a gene that determines response to elicitins (Vleeshouwers et al. 2006). We screened a collection of wild *Solanum* germplasm by *Potato virus X* (PVX) agroinfection for responses to INF1, a secreted elicitin of *P. infestans* (**Figure 1A**). *Solanum microdontum* genotype 360-1 consistently responded to INF1 with a cell death response (**Figure 1B**). We crossed mcd360-1 with *S. microdontum* ssp *gigantophyllum* 714-1 that does not respond to INF1. The F1 population segregated for response to INF1 in a 1:1 ratio, which suggests that ELR is a single dominant gene.

Genetic mapping placed ELR on the South Arm of chromosome 12 of potato, in a region corresponding to contig 167 in the reference potato genotype RH (**Figure S1A, Table S1)**. BAC end sequences were used to develop CAPS markers, Rhl8 and Rhr0 of RH (van Os et al. 2006, Xu et al. 2011), and a co-

localizing tomato BAC clone sequence led to marker LBC (**Figure S1B**). The RH contig 167 spans the ELR locus and contains sequences with homology to RLPs (**Figure S1C**). By screening a BAC library of mcd360-1 with Rhl8, Rhr0 and LBC, four BAC clones that cover a major part of the Rhl8-Rhr0 interval were identified (**Figure S1D**). Sequencing of these BACs revealed a cluster of 13 RLPs. Further fine mapping ultimately resulted in two candidate genes, RLP85 and RLP207 (**Figure 1C, Figure S1E**).

To determine whether RLP85 or RLP207 confer recognition of INF1, transient expression assays were performed in *Solanum hjertingii* 349-3. *Agrobacterium tumefaciens*-mediated co-expression (agroinfiltration) of RLP85 with INF1 led to cell death, whereas RLP207 did not (**Figure 1D**). We generated stable transgenic potato cultivar Désirée plants expressing RLP85 and RLP207 and subjected the regenerants to PVX agroinfection with INF1. Désirée-RLP85 transgenic plants, but not Désirée-RLP207, showed specific cell death to PVX-INF1 (**Figure 1E)**. We designated RLP85 as ELR. The *Elr* gene has an ORF encoding a protein of 1094 amino acids predicted to contain a signal peptide, 36 extracellular LRR domains, a transmembrane domain and a short cytoplasmic tail (**Figure S2**). The ELR protein shares between 28 and 36% amino acid sequence identity with tomato RLP proteins implicated in immunity to fungal pathogens, namely Cf4/Cf9, Cf2/Cf5, Ve1 and LeEix1.

To study the recognition spectrum of ELR, we explored its ability to induce cell death in response to a broad range of elicitins of oomycetes that infect various host plants (**Figure 2A-C**). We tested six *Inf* elicitins of *P. infestans*, two elicitin-likes, seven elicitins from six other *Phytophthora* species and one from *Pythium ultimum* (**Table S2**). PVX agroinfection in Désirée-ELR#34 showed high (>40%) frequencies of cell death for four elicitins, i.e. INF1, INF2A, INF5 and INF6 of *P. infestans* and to eleven elicitins of diverse other *Phytophthora* species (**Figure 2D, Figure S3)**. This broad spectrum of elicitin recognition by ELR was confirmed by agroinfiltration assays (**Figure S4**). Remarkably, the amino acid sequence of the four recognized elicitins of *P. infestans* is diverse, ranging from as low as 45% to 65% sequence identity (**Figure S3**). In addition, these four elicitins are all expressed during infection in all examined *P. infestans* strains suggesting that they are potentially recognized by ELR during host colonization (**Figure S5**). These data show that ELR induces defense responses upon recognition of a broad spectrum of elicitins, even extending beyond the species of *P. infestans*.

A central regulator of PTI in plants is the BRI1 associated kinase 1 (BAK1), also known as SERK3 in solanaceae plants. This cell surface protein undergoes complex formation with PRRs upon ligand binding (Chinchilla et al. 2007, Heese et al. 2007). Response to INF1 is dependent on BAK1/SERK3 (Chaparro-Garcia et al. 2011). Co-immunoprecipitation experiments revealed that various GFP-tagged ELR fusion proteins associate with BAK1/SERK3 homologs from potato and Arabidopsis (**Figure 3, Figure S6).** The association can be enhanced after elicitation with INF1, suggesting that ELR might associate in an activated receptor complex, similar to enhanced FLS2 association with BAK1/SERK3 after treatment with flagellin flg22 (Chinchilla et al. 2007). Control experiments showed that ELR-eGFP was still able to induce INF1-mediated cell death (**Figure S7**). eGFP- and GFP-tagged ELR

localized to the plasma membrane *in planta* similar to other RLPs, such as Cf9 and LeEIX (van der Hoorn et al. 2001, Bar and Avni 2009) (**Figure S8**). These experiments indicate that ELR most likely functions at the cell surface where it associates with BAK1/SERK3 to activate defense responses.

To test whether ELR enhances resistance to *P. infestans*, we subjected Désirée and seven Désirée-ELR transformants to disease tests. Detached leaves of potato plants were spot-inoculated with zoospore suspensions of two *P. infestans* strains 88069 and EC1_DC2005, in three independent experiments. Lesion sizes were measured from three to six days after inoculation, and lesion growth rates (LGR) were statistically analysed. For both *P. infestans* strains, lesions expanded at slower rates in the Désirée-ELR transformants compared to Désirée and Désirée-RLP207 control plants (**Figure 4, Table S3 - S5).** Independent assessment of pathogen colonization with a *P. infestans* strain 88069tdtom, expressing a variant of the red fluorescent protein, confirmed that ELR confers enhanced resistance to *P. infestans* strains (**Figure S9)**.

Our study demonstrates that the BAK1/SERK3-dependent ELR has the potential to contribute to disease resistance of agronomically important crops. ELR recognizes multiple elicitins, a family of evolutionary conserved proteins under purifying selection, which sharply contrast with the rapidly evolving RxLR effectors that are recognized by NB-LRR immune receptors (Jiang et al. 2006, Haas et al. 2009, Raffaele et al. 2010). Breeding potatoes with immune receptors that target conserved proteins, such as elicitins, is expected to maximize the potential for disease resistance durability. This can efficiently be achieved by effector-based strategies. An attractive approach would be to pyramid the layer of broad-spectrum quantitative resistance conferred by surface receptors, such as ELR, with cytoplasmic NB-LRR receptors that provide a higher degree but narrower spectrum of resistance. In addition, given that ELR recognizes the elicitin molecular pattern of a wide range of *Phytophthora* species, this receptor-like protein could potentially enhance disease resistance to a number of oomycete plant pathogens, many of which are severe threats to a variety of crops and to world food security (Lacombe et al. 2010).

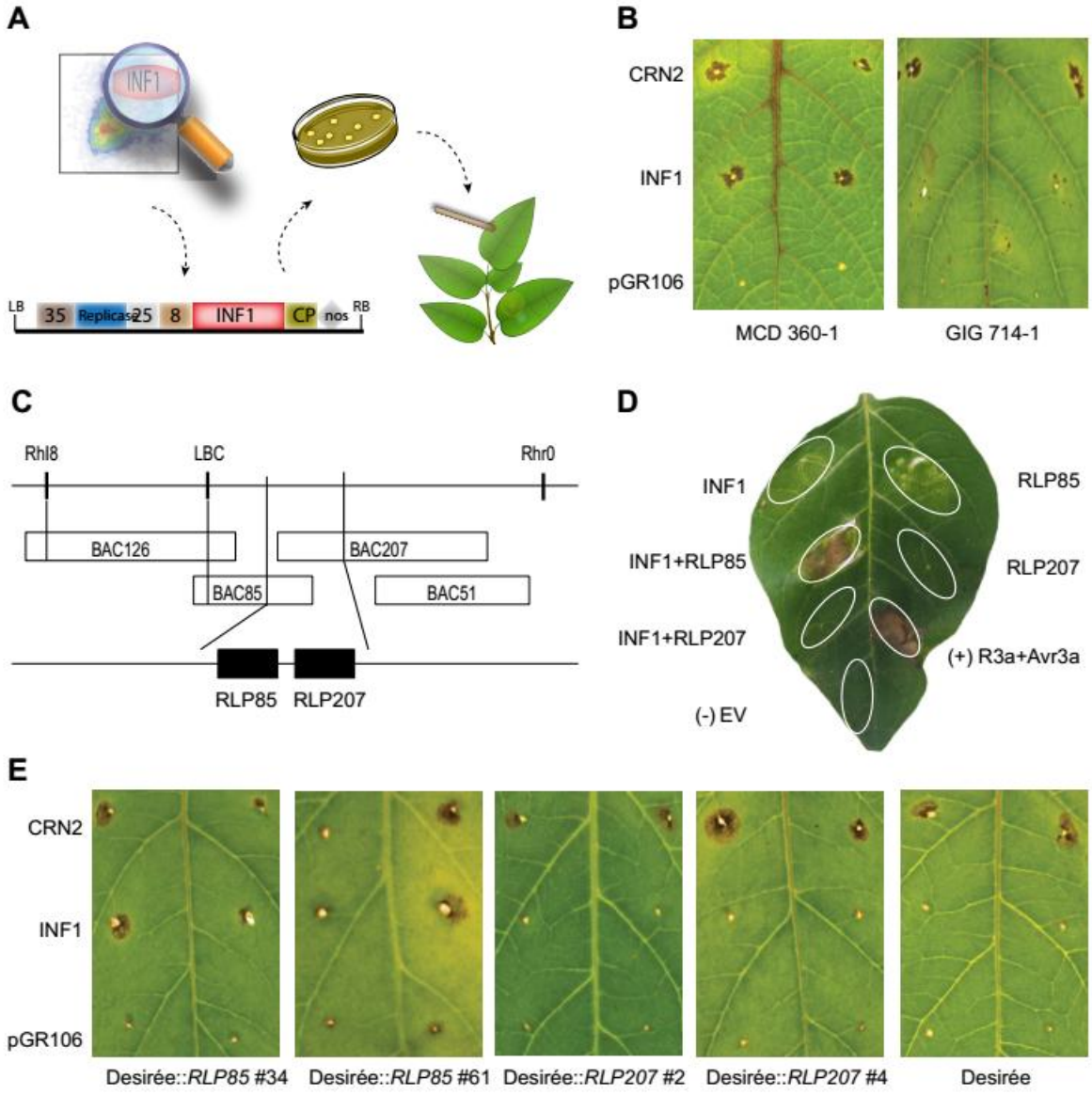


Figure 1. *Solanum microdontum* ELR confers response to elicitins.

(**A**) ELR was identified using effector-based phenotyping (Vleeshouwers et al. 2006). *Inf1* elicitin of *Phytophthora infestans* was transiently expressed by PVX agroinfection in potato germplasm. (**B**) *Solanum microdontum* 360-1 shows cell death response to INF1, *S. microdontum* spp. *gigantophyllum* 714-1 does not. (**C)** Genetic mapping places the gene conferring elicitin response (ELR) on chromosome 12 between flanking markers Rhl8/LBC and Rhr0. RLP85 and RLP207 (RLP85-1 and RLP207-1, respectively in Suppl Figure Mapping ELR) are identified as candidate genes. (**D**) Complementation test in *Solanum hjertingii*. Agro-co-infiltration of INF1 with 35S-RLP85 or with 35S-RLP207 shows that RLP85 induces a specific response to INF1. Photo was taken at 3 days post inoculation. (**E**) Complementation test with PVX-INF1 on transgenic potato cv. Désirée. Two Désirée transformants expressing 35S-RLP85 (ELR#34, ELR#61) show specific cell death responses to pGR106-INF1, two RLP207 transformants and control Désirée do not show responses to INF1.

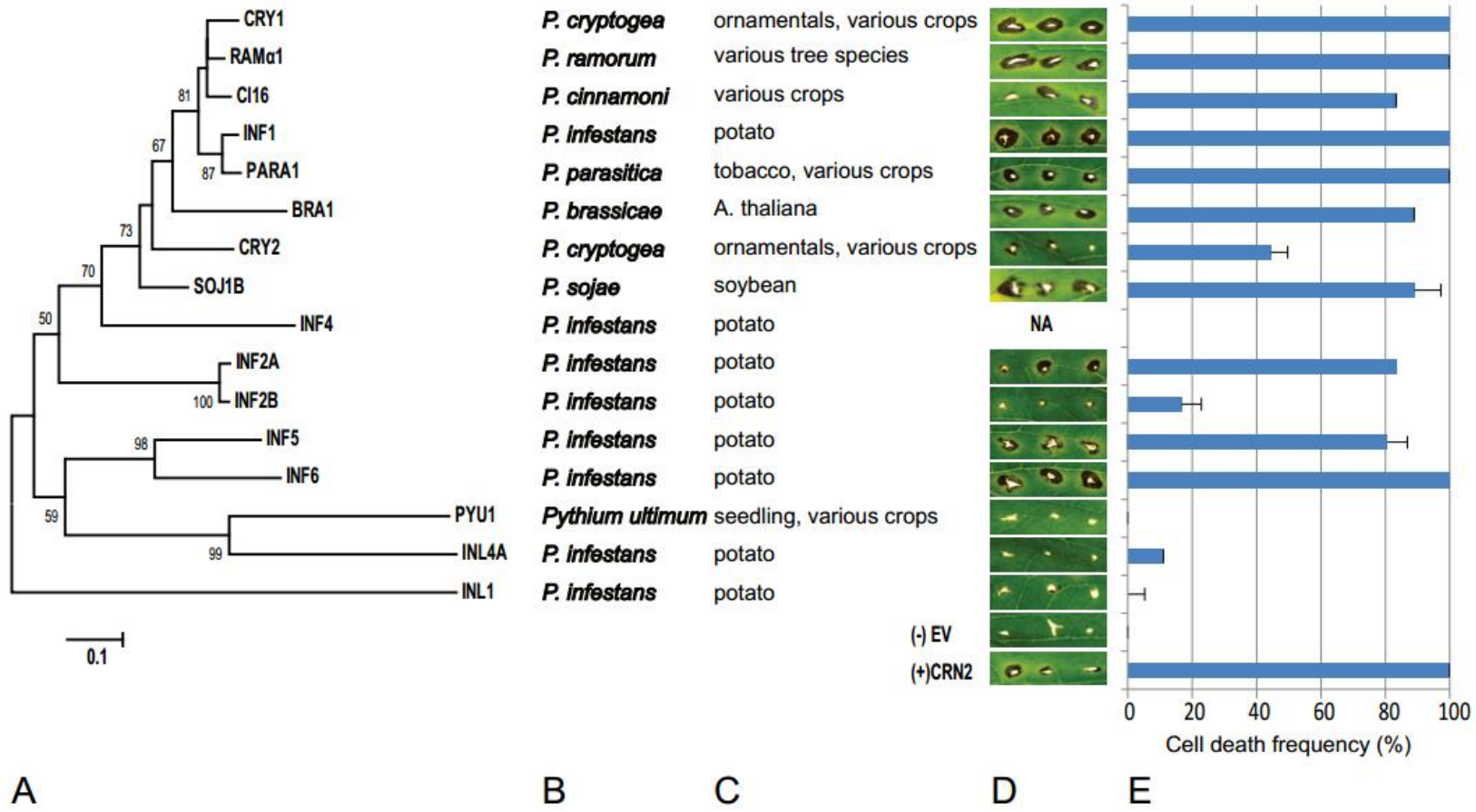


Figure 2. ELR mediates broad-spectrum response to elicitins of oomycetes.

(**A**) Neighbor-joining tree of elicitins. The tree was based on the 98 amino acid elicitin domain that is conserved in oomycete elicitins. Bootstrap values exceeding 50% are indicated at the nodes, branch lengths represent weighted amino acid substitutions. The tested elicitins belong to (**B**) Oomycete species, including various *Phytophthora* species and *Pythium ultimum*. (**C**) Host plants. (**D**) PVX agroinfection with pGR106-elicitins in potato transformant Désirée ELR#34, representative photographs of triplicate inoculations. The empty pGR106 vector and pGR016-CRN2 were used as negative and positive control, respectively. (**E**) Histogram of frequencies of cell death responses upon PVX agroinfection.

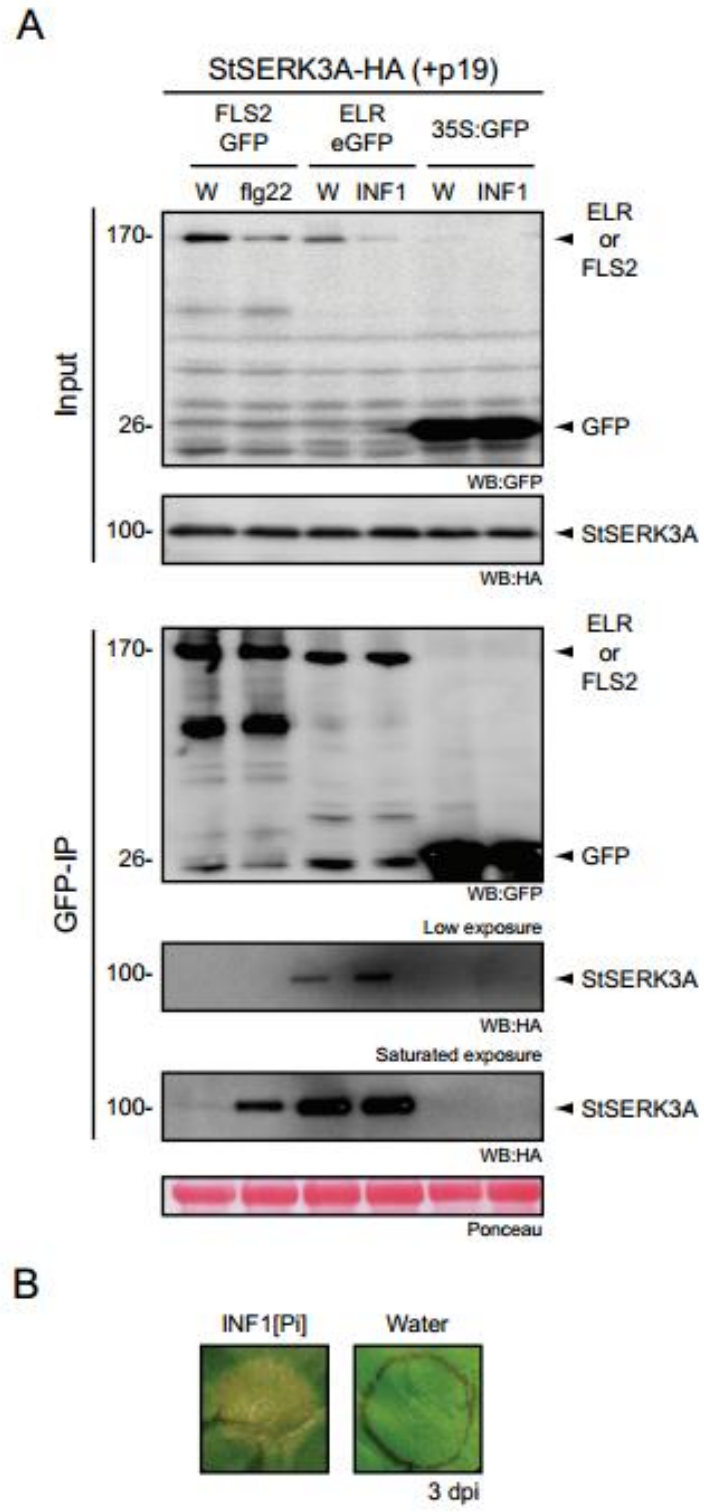


Figure 3. ELR associates with the immune co-receptor BAK1/SERK3.

(**A**) ELR and FLS2 co-immunoprecipitate with StSERK3A and the association is enhanced upon elicitor treatment. ELR-GFP or FLS2-GFP was transiently co-expressed with StSERK3A-HA in *N. benthamiana*. Leaves were treated with water (W), 100 nM flg22 or 10 µg/ml INF1 elicitin for 15 minutes. Proteins were extracted enriching for membrane proteins and subjected to immunoprecipitation (IP) with anti-GFP. Ponceau stain of the total extract blot indicates equal loading (bottom). (**B**) Leaf panels of *N. benthamiana* show cell death to infiltrated INF1 but not to water control at 3 dpi.

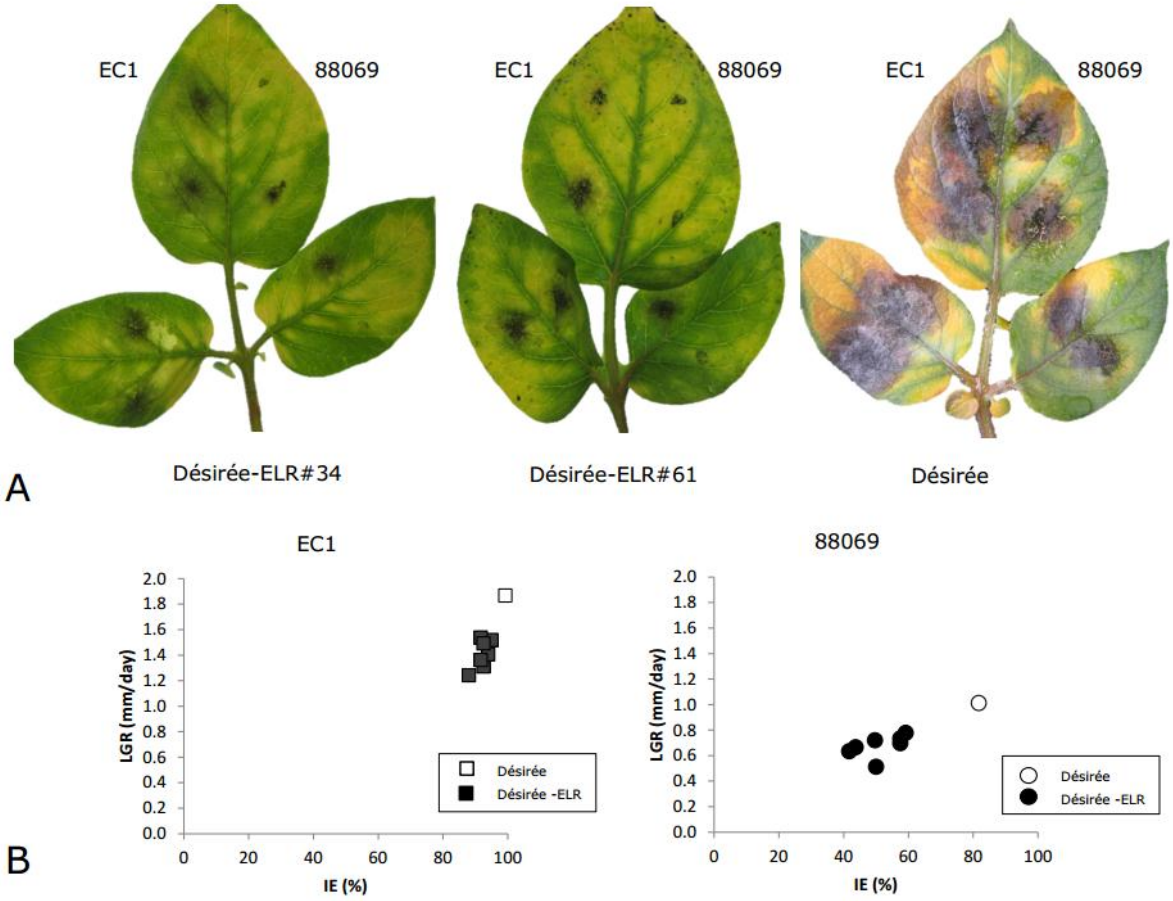


Figure 4. ELR confers enhanced resistance to *P. infestans* in potato.

(A) Désirée-ELR transformants (filled circles) display lower lesion growth rates (LGR) and lower infection efficiencies (IE) than Désirée control (open circles). Leaves were spot-inoculated with two *P. infestans* strains EC1_DC2005 (EC1) and 88069 on the left or right side of the mid vein, respectively. LGR was significantly lower in all seven Désirée-ELR transformants, for both tested *P. infestans* strains (**Table S3, Table S4, Table S5).** The least aggressive *P. infestans* strain 88069 shows less lesions in Désirée-ELR transformants. Representative photographs are shown for Désirée control (**B**), Désirée -ELR#34 (**C**) and Désirée -ELR#61 (D).

Supplementary Materials and Methods:

Plant material

Solanum microdontum (mcd) genotype 360-1 (Vleeshouwers et al. 2006, Vleeshouwers et al. 2011), *S. microdontum* ssp *gigantophyllum* (gig) 714-1 and recombinants of the F1 population 7661 (mcd360-1 x gig714-1) were clonally propagated *in vitro* on MS medium supplemented with 20% sucrose (Murashige and Skoog 1962) at 18 ℃. For experiments, top shoots were transferred to fresh medium at 25 ℃. After allowing 1–2 weeks for root formation, plantlets were transferred to sterilized soil and grown in regulated greenhouse compartments on a 22 ℃/18 ℃ day/night regime. Well-controlled greenhouse conditions were found essential for obtaining good quality of potato plants and reproducible results. *Nicotiana benthamiana* plants were generated from seeds and grown under the same greenhouse conditions.

Genetic mapping

The F1 population 7661 (mcd360-1 x gig714-1) was subjected to a genome-wide SSR markers screen (Bakker et al. 2011) on the parental genotypes and a subset of 51 F1 individuals. CAPS markers from chromosome 12 were tested and placed on the genetic map.

High-resolution mapping

The F1 population was extended to 3600 offspring genotypes, which were tested with flanking markers GB1755 and IPM5. Additional CAPs markers Rhl8 and Rhr0 were developed based on BAC clones at the extremities of RH contig 167 (Tang et al. 2009). BlastN of the RH BAC end sequences from contig 167 revealed homology with a tomato BAC clone LE_HBa0146I19, and an additional CAPS marker LBC was developed from tomato. The recombinants of the GB1755 - IPM5 interval were tested with Rhl8, LBC and Rhr0 and phenotyped for the response to INF1 elicitin by PVX agroinfection. A high-resolution map was constructed.

Physical mapping

A BAC library of mcd360-1 was constructed. The library that consisted of 110592 clones with an average insert size of 110 kb was screened with the CAPS markers Rhl8, LBC and Rhr0. Sequencing the BAC ends of identified BAC clones led to the development of the coupling phase marker T85. A second BAC library screen was performed with T85, and subsequent BAC end marker development resulted in the marker T207, which was also screened on the library. Identified BAC clones MCD126, MCD85, MCD207 and MCD51 were sequenced. Markers C95 and C12 were developed on the different candidate genes.

Expression of *P. infestans* effectors and plant genes in *Agrobacterium*

Elicitins (**Table S2)** were cloned using the Gateway technology (Invitrogen, SanDiego, CA, USA). Full-length Elicitins flanked with attL1 sequence before the start codon (5'-AAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAAT

GCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGC-3') and attL2 sequence after the stop codon (5'-CAGCTTTCTTGTACAAAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACG AACAGGTCACTATCAGTCAAAATAAAATCATTATTT-3') were synthesized into the pUC57 vector by GenScript (US). The ORFs of the elicitins were then transferred into destination vectors pK7WG2 (Karimi et al. 2002) and pGWC-PVX (Valli et al. 2008) by LR-Reaction II (Untergasser 2006), respectively. The elicitin domain of Inf1 elicitin was also cloned in pCB302-3 and pGR106, as described previously in (Xiang et al. 1999, Lu et al. 2003, Vleeshouwers et al. 2006). ELR (RLP85) and RLP207 were transferred into destination vectors pK7WG2, behind the 35S promoter. The R3a and Rpi-vnt1 resistance genes (Huang et al. 2005, Foster et al. 2009, Pel 2010) were expressed in pBINplus and pGRAB, respectively. Isolated plasmids for each construct were introduced by electroporation into *A. tumefaciens* strain GV3101 or AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG (pBBR1MCS-5.virGN54D) (van der Fits et al. 2000).

PVX agroinfection

PVX agroinfection was performed on 4-5 week old potato plants in *A. tumefaciens* strain GV3101 as previously described (Vleeshouwers et al. 2006, Du et al. 2013). Crinkler Crn2, a nonspecific cell death inducing gene of *P. infestans* was included as a positive control, and the pGR106 or pGWC-PVX empty vectors as negative control. Briefly, cultures of recombinant *A. tumefaciens* strains carrying elicitins (**Table S2)** were grown for 2 days at 28 ℃ on solid agar LB medium supplemented with antibiotics (Jones et al. 1999, Takken et al. 2000, Torto et al. 2003). Excess bacteria was inoculated by piercing the leaf at both sides of the mid-vein. Local symptoms were visually scored at 12-16 dpi.

Agroinfiltration

Agroinfiltration of *Agrobacterium tumefaciens* strains AGL1 was performed on leaves of 4–5 week old *Solanum hjertingii* 349-3 plants, using routine protocols (van der Hoorn et al. 2000, Du et al. 2013). Briefly, *A. tumefaciens* strains were grown to an optical density at 600 nm of 0.3 and leaf panels were infiltrated with the *A. tumefaciens* suspensions. Symptoms were monitored from 2 to 6 dpi. For co-infiltrations, *A. tumefaciens* carrying effector genes were mixed with *A. tumefaciens* strains carrying plant RLP or *R* genes in a 1:1 ratio.

Complementation studies

Stable transformation of potato cultivar Désirée with 35S-ELR and 35S-RLP207 in pK7WG2 was carried out using routine potato transformation protocols (Filati et al. 1987, Hoekema et al. 1989). Independent primary transformants were selected and cultured in the greenhouse and subsequently tested for response to INF1 elicitin using PVX agroinfection with pGR106-Inf1.

Confocal microscopy

Confocal microscopy was carried out as previously described (Bozkurt et al. 2011). Leaf patches of *N. benthamiana* were cut and mounted in water after transient *A. tumefaciens*-mediated expression of the fluorescent proteins. Confocal analysis was performed on a Leica DM6000B/TCS SP5 microscope (Leica Microsystems) using the following excitation wavelengths: eYFP (458 nm), GFP (488 nm) and RFP (561 nm). Same microscope settings were applied to acquire all individual images shown in a given Figure for comparison between samples.

Co-immunoprecipitation experiments

StSerk3A was amplified from *Solanum tuberosum* cultivar Désirée cDNA, using the conserved primers described in (Chaparro-Garcia et al. 2011). Fusion proteins StSERK3A-3×HA and StSERK3A-GFP were cloned into Gateway binary vectors pGWB14 and pK7FWG2 respectively using the conserved primers described in (Chaparro-Garcia et al. 2011) and transformed in *A. tumefaciens* GV3101. *ELR* was cloned into pENTR and the GFP fusions were generated by Gateway LR recombination (Invitrogen) of pENTR: *ELR* and pK7FWG2: (*ELR-GFP*). *ELR-eGFP* was cloned into the binary vector pBIN-KS-35S::GWY-eGFP as described in (Liebrand et al. 2014). Both *ELR-GFP* constructs were transformed in *A. tumefaciens* GV3101. Total proteins were extracted from *N. benthamiana* leaves at 2.5 days after transient co-transformation of StSERK3A-3×HA or AtBAK1-3×HA and ELR-GFP or ELR-eGFP and the p19 silencing suppressor (Voinnet et al. 2003) in a 1:1 ratio. AtFLS2-GFP and 35S:GFP were used as controls. Leaves were subjected to water or elicitor treatment as stated in each Figure and protein extraction was done as described in (Win et al. 2011). Co-immunoprecipitation experiments were performed using the GFP-affinity matrix (Chromotek) in a 1.5 ml low-bind eppendorf. Non-diluted samples were incubated on roller mixer for 4 hours at 4°C and washed with 1 ml of washing buffer (TBS + 0.5 % NP40) at least five times. Pelleted samples were resuspended in 1xSDS buffer, denatured at 70°C for 10 minutes and loaded in an SDS-PAGE gel for western blot analysis. Blots were pre-blocked in 3% BSA and probed with anti-GFP and anti-HA (Santa Cruz) antibody. Proteins were visualized with ECL (Amersham Biosciences).

***Phytophthora infestans* strains**

Phytophthora infestans strains that were used for most disease tests in this study are 88069, EC1_DC2005 (abbreviated EC1 in the Figures) and the transgenic strain 88069tdtom that expresses a red fluorescent protein in 88069 genetic background. Strain 88069 (race 1.3.4.7) originates from the Netherlands in 1988. Strain EC1_DC2005 (race 1.3.4.7.10.11) originates from Ecuador. Strains were stored in liquid nitrogen stocks, and grown on rye sucrose medium (Caten and Jinks 1968).

Disease tests

Phytophthora infestans infection assays were performed on detached leaf tests, in two platforms, i.e. 1) a routine high throughput assay by epidemic wildtype *P. infestans* strains measuring lesion sizes using a digital caliper and 2) an infection image processing assay using transgenic tdtom-labeled *P. infestans*.

1) The high throughput disease assay with digital caliper was performed as previously described with slight modifications (Vleeshouwers et al. 1999). Briefly, two well-developed compound leaves were detached from mature greenhouse-grown plants, placed in water-saturated florists foam (Oasis) in a tray. Spore suspensions of *P. infestans* strains 88069 and EC1_DC2005 were prepared by rinsing a plate covered with mycelium with water and incubating the sporangiophore at 4 ℃ for 1-2 hours. After release of zoospores, the concentration was adjusted to 5 × 10^4 spores/ml. *P. infestans* strains were spot-inoculated on the abaxial side of the same compound leaf, by placing 10 µl droplets i.e. on the various leaflets right and left of the midvein, for each strain, respectively. The trays were covered with transparent bags and incubated in a climate chamber at a photoperiod 16 and 8 h (day and night, respectively) at 15 ℃. Double-blind disease assessments were performed at 3 - 6 days post inoculation (dpi). Lesion diameters were measured using a digital calliper connected to a personal computer. The infection efficiency (IE) was calculated as the fraction of successful inoculations (i.e., fraction of expanding lesions, >25 mm^2, to the total number of inoculations) per leaf. These were averaged across plants and subsequently averaged across the experiments for genotype by strain. The lesion growth rate (LGR) was statistically analysed with a mixed model using REML of Genstat (International 2013) using the following model:

$$\underline{y_{lmno}} = \mu + \alpha_i + \beta_j + \gamma_k + \beta\gamma_{jk} + \underline{\tau_{il}} + \underline{\tau\upsilon_{ilm}} + \underline{\tau\upsilon\varphi_{ilmn}} + \underline{\varepsilon_{ilmno}}$$

Fixed: ?? = base level,

α_i = experiment (i=1...3),

?2$_j$ = genotype (j=1…8),

?3$_k$ = strain (k=1,2),

?2?3$_{jk}$ = genotype.strain interaction

Random: ??$_{il}$ = tray (l=1…nt; nt=10 or 20) ~ N(0,σ_τ),

$\tau\upsilon_{ilm}$ = tray.plant (m=1...np; np=2 or 4) ~ N(0,$\sigma_{\tau\upsilon}$),

$\tau\upsilon\varphi_{ilmn}$ = tray.plant.leaf (n=1…nl; nl=2 or 4) ~ N(0,$\sigma_{\tau\upsilon\varphi}$),

ε_{ilmno} = spot or residual/experiment (o=1…ns; ns=4 or 8) ~ N(0,$\sigma_{\varepsilon,i}$)

The residual level is the spot inoculation level, a different residual per experiment. Experience from the past have shown that the leaflet factor within a leaf is small compared to the spot inoculation variation and by omitting it in the model its variation is included in the residual. In general the residuals are comparable and exceeded several times the other random components, indicating that the main source of random variation is the biological variation which is reflected in the spot inoculation unit, while tray, plant and leaf variation are well controlled.

2) For the infection image processing assay, plants and inoculum were prepared as described above. Leaves were droplet (10 µl, 6 spots per leave) inoculated with the zoospore suspension of transgenic *P. infestans* 88069tdtom on the abaxial side and incubated at 16-18 ℃. Disease lesions were measured (in

mm) starting at 2 days post inoculation (dpi). Lesion area was measured with a Leica Stereomicroscope (Leica Microsystems CMS GmbH) mounted with a CCD camera under UV LED illumination and filter settings for DsRed. Images were processed using an algorithm for the Acapella™ software.

The algorithm reads a series of TIFF files into the Acapella™ image analysis platform. TIFF images are split into three planes – hue, saturation, and intensity value. Only intensity plane is used in the image analysis. Whilst splitting the image, a convolution method is used to harmonise intensity values. Image masks are applied to identify regions with high intensity/contrast values – generated masks are randomly coloured and treated as a set of image objects. A filtering system is used to detect the scale (pixel to μm) according to its unique intensity, contrast, and width/length ratio. Another filtering system is used to filter out objects such as letters and experiment errors (inappropriate intensity, size, contrast, and location (attached to the image border). After screening, only genuine infected areas are retained.

To measure the area and the perimeter of the infected area, the algorithm firstly splits the detected infected areas into many smaller objects. Based on the split objects, the algorithm detects the centre as well as it finds the most left/top/bottom/right pixels of the infection areas. Based on coordinates of those most left/top/bottom/right pixels, minor radius and major radius of the infection areas are calculated and refined. The area and the perimeter of the infected regions are based on the calculated minor radius and major radius. The formula used for computing the area is: $Area = \pi ab$ (a, major radius; b, minor radius).

Disease scoring data were subjected to statistical analysis using one-way ANOVA and T-test with R package.

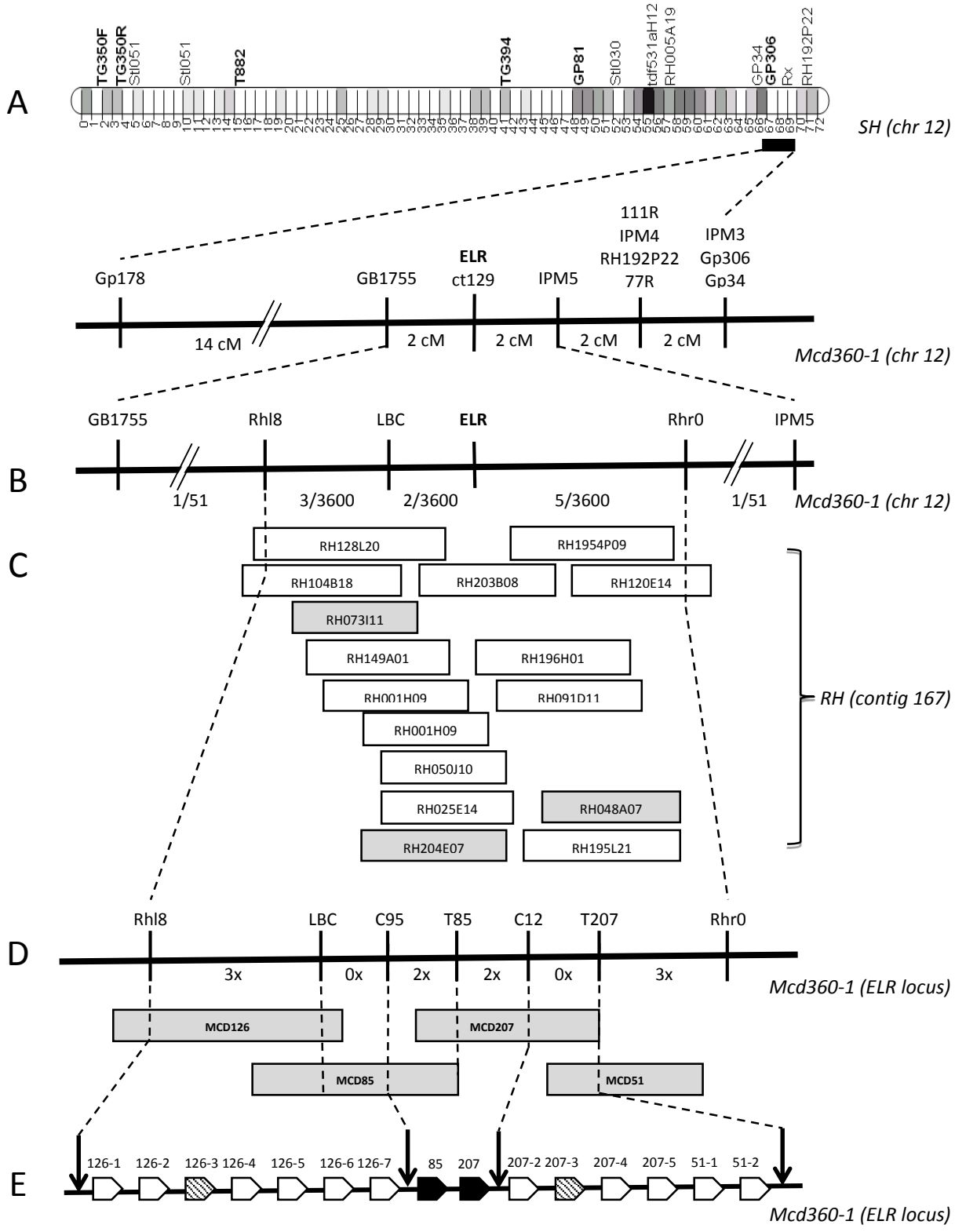


Figure S1. Map-based cloning of ELR from *Solanum microdontum*.

(**A**) Genetic map of ELR locus on chromosome 12 of potato. In a population of 51 F1 individuals of the mcd360-1 x gig714-1 population, the SSR marker RH192P22 is linked to ELR with two recombinants. RH192P22 genetically maps in bin 70/71 in the SH83-92-488 (SH) genetic map (van Os et al. 2006, Tang et al. 2009, de Boer et al. 2011), approximately 6 cM proximal to the *Rx/Gpa2* cluster on the SH83-92-488 (SH) genetic map (van der Voort et al. 1999). Ten CAPS markers that localize in this regions were also

placed on the genetic map, seven are distal, two proximal, and one co-segregates with ELR. (**B**) High resolution map of the ELR locus. The screening of recombinants from an extended population of 3600 individuals revealed the genetic location of marker Rhl8 five recombinants proximal, Rhr0 five recombinants distal, and the tomato LBC two recombinants proximal to ELR. (**C**) RH contig 167 is spanning the ELR locus, which contains BAC clones that harbor RLP-like sequences (grey). (**D**) Physical map of the ELR locus. The coupling-phase BAC clones MCD126 and MCD85 were identified by markers Rhl8 and LBC, BAC clone MCD207 by marker T85 and BAC clone MCD51 by marker T207. The ELR locus is spanned by these four MCD BAC clones. Markers C95 and C12 were developed from the BAC clone sequences to exclude some candidate genes. (**E**) Relative position of RLP candidate genes. The ELR locus comprises at least 13 predicted RLP genes (block arrow) and two pseudogenes (hatched). The markers C95 and C12 restrict the ELR interval to approximately 100 kb, in which the RLP85 and RLP207 (black) are located.

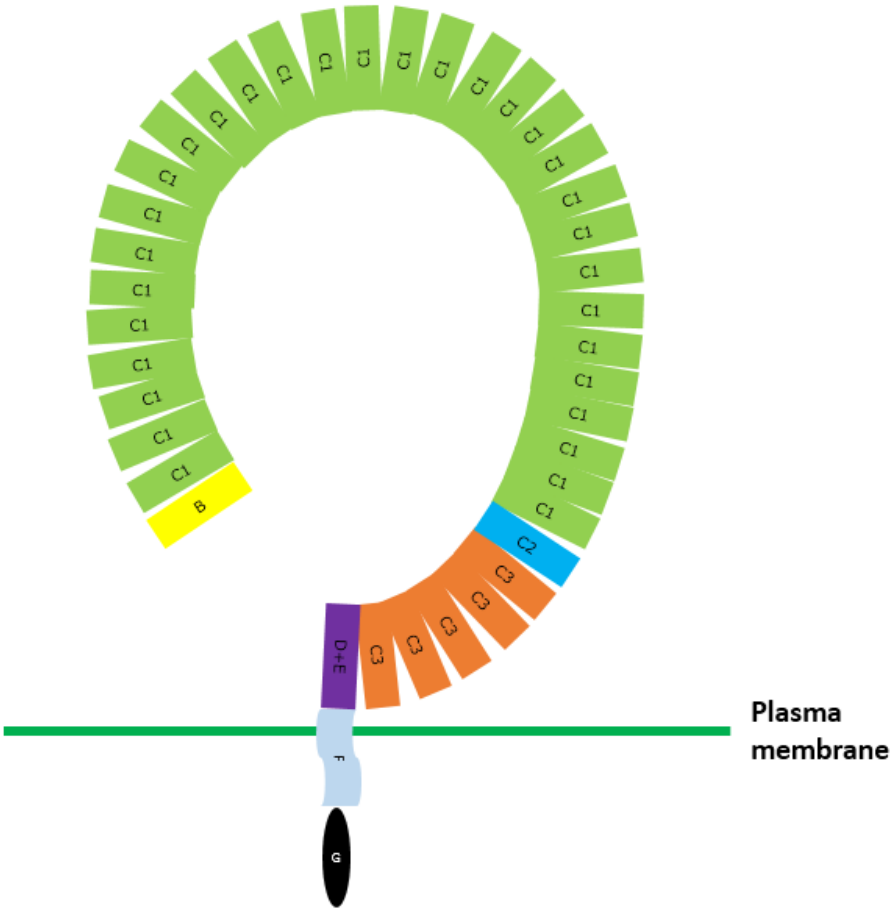
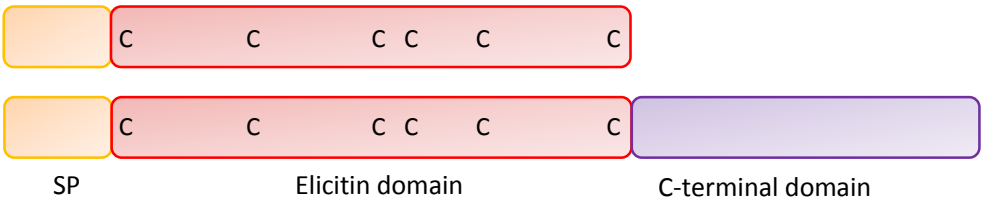


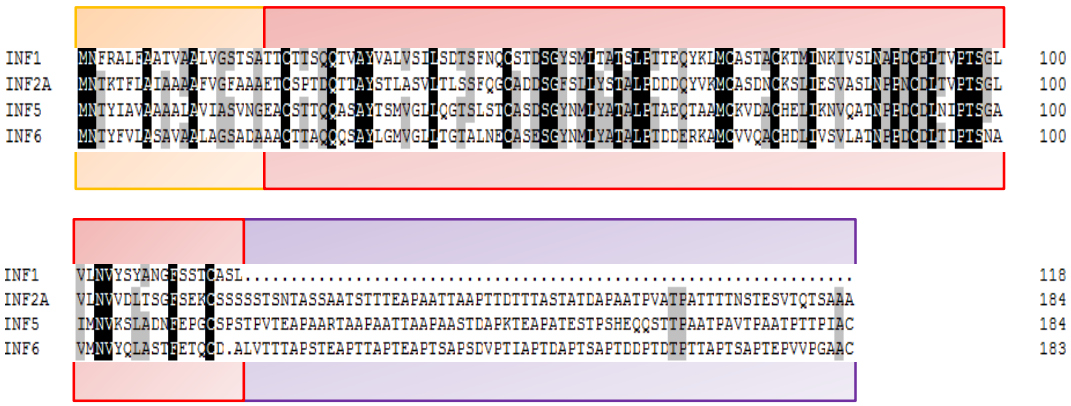
Figure S2. ELR encodes a receptor-like protein (RLP).

The *ELR* gene encodes a predicted polypeptide of 1094 amino acids from a single ORF and consists of domains typical for RLP. Domain A (not depicted) contains a putative signal peptide of 23 amino acids (SignalP) and is cleaved in the predicted mature protein. Domain B contains a Cys-rich or mature N-terminus that contains three conserved structural motifs LLxxK, LssW and CxWxGVxC (Rivas and Thomas 2005, van der Hoorn et al. 2005). Domain C consists of 36 imperfect repeats of the consensus sequence LxxLxxLxxLDLSSNNLxGxIPxx (Jones et al. 1994), and is divided in three subdomains, where C1 and C3 are the LRR regions and C2 represents a non-LRR island. Domains D is a spacer. Domain E is an acidic domain. Domain F is a transmembrane domain. Domain G represents a short cytoplasmic tail that does not contain any known motif related to endocytosis present in *Cf* homologs (Geldner and Robatzek 2008).

A



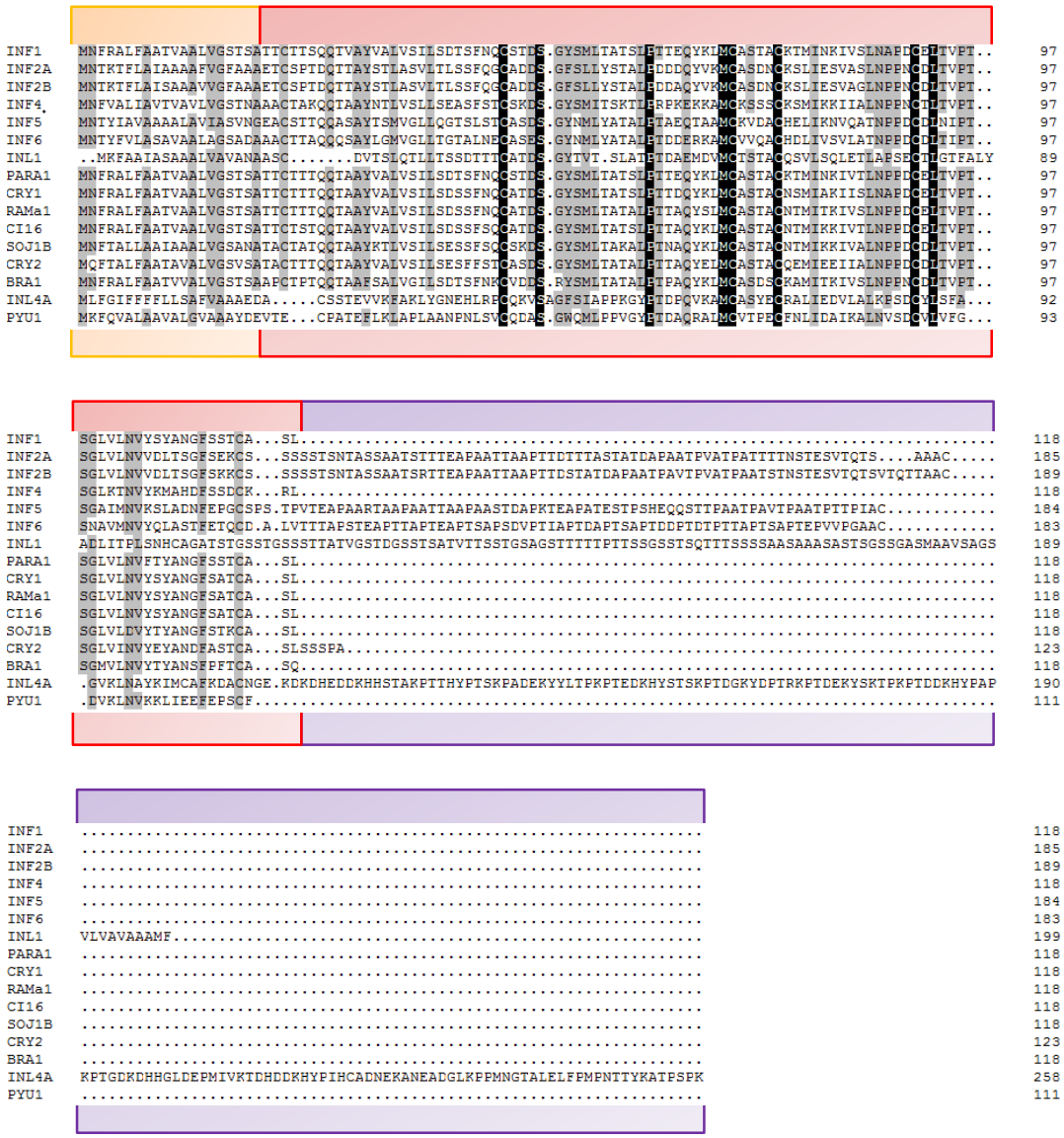
B



C

Sequence	INF1	INF2A	INF5	INF6
INF1	ID			
INF2A	52%	ID		
INF5	45%	46%	ID	
INF6	45%	43%	65%	ID

D



E

Sequence	INF1	INF2A	INF2B	INF4	INF5	INF6	INL1	PARA1	CRY1	RAMα1	CI16	SOJ1B	CRY2	BRA1	INL4A	PYU1
INF1	ID															
INF2A	52%	ID														
INF2B	51%	97%	ID													
INF4	57%	46%	46%	ID												
INF5	45%	46%	46%	43%	ID											
INF6	45%	43%	42%	42%	65%	ID										
INL1	23%	27%	28%	27%	24%	25%	ID									
PARA1	94%	53%	53%	58%	46%	45%	26%	ID								
CRY1	90%	56%	54%	57%	45%	46%	27%	88%	ID							
RAMα1	89%	56%	56%	55%	47%	46%	29%	89%	93%	ID						
CI16	88%	54%	55%	58%	47%	44%	29%	90%	92%	94%	ID					
SOJ1B	77%	54%	55%	65%	47%	46%	28%	80%	78%	82%	84%	ID				
CRY2	74%	52%	53%	60%	52%	49%	30%	77%	79%	81%	80%	78%	ID			
BRA1	71%	54%	54%	55%	44%	45%	23%	73%	70%	74%	71%	69%	67%	ID		
INL4A	26%	30%	30%	29%	27%	28%	22%	27%	27%	27%	29%	29%	28%	27%	ID	
PYU1	27%	28%	29%	26%	32%	31%	19%	28%	28%	29%	30%	30%	29%	28%	43%	ID

Figure S3. Sequence alignment of elicitins of oomycetes.

(**A**) Elicitins are modular proteins consisting of a signal peptide (orange) and an elicitin domain (red); some elicitins also contain a C-terminal domain (purple). The conserved 98-acid elicitin contains six cysteine residues in a typical spacing pattern; this elicitin domain is required for recognition. C-terminal tails of variable length are most likely involved in localization to e.g. cell wall (Huitema et al. 2005, Jiang et al. 2006). (**B**) Sequence alignment of four *P. infestans* elicitins that are recognized by ELR. Identical amino acids are marked (black), amino acids with >75% sequence identity are shaded (grey) (**C**) Percentages of amino acid sequence identity of the elicitin domain of the four recognized elicitins of *P. infestans*. (**D**) Sequence alignment of 16 elicitins of diverse oomycete species. (**E**) Percentages of amino acid sequence identity of the elicitin domain of 16 elicitins of diverse oomycetes.

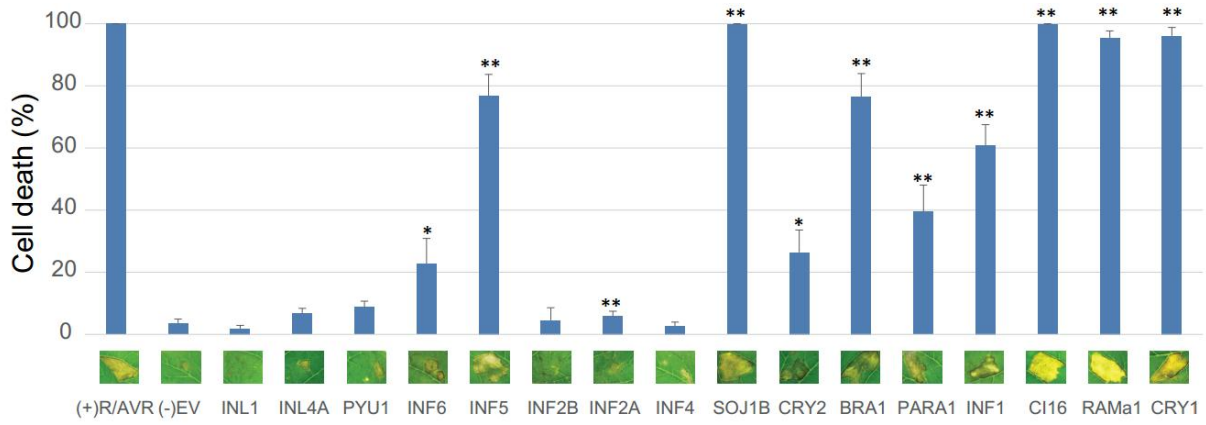


Figure S4. A broad spectrum of elicitins induce cell death in Désirée-ELR.

(**A**) Agroinfiltration of a diverse set of oomycete elicitins **(Figure 2, Table S2)**. The elicitins were expressed in pK7WG2 and agro-infiltrated in leaves of transgenic Désirée-ELR#34. Representative photos of infiltrated leaf panels at 5 days post infiltration (dpi) are shown. (**B**) Histogram of level of cell death response which was quantified by macroscopic scoring for cell death intensity at 5 dpi. Results are averages, ± s.e.m. (n=11), * indicates significantly different (P<0.05) using Student's *t* test. Agro-infiltration is a slightly less sensitive assay than PVX agroinfection (Figure 2, Main text) (Vleeshouwers and Rietman 2009), still the broad spectrum of elicitin recognition by ELR upon is confirmed.

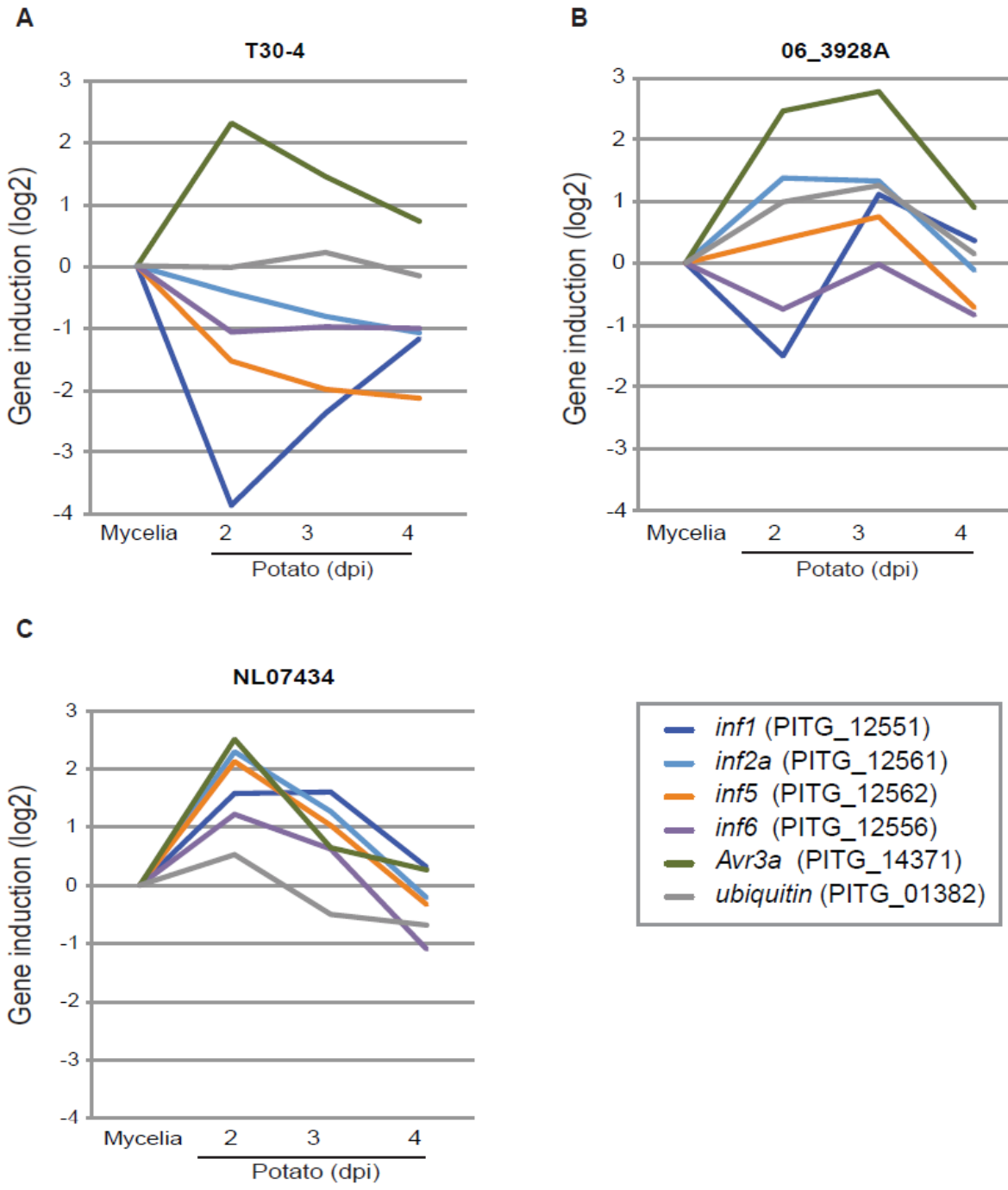


Figure S5. Gene expression of elicitins.

(**A, B, C**) A time course expression pattern of elicitins during infection of potato for the *P. infestans* strains T30-4 (**A**), 06_3928A (**B**), and NL07434 (**C**). The y-axis is showing gene induction (log2) which was calculated for each time point of the infected material from 2 to 4 dpi. The induction values were normalized against the mycelia samples (Cooke et al. 2012). Genes encoding recognized elicitins (Figure 2.), i.e. *Inf1* (dark blue), *Inf2* (light blue), *Inf5* (orange) and *Inf6* (purple) are shown. The cytoplasmic RXLR effector *Avr3a* (green) is shown as an example of a typical induced gene during infection and the ubiquitin gene (grey) as a control.

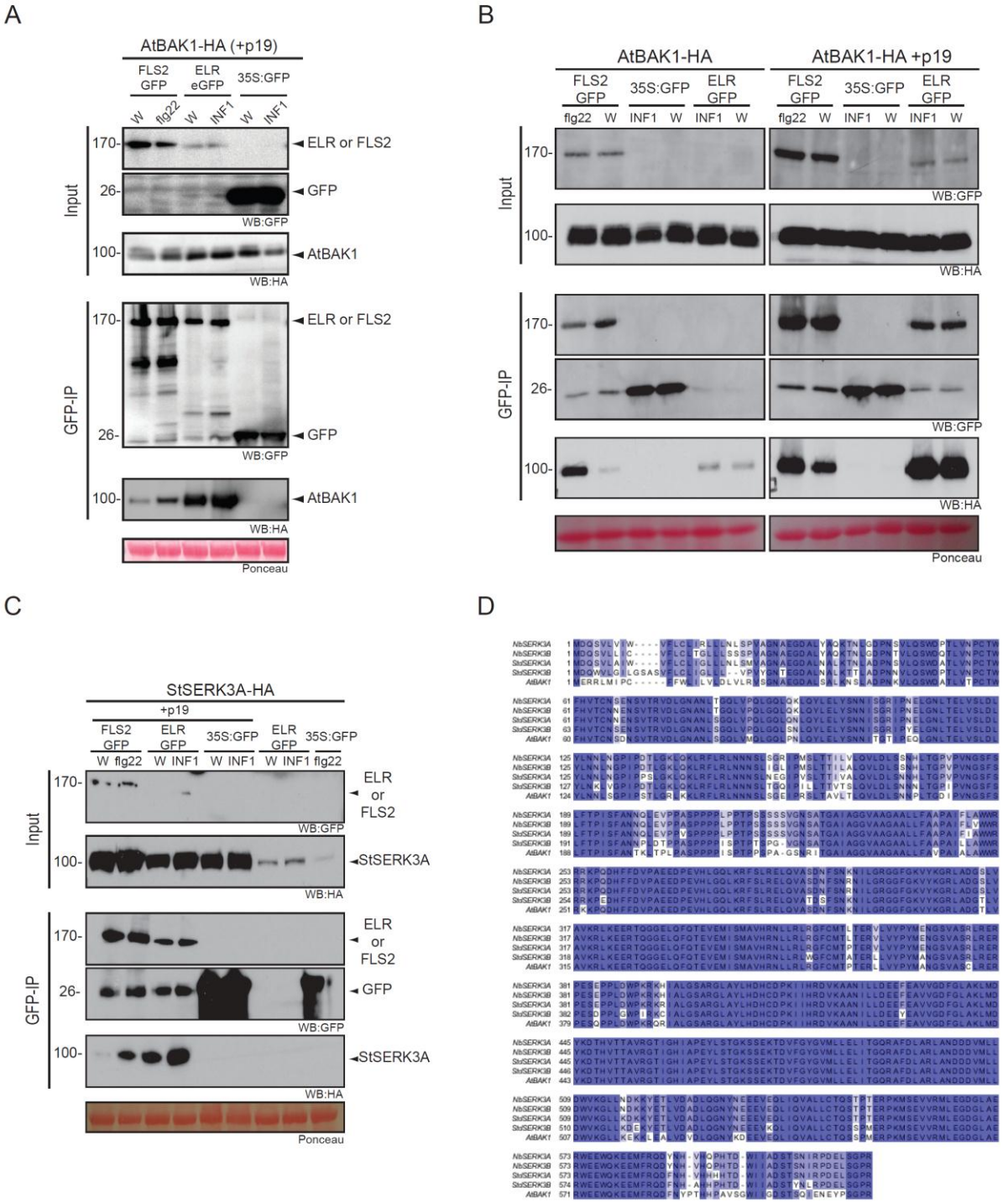

A
AtBAK1-HA (+p19)
FLS2 GFP
ELR eGFP
35S:GFP
W flg22 W INF1 W INF1
Input
170-
26-
100-
ELR or FLS2
GFP
AtBAK1
WB:GFP
WB:HA
GFP-IP
Ponceau
B
AtBAK1-HA
AtBAK1-HA +p19
FLS2 GFP
35S:GFP
ELR GFP
flg22 W INF1 W INF1 W
C
StSERK3A-HA
+p19
W flg22 W INF1 W INF1 W INF1 flg22
StSERK3A
D

Figure S6. BAK1/SERK3 co-immunoprecipitates with GFP-tagged ELR.

(**A, B, C**) ELR-eGFP and ELR-GFP co-immuno-precipitate with AtBAK1 and StSERK3a. ELR-eGFP (A) or ELR-GFP (B, C) was transiently co-expressed with AtBAK1-HA (A, B) or StSERK3A (C) in the absence or presence of the post-transcriptional silencing-suppressor p19 and challenged with INF1[Pi] (10 µg/ml) or water for 15 minutes. Immunoprecipitation (IP) was carried out with GFP beads and total protein extracts and IP were blotted with the appropriate antisera as indicated. As negative control AtBAK1-HA and StSERK3a-HA were also co-expressed with 35S:GFP and subjected to the same treatment as described above. FLS2-GFP, AtBAK1-HA and StSERK3A treated with flg22 (100 nM) or water for 15 minutes is shown on the left side of each panel as a positive control. Presence of p19 enhances the detectability of the ELR protein. Ponceau stain of the total extract blot indicates equal loading (bottom). (**D**) ClustalW alignment of homologs of AtBAK1 in *N. benthamiana* and *S. tuberosum* cv. Désirée. Tblastn search in the potato genome (PGSC DM v. 3.4) using AtBAK1 as a query revealed two copies in potato *StSerk3A* and *StSerk3B*, which share a high sequence identity to *NbSerk3A*/*B* of *Nicotiana benthamiana* of 95% and 88%, respectively. StSERK3A was used for all experiments. Amino acid residues are shaded dark blue if identical and a lighter shade of blue if similar. Sequences were viewed in Jalview.

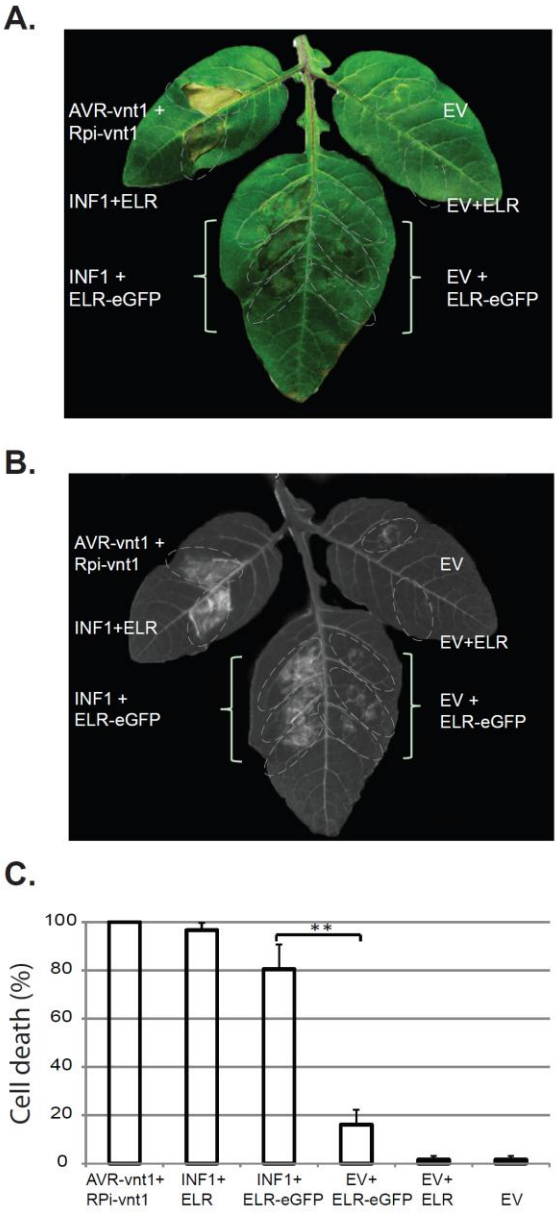


Figure S7. eGFP-tagged ELR causes cell death with INF1 in potato.

(**A, B**) Agro-co-infiltration of ELR-eGFP with INF1 in *S. hjertingii* 349-3 shows that eGFP-tagged ELR is a functional protein and causes cell death with INF1. Cell death is visualized as collapsed leaf tissue (A; normal light) and autofluorescence (B; UV light). (**C**). Quantification of cell death responses after macroscopic scoring for cell death intensity at 5 dpi. Results are averages, ± s.e.m. (n=6), ** indicates significantly different levels of cell death between INF1 vs. empty vector (EV) co-infiltrations with ELR-eGFP (pairwize *t* test, P<0.001). Agro-co-infiltration of Rpi-vnt1 with Avrvnt1 and of untagged ELR with INF1 are included as positive controls, the empty vector (pK7WG2) and co-infiltrations with ELR-GFP and with ELR-eGFP as negative controls.

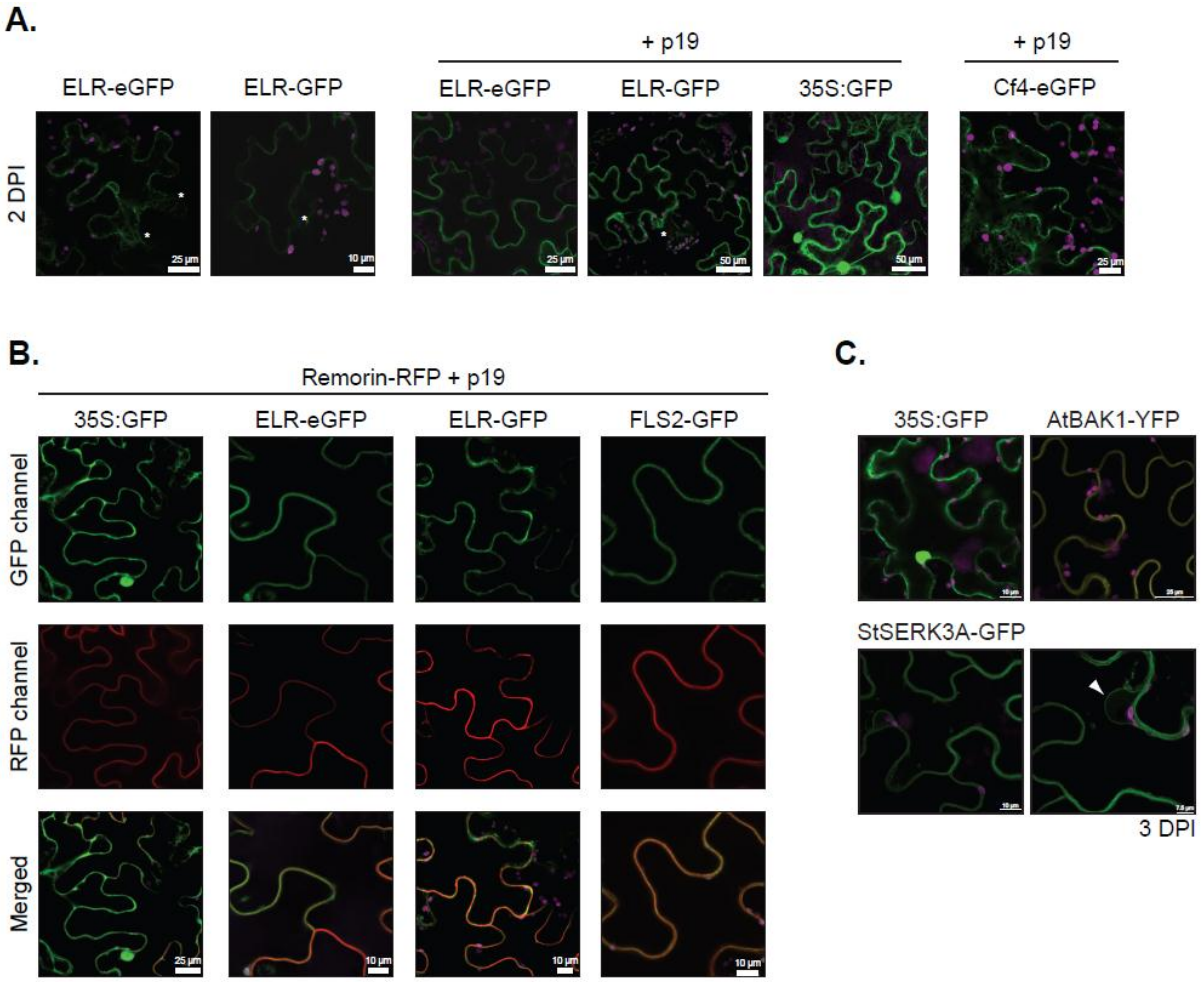


Figure S8. ELR and StSERK3A mainly localize to the plasma membrane.

(**A**) *Agrobacterium tumefaciens*-mediated expression of ELR-eGFP and ELR-GFP fusion proteins with and without p19 revealed that ELR mostly has plasma membrane subcellular distribution with a small fraction accumulating at the ER (white asterisks). *A. tumefaciens*-mediated expression of 35S-GFP and Cf4-eGFP were included as controls. Images in this Figure were taken at 2 DPI. (**B**) ELR peripheral localization was confirmed by co-expression of ELR-eGFP and ELR-GFP with a plasma membrane marker (remorin-RFP); 35S-GFP and the plasma membrane localized receptor FLS2-GFP were included as controls. All images in this Figure were taken at 2.5 DPI and co-expressed with p19 in all cases. (**C**) Plasma membrane localization of StSERK3A-GFP and AtBAK1-YFP after transient expression in *N. benthamiana* at 3 dpi without p19. The white arrowhead in the close up image indicates stSERK3A-GFP localization at the tonoplast. Magenta corresponds to autofluorescence of plastids.

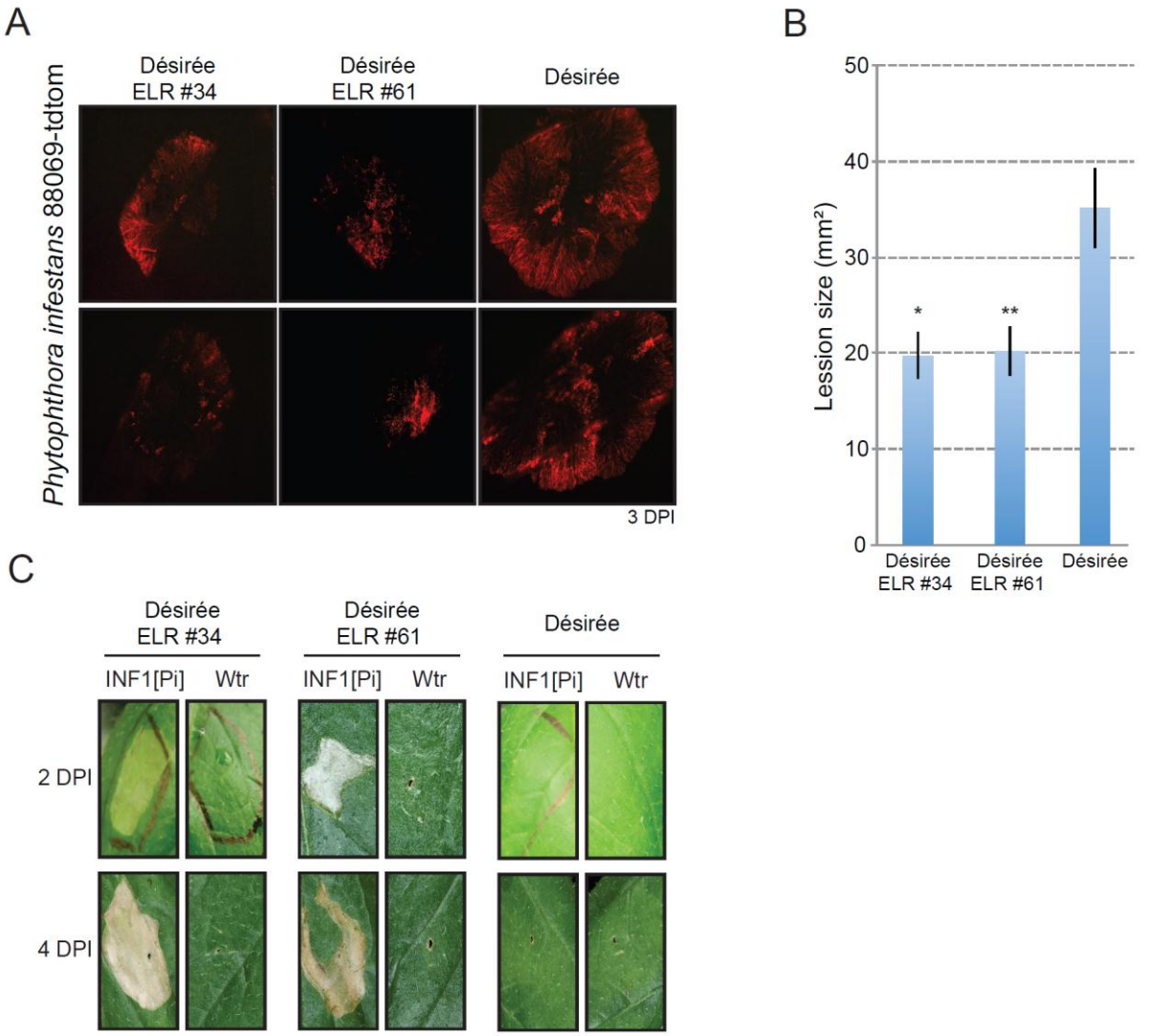


Figure S9. ELR reduces the growth of *P. infestans* in *S. tuberosum* cv. Désirée.

(**A**) Two stable transformants, Désirée-ELR#34, Désirée-ELR#61 and Désirée controls were subjected to detached leaf tests with *P. infestans* 88069tdtom. Infection was followed in 6 leaves per genotype and 4 droplets per leaf at 2, 3, and 4 DPI. (**B**) The histogram shows the average infection area (n=24) in mm^2 at 3 DPI measured from the pictures. Asterisks represent statistically significant difference at $P < 0.05$ (*) and $P < 0.01$ (**) (ANOVA, Tukey post-test). (**C**) Cell death response to infiltrated INF1 protein (10 µg/ml) in Désirée-ELR#34 and Désirée-ELR#61. Potato plants were infiltrated with INF1 [Pi] or water to confirm the expression of the ELR transgene. All experiments were repeated three times with similar results.

Tables S1. Genetic markers for mapping, fine-mapping and physical mapping.

Study	Marker	Type	Sequence	Reference	TM	Enzyme
Mapping	Rh192P22	SSR	F aattctttgaaattggcccc R cacacccaacaatctttccc	(Bakker et al. 2011)	56	a.s.[1]
	GB1755	CAPS / HRM	F ttaacgaactagcagtttatagacgc R ttgcttggactcttcataaaaca		52	DdeI
	Ct129	CAPS	F gtctaagaagatgaaaaggggtgc R ttggagtttgttaaggacttcgattgc	(Bendahmane et al. 1997)	52	XapI
	111R	CAPS	F ccactgtgtaaggggtcaactatagtc R gagatgaagattttcttgtctgatgg	(van der Voort et al. 1999)	52	MspI
	IPM5	CAPS / HRM	F agctccattcgtgacgat R agcttcgataattctaaatttg	(Bendahmane et al. 1997)	52	DdeI
	IPM4	CAPS	F gtactggagagctagtagtgatca R accactggcaaatggccatacga	(Bendahmane et al. 1997)	52	RsaI
	77R	CAPS	F ctcgagggattgaatccaaattat R ggaagcagaatactcctgactact	(van der Voort et al. 1999)	52	Hin1I
	IPM3	CAPS	F agtagtttcaggctagtg R caacatcacttgatcagac	(Bendahmane et al. 1997)	52	XapI
	Gp34	CAPS	F cgttgctaggtaagcatgaagaag R gttatcgttgatttctcgttccg	(Bendahmane et al. 1997)	52	HaeIII
	Gp178	CAPS	F tgcactttaagagaggagaaaaga R ctgcagcttactcggaatgc		52	MwoI
	Gp306	CAPS	F cgttgctaggtaagcatgaaga R ctgcaggttggattttgtga		52	AluI
Fine mapping, physical mapping	Rhl8	CAPS	F cttccaaatttcccggattgg R gtaacattggctctgagcctc		55	MnlI
	Rhr0	CAPS	F ttgggtaagtggagcaggg R ggcttggaatctcggactatg		55	MnlI
	LBC	CAPS	F tgaatcagctgaagcagtcg R tgttgaacatcttcttaacagca		45	ScrFI
	C95	CAPS	F tgagccaccagtaggtaggg R aaaccaaaaagcccaaagt		57	Hin1II
	T85	CAPS	F ggttccattgaagcctagca R agccctctttttccctacca		57	MwoI
	C12	CAPS	F cactcggattgacctttctg R tgaatcgggactgatgaaca		58	ScrFI
	T207	CAPS	F ataattactggcagataaacc R gtacttacagatatgagagcg		55	a.s.

[1] a.s.; allele specific

Table S2. Elicitins genes from diverse oomycete species.

Elicitin	Gene identity	Type	Species	Reference	Genbank
INF1	PITG_12551	Elicitin	*Phytophthora infestans*	(Kamoun et al. 1997, Kamoun et al. 1998, Jiang et al. 2006, Haas et al. 2009)	XM_002900382.1
INF2A	PITG_12561	Elicitin	*Phytophthora infestans*	(Kamoun et al. 1997, Kamoun et al. 1998, Jiang et al. 2006, Haas et al. 2009)	AY693804.1
INF2B	nd	Elicitin	*Phytophthora infestans*	(Kamoun et al. 1997, Kamoun et al. 1998, Jiang et al. 2006, Haas et al. 2009)	AF004952.1
INF4	PITG_21410	Elicitin	*Phytophthora infestans*	(Jiang et al. 2006, Haas et al. 2009)	XM_002895013.1
INF5	PITG_12562	Elicitin	*Phytophthora infestans*	(Jiang et al. 2006, Haas et al. 2009)	AF419842.1
INF6	PITG_12556	Elicitin	*Phytophthora infestans*	(Jiang et al. 2006, Haas et al. 2009)	XM_002900387.1
INL1[a]	PITG_12599	Elicitin-like	*Phytophthora infestans*	(Jiang et al. 2006, Haas et al. 2009)	AF419844.1
INL4A	PITG_02525	Elicitin-like	*Phytophthora infestans*	(Jiang et al. 2006, Haas et al. 2009)	XM_002907398.1
PARA1	PPTG_19861	Elicitin	*Phytophthora parasitica*	(Kamoun et al. 1993, Kamoun et al. 1998)	S67432.1
CRY1	nd	Elicitin	*Phytophthora cryptogea*	(Panabieres et al. 1995)	Z34462.1
RAMa1	nd	Elicitin	*Phytophthora ramorum*	(Tyler et al. 2006, Manter et al. 2007)	DQ680026.1
CI16	nd	Elicitin	*Phytophthora cinnamomi*	(Duclos et al. 1998)	AJ000071.1
SOJ1B	nd	Elicitin	*Phytophthora sojae*	(Mao and Tyler 1996, Qutob et al. 2003, Jiang et al. 2006, Tyler et al. 2006)	AY183409.1
CRY2	nd	Elicitin	*Phytophthora cryptogea*	(Panabieres et al. 1995)	Z34460.1
BRA1	nd	Elicitin	*Phytophthora brassicae*	(Jiang et al. 2006)	AY244545.1
PYU1	nd	Elicitin	*Pythium ultimum*	(Cheung et al. 2008, Levesque et al. 2010)	T009389[b]

[a] Previously known as *INF7*

[b] PYU1 from http://supfam2.cs.bris.ac.uk/SUPERFAMILY/cgi-bin/gene.cgi?genome=Pu&seqid=PYU1_T009389.

[c] nd, no data.

Table S3. ELR confers resistance to *P. infestans* in transgenic potato.

Three independent double blind disease testing experiments of potato cultivar Désirée and seven Désirée -ELR transformants were performed with *P. infestans* strains 88069 and EC1. Lesions were measured using a digital caliper. Lesion growth rates (LGR) were statistically analyzed using REML variance components analysis; the estimated variance components, residual models for each experiment and statistical tests for fixed effects are presented. The effect of ELR (genotype) and inoculated strain were statistically significant.

Estimated variance components		
Random term	component	s.e.
exp.tray	0.0387	0.0132
exp.tray.plant	0.0033	0.0057
exp.tray.plant.leaf	0.0182	0.0072

Residual model for each experiment					
Experiment factor: exp					
Experiment	Term	Factor	Model(order)	Parameter Estimate	s.e.
2	Residual	Identity	Variance	0.195	0.013
3	Residual	Identity	Variance	0.620	0.037
4	Residual	Identity	Variance	0.333	0.020

Tests for fixed effects				
Fixed term	n.d.f.	d.d.f.	F statistic	P
Exp	2	32.1	125.70	<0.001
genotype	7	68.3	6.41	<0.001
Strain	1	1229.3	896.07	<0.001
genotype.strain	7	1229.3	2.31	0.024

Table S4. Infection efficiencies and lesion growth rates on Désirée-ELR.

Seven Désirée-ELR transformants were assessed for lesion growth rates (LGR) and infection efficiencies (IE) to *Phytophthora infestans* strains EC1 and 88069, and compared to Désirée wildtype. Désirée-ELR transformants show slower expanding lesions than Désirée controls (P<0.001). *P. infestans* strain EC1 causes faster growing lesions than strain 88069 (P<0.001). The average LGR over three independent experiments are presented. Pairwise testing in homogenous groups was performed.

Plant genotype	*P. infestans* strain					
	88069			EC1		
	IE (%)	LGR (mm/day)		IE (%)	LGR (mm/day)	
Désirée-ELR#02	59	1.05	b	93	1.58	de
Désirée-ELR#26	58	0.97	b	95	1.76	e
Désirée-ELR#34	50	0.96	b	94	1.67	e
Désirée-ELR#45	42	0.86	ab	92	1.58	de
Désirée-ELR#46	58	0.92	b	92	1.79	e
Désirée-ELR#54	44	0.90	b	93	1.72	e
Désirée-ELR#61	50	0.67	a	88	1.42	cd
Désirée	82	1.30	c	99	2.18	f

Table S5. Infection efficiencies and lesion growth rates on Désirée-ELR and Désirée-RLP207.
Désirée-ELR transformants, Désirée-RLP207 transformants and Désirée wildtype were assessed for infection efficiencies (IE) and lesion growth rates (LGR) to *Phytophthora infestans* strains EC1 and 88069. Désirée-ELR transformants show slower expanding lesions than Désirée-RLP207 and Désirée controls (P=0.005). *P. infestans* strain EC1 causes faster growing lesions than strain 88069 (P<0.001). The average LGR are presented, calculated over two independent experiments that are part of the three experiments in **Table S4**. Pairwise testing in homogenous groups over the two experiments was performed.

Plant genotype	*P. infestans* strain					
	88069			EC1		
	IE (%)	LGR (mm/day)		IE (%)	LGR (mm/day)	
Désirée-ELR#02	69	1.27	bc	100	2.04	ef
Désirée-ELR#26	59	0.94	ab	100	2.40	fg
Désirée-ELR#34	63	1.00	ab	99	2.20	ef
Désirée-ELR#45	55	1.00	ab	100	2.14	ef
Désirée-ELR#46	81	1.25	bc	100	2.44	fg
Désirée-ELR#54	54	0.90	ab	100	2.23	f
Désirée-ELR#61	70	0.72	a	98	1.84	de
Désirée-RLP207#2	78	1.49	cd	100	2.76	g
Désirée	80	1.15	bc	100	2.67	g

Acknowledgments

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DNA and protein sequence of ELR will be deposited in Genbank.

Chapter 5

ELR is conserved in *Solanum* species

-Functional characterization and evolutionary analysis

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ELR is conserved in *Solanum* species

-Functional characterization and evolutionary analysis

Abstract

Pattern recognition receptors (PRR) perceive conserved PAMPs of microbes. So far, relatively few PRR from plants have been identified, and those are represented by receptor-like proteins (RLP) and receptor-like protein kinases (RLK). ELR from *Solanum microdontum* is the first RLP against oomycetes and recognizes elicitins, which are known as PAMPs of *Phytophthora* and *Pythium* species. In this study, we investigate the evolutionary origin of ELR among wild *Solanum* species. We found that ELR is present in various phylogenetic clades of tuber-bearing *Solanum*. Geographically, those species occur in both centres of diversity of tuber-bearing *Solanum*, i.e. South and Central America. Functional characterizations of eight ELR orthologs showed that their specificity to recognize elicitins from diverse *Phytophthora* species is highly conserved. The *Solanum* ELR orthologs also showed similar response specificity in *Nicotiana* species. Database searches suggest that ELR may be restricted to *Solanaceae* as no close homologs were identified in other families. Comparing the obtained ELR sequences within tuber-bearing *Solanum* pointed to a subdomain of four LRRs that appears correlated to the ability to respond to INF1. We conclude that *ELR* is an ancient gene under purifying selection, which may have evolved in *Solanaceous* plant species.

Introduction

Phytophthora infestans is one of the most devastating pathogens in the world and causes huge economic losses in potato production every year. During past years, the potato - *P. infestans* interaction has been extensively studied in order to solve the potato's biggest problem. As far as known, to colonize the host potato, *P. infestans* secretes a large amount of effectors both inside and outside the plant cells. During the coevolution, potato also evolves intracellular and extracellular immune receptors that can recognize those effectors. The most widely studied class of receptors is represented by the coiled-coil, nucleotide-binding, leucine-rich repeat (NB-LRR) class of intracellular immune receptors, which can recognize the cytoplasmic RXLR effectors and mount defense responses. Till now, more than 21 *R* genes of the NB-LRR class have been cloned, but most of them have been defeated by *P. infestans* because of the fast evolving RXLR effectors (Vleeshouwers et al. 2011).

Compared to RXLR effectors, apoplastic effectors are more conserved. Some of the apoplastic effectors have been considered as pathogen-associated molecular patterns (PAMPs), which are conserved

features or molecules of pathogens. Extracellular PAMPs can be detected by plant apoplastic receptors, also called pattern recognition receptors (PRRs). PAMP-triggered immunity (PTI) based on PRRs has great promise to confer a broad spectrum and durable resistance (Lacombe et al. 2010). In **Chapter 4**, we reported the cloning of *ELR*, a PRR that recognizes elicitins, a class of apoplastic effectors secreted by *P. infestans. ELR* was cloned from the wild potato species *Solanum microdontum.* We showed that ELR can also induce cell death response upon recognition of elicitins from other *Phytophthora* species, which indicates a broad-spectrum recognition that acts beyond the species of *P. infestans*. Besides, overexpression of *ELR* in a susceptible potato cultivar (Désirée) conferred enhanced resistance to late blight.

Having collected some insights on the conservation of the effectors (**Chapter 4**) (Jiang et al. 2006), we now aim at investigating the evolutionary origin of the receptor. Similar to PAMPs being more conserved than RXLR effectors, are the matching PRR more conserved than *R* genes? In this study, we investigate the genetic variation of *ELR* in wild tuber-bearing *Solanum* species, and perform database searches beyond those species. In addition, we performed functional studies with identified *ELR* orthologs and hypothesize that *ELR* is an ancient gene in *Solanum* or *Solanaceae*.

Results

INF1-recognizing *Solanum* species are distributed in both Central and South America.

ELR originates from *Solanum microdontum*, a diploid wild tuber-bearing *Solanum* species from South America (**Chapter 4**). To test whether ELR is conserved in other *Solanum* species, 115 *Solanum* genotypes of section *Petota* were subjected to functional screens for response to INF1 by PVX agroinfection. In total, 32 *Solanum* genotypes showed necrosis response to INF1 (Rietman 2011) and these belong to 19 *Solanum* species that are classified in 7 different taxonomic series (Table 1).

The relationship between these INF1-responding *Solanum* genotypes is presented in a phylogenetic tree, which is based on AFLP analysis (Jacobs 2008) and reflects their taxonomic diversity (Figure 1A) (Hawkes 1990, Jacobs et al. 2008). The geographic origin of the INF1-responding *Solanum* was retrieved from the Sol*R*gene database (Vleeshouwers et al. 2011), and shows that INF1-recognizing species are widely distributed in at least 5 countries of both Central and South America (Figure 1B).

Table 1. List of *Solanum* genotypes that show cell death to INF1 after PVX agroinfection with pGR106-INF1. The *Solanum* accessions originate from diverse geographic locations and belong to various taxonomic series of *Solanum* section *Petota* (Hawkes 1990, Jacobs 2008, Jacobs et al. 2008). Presence (+) or absence (-) of response to PVX agroinfection is indicated.

Series	*Solanum* species	Abbreviation	Genotype	Country of origin	PVX agroinfection		
					pGR106-INF1	pGR106	pGR106-CRN2
Demissa	*S. demissum*	DMS	582-1	Mexico	+	-	+
Demissa	*S. demissum*	DMS	364-1	Mexico	+	-	+
Demissa	*S. demissum*	DMS	585-1	Mexico	+	-	+
Demissa	*S. edinense*	EDN	150-4	Mexico	+	-	+
Demissa	*S. edinense*	EDN	151-1	Mexico	+	-	+
Demissa	*S. semidemissum*	SEM	295-1	na	+	-	+
Demissa	*S. species*	SPEC	891-1	Bolivia	+	-	+
Longipedicellata	*S. papita*	PTA	369-1	Mexico	+	-	+
Longipedicellata	*S. papita*	PTA	767-1	Mexico	+	-	+
Longipedicellata	*S. stoloniferum*	STO	389-4	Mexico	+	-	+
Megistacroloba	*S. astleyi*	AST	114-5	Bolivia	+	-	+
Megistacroloba	*S. megistacrolobum toralapanum*	TOR	705-2	Bolivia	+	-	+
Megistacroloba	*S. megistacrolobum toralapanum*	TOR	704-4	Bolivia	+	-	+
Piurana	*S. piurana*	PUR	7782-24 [1]	Peru	+	-	+
Piurana	*S. piurana*	PUR	206-2	Peru	+	-	+
Piurana	*S. piurana*	PUR	206-1	Peru	+	-	+
Piurana	*S. tuquerrense*	TUQ	299-4	na	+	-	+
Polyadenia	*S. lesteri*	LES	358-2	Mexico	+	-	+
Tuberosa	*S. huancabambense*	HCB	354-1	Mexico	+	-	+
Tuberosa	*S. huancabambense*	HCB	353-8	Mexico	+	-	+
Tuberosa	*S. microdontum*	MCD	360-1	Argentina	+	-	+
Tuberosa	*S. microdontum*	MCD	360-8	Argentina	+	-	+

[1] F1 offspring of *S. piurana* 206-1 *x S. chomatophilum* 559-1

Continued Table 1. List of *Solanum* genotypes that show cell death to INF1 after PVX agroinfection with pGR106-INF1. The *Solanum* accessions originate from diverse geographic locations and belong to various taxonomic series of *Solanum* section *Petota* (Hawkes 1990, Jacobs 2008, Jacobs et al. 2008). Presence (+) or absence (-) of response to PVX agroinfection is indicated.

Series	*Solanum* species	Abbreviation	Genotype	Country of origin	PVX agroinfection		
					pGR106-INF1	pGR106	pGR106-CRN2
Tuberosa	*S. mochiquense*	MCQ	186-2	Peru	+	-	+
Tuberosa	*S. venturii*	OKA	741-1	Argentina	+	-	+
Tuberosa	*S. venturii*	OKA	366-2	Argentina	+	-	+
Tuberosa	*S. venturii*	OKA	367-1	Argentina	+	-	+
Tuberosa	*S. venturii*	VNT	250-2	Argentina	+	-	+
Tuberosa	*S. microdontum gigantophyllum*	GIG	712-6	Bolivia	+	-	+
Tuberosa	*S. phureja*	PHU	371-7	Colombia	+	-	+
Tuberosa	*S. phureja*	PHU	200-4	Colombia	+	-	+
Tuberosa	*S. species*	SPEC [2]	253-1	Peru	+	-	+
Yungasensa	*S. chacoense*	CHC	543-5	Bolivia	+	-	+

[2] Unclassified *Solanum* species (Jacobs 2008)

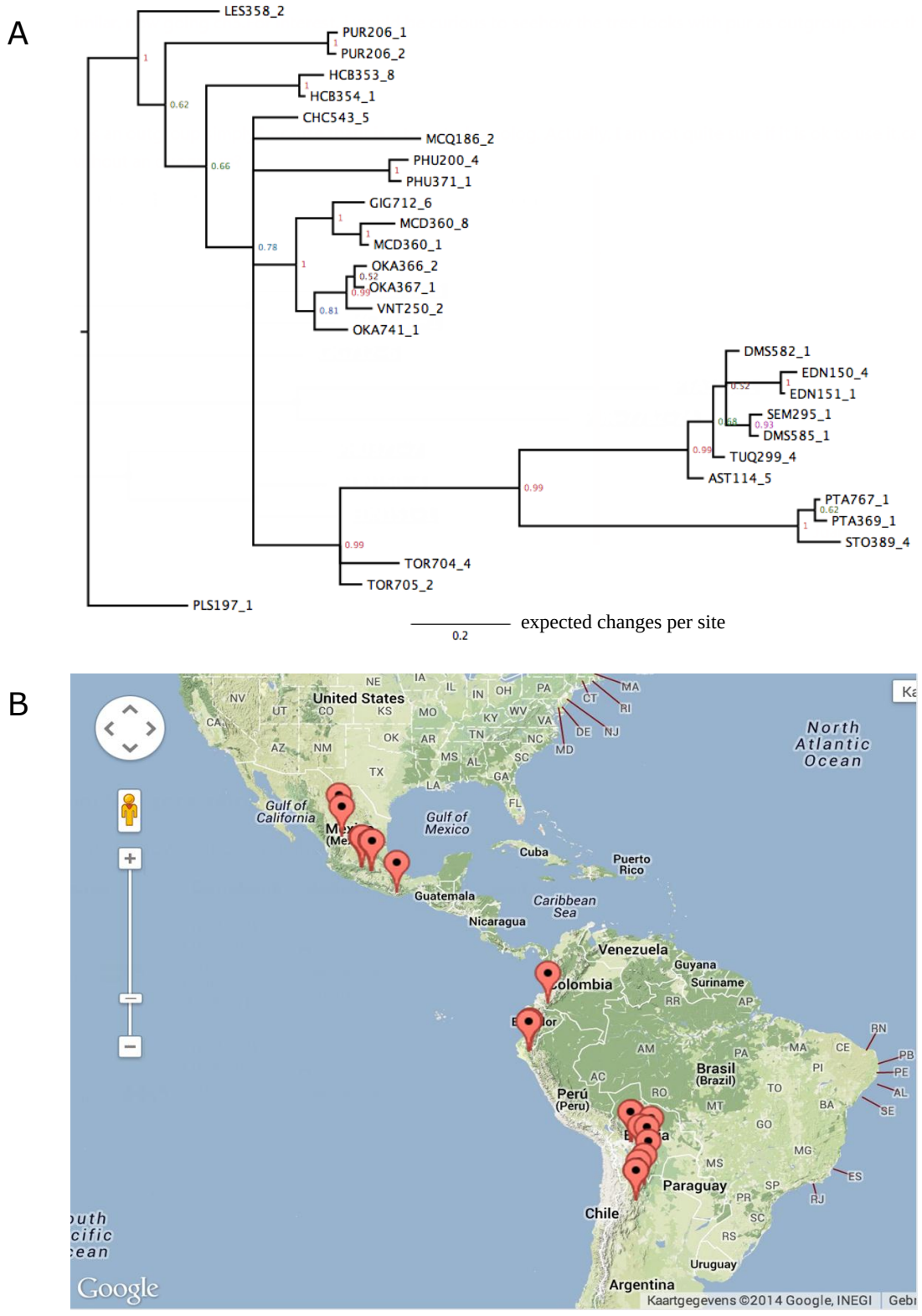


Figure 1. INF1-recognizing species are widely distributed and phylogenetically diverse.

(A) A phylogenetic tree of 28 identified INF1-recognizing genotypes created by MrBayes 3.2 (Table 1). The branch length represents expected changes per site and the number in each branch note is the prior probability. *S. palustre* (PLS) 197-1 is used as an outgroup. **(B)** Natural distribution of 21 identified INF1-recognizing *Solanum* accessions (Vleeshouwers et al. 2011).

Seven INF1-recognizing *ELR* orthologs have been identified

Since the response to INF1 is widely conserved in tuber-bearing *Solanum* species, we hypothesized that those species contain homologs of *ELR*. To explore the natural variation of *ELR* homologs, we mined the *ELR* alleles from 20 identified INF1-recognizing *Solanum* genotypes of 15 species (Table 2). Genomic DNA was used as PCR templates and conserved primers designed for cloning the full ORF of *ELR* homologs. Then PCR products were cloned into a Gateway vector (pDonr221) and transferred into *E. coli*. For each genotype, 24 *E. coli* colonies were subjected to qPCR. High resolution melting curves were compared and grouped based to their profiles (Hofinger et al. 2009). Based on these data, a few amplicons were selected and cloned into the expression vector pK7WG2 for functional tests (Table 2). Agro-co-infiltration with pK7WG2-INF1 was performed for each recombinant vector in *Solanum hjertingii* (HJT) 349-3, a wild *Solanum* genotype that does not respond to INF1. Confluent cell death was found in the leaf panel co-infiltrated with the mixture of pK7WG2-INF1 and 7 pK7WG2-amplicons that were cloned from *Solanum chacoense* (CHC543-5), *S. piurae* (PUR7782-24), *S. edinense* (EDN151-1), *S. microdontum* spp. *gigantophyllum* (GIG712-6), *S. papita* (PTA369-1), *S. edinense* (EDN150-4) and *S. phureja* (PHU371-7) (Figure 2). The remaining 29 *ELR* homologs did not show cell death when coexpressed with INF1, and these show amino acids sequence identities ranging from 72% - 93% (Table 2 and Table S1). We conclude that 7 *ELR* orthologs that recognize INF1 have been identified.

ELR is under purifying selection

To further study the INF1-recognizing ELR orthologs, we generated an amino acids sequence alignment. The newly identified ELR homologs are highly similar to ELR with sequence identities ranging from 93% - 99% to ELR, and 93% - 100% to each other (Table 3 and Figure 3). Most of the polymorphic amino acids occur in the non-LRR island domain between LRR31 and LRR32, C-terminal of LRR36 and in the B domain at N-terminal of LRR1 (Figure 3). In contrast, regions between LRR4 - LRR7, LRR13 - LRR14 and LRR1, 3, 8-10, 15, 16, 21-22, 25-26, 30-31 and 34 are 100% conserved.

The phylogenetic relationship between the INF1-responding ELR orthologs was visualized by generating a phylogenetic tree. As an outgroup, we included ELR ortholog cloned from PUR7782-24 (Figure 4). The gene tree of ELR reflects the phylogenetic structure of *Solanum* species (Jacobs et al. 2008). This suggests that ELR evolved early in or before the divergence of the included *Solanum* section *Petota* species. Furthermore, Ka/Ks analysis shows that ELR is under purifying selection (Xiao Lin, data not shown).

Table 2. *ELR* homologs were amplified from diverse *Solanum* species and subjected to agro-co-infiltrations with INF1 in *Solanum hjertingii* 349-3. Seven ELR homologs showed response to INF1.

Series	*Solanum* species	Genotype	Clone no.	Response to INF1
Demissa	*S. demissum*	DMS582-1	21	NO
	S. edinense	EDN150-4	4A-1	NO
			19	**YES**
		EDN151-1	9-1	**YES**
			9-11	NO
	S. semidemissum	SEM295-1	16	NO
Longipedicellata	*S. papita*	PTA369-1	8-16	**YES**
		PTA767-1	10	NO
Megistacroloba	*S. megistacrolobum toralapanum*	TOR704-4	V11-3	NO
Piurana	*S. piurana*	PUR7782-24	V3-8	**YES**
		PUR7782-27	V4-1	NO
			V4-9	NO
		PUR206-2	11	NO
Polyadenia	*S. lesteri*	LES358-2	6	NO
Tuberosa	*S. microdontum gigantophyllum*	GIG712-6	1A-2	NO
			1A-5	NO
			1A-16	NO
			1A-24	**YES**
	S. huancabambense	HCB354-1	7-1	NO
	S. microdontum	MCD360-1	2	NO
			5	NO
		MCD360-8	V14-1	NO
			V14-14	NO
	S. mochiquense	MCQ186-2	V9-1	NO
	S. venturii	OKA366-2	V15-2	NO
			V15-4	NO
			V15-6	NO
	S. phureja	PHU371-7	V13-1	NO
			V13-6	NO
			V13-10	**YES**
			V13-13	NO
	S. species	SPEC253-1	18	NO
			12-1	NO
			12-9	NO
			12-11	NO
Yungasensa	*S. chacoense*	CHC543-5	10-2	**YES**

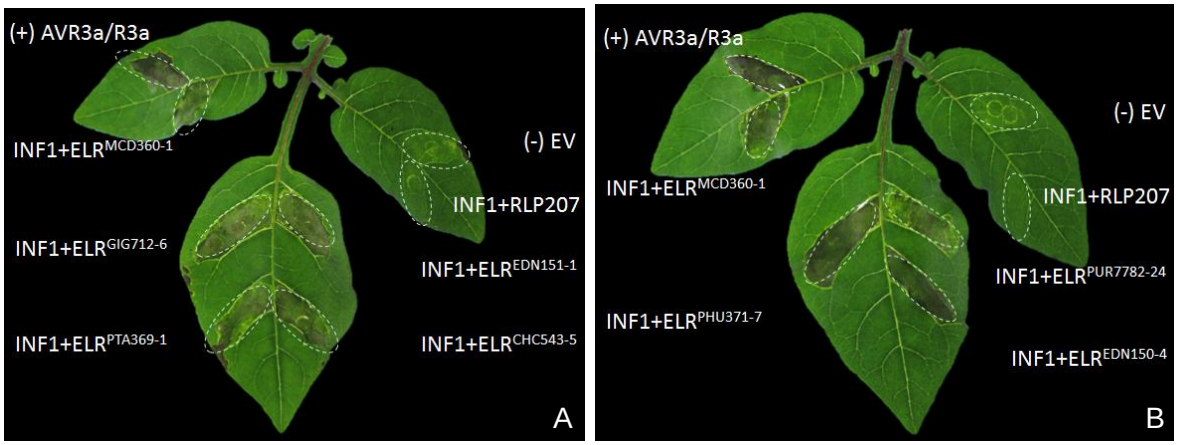


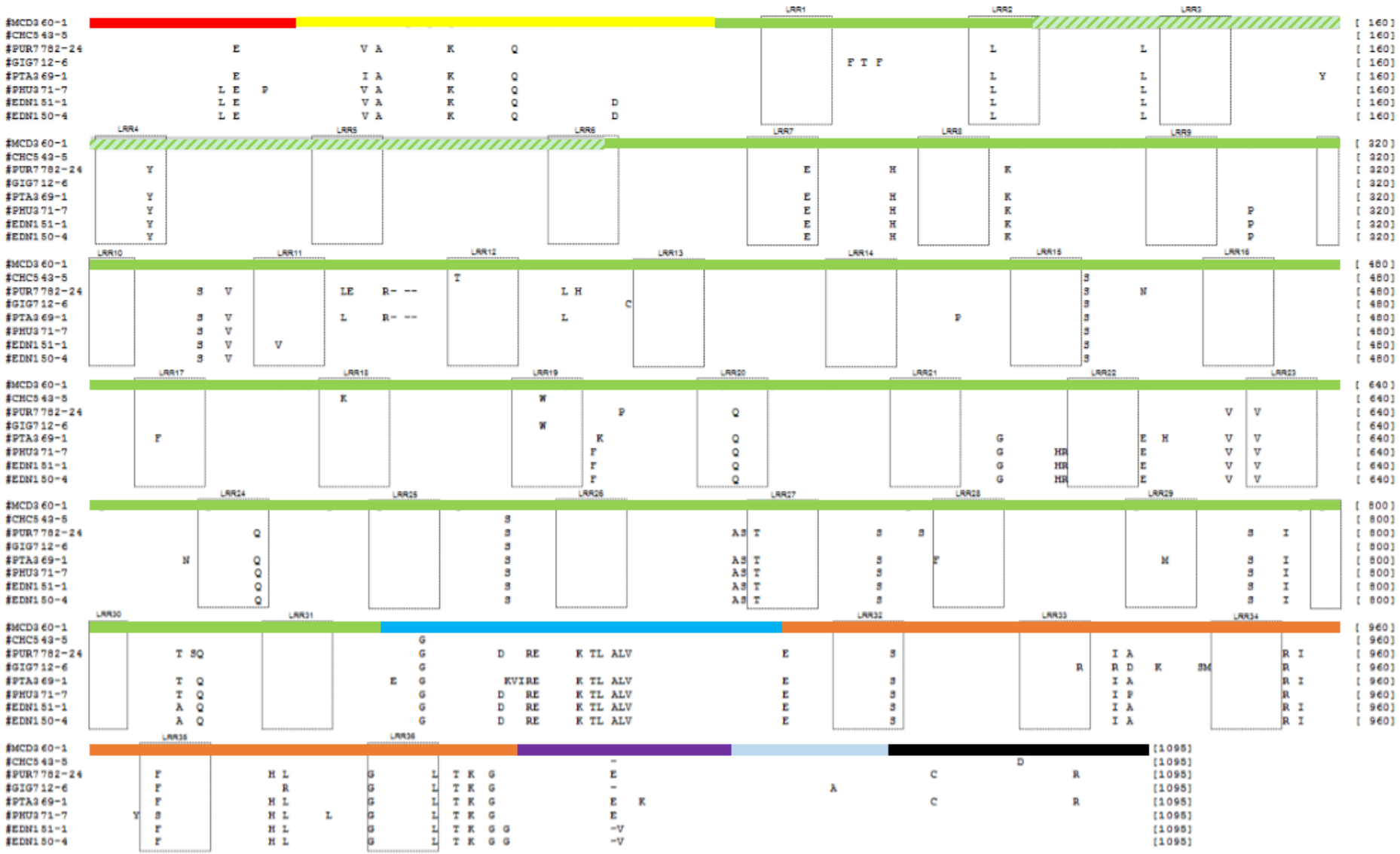
Figure 2. Seven *ELR* homologs respond to INF1 by agro-co-infiltration in the wild potato HJT349-3.

Agro-co-infiltration of pK7WG2-ELR homologs with pK7WG2-INF1 in HJT349-3 identified 7 INF1-recognizing *ELR* orthologs. 4-5-week-old plants were agro-co-infiltrated with the different recombinant *A. tumefaciens*. Confluent cell death was detected in the leaf panels co-infiltrated with a mixture of pCB302-3-INF1 and 7 pK7WG2-ELR homologs, which are cloned from GIG712-6, PTA369-1, EDN151-1, CHC543-5 **(A)**, and PHU371-7, PUR7782-24, EDN150-4 **(B)**. Confluent cell death also showed in the leaf panels co-infiltrated with a mixture of pBINplus-R3a and pK7WG2-AVR3a (Armstrong et al. 2005, Huang et al. 2005) or a mixture of pCB302-3-INF1 and pK7WG2-ELR (positive controls). No symptom showed to agroinfiltrated empty pK7WG2 or agro-co-infiltrated pCB302-3-INF1 and pK7WG2-RLP207 (negative controls). Photos were taken at 3 d after infiltrations.

Table 3. Sequence identity matrix of ELR orthologs

Amino acid sequences were aligned by BioEdit. Data in the table denote sequence identities between each two ELR orthologs. ID means identical.

Sequence	MCD 360-1	CHC 543-5	PUR 7782-24	GIG 712-6	PTA 369-1	PHU 371-7	EDN 151-1	EDN 150-4
MCD360-1	ID							
CHC543-5	99%	ID						
PUR7782-24	94%	94%	ID					
GIG712-6	98%	98%	94%	ID				
PTA369-1	93%	93%	98%	93%	ID			
PHU371-7	94%	94%	98%	94%	97%	ID		
EDN151-1	94%	94%	98%	94%	97%	99%	ID	
EDN150-4	94%	94%	97%	94%	97%	99%	100%	ID


LRR1
LRR2
LRR3
LRR4
LRR5
LRR6
LRR7
LRR8
LRR9
LRR10
LRR11
LRR12
LRR13
LRR14
LRR15
LRR16
LRR17
LRR18
LRR19
LRR20
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LRR22
LRR23
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LRR25
LRR26
LRR27
LRR28
LRR29
LRR30
LRR31
LRR32
LRR33
LRR34
LRR35
LRR36
#MCD3 60-1 [160]
#CHC5 43-5 [160]
#PUR7 782-24 [160]
#GIG7 12-6 [160]
#PTA3 69-1 [160]
#PHU3 71-7 [160]
#EDN1 51-1 [160]
#EDN1 50-4 [160]
#MCD3 60-1 [320]
#CHC5 43-5 [320]
#PUR7 782-24 [320]
#GIG7 12-6 [320]
#PTA3 69-1 [320]
#PHU3 71-7 [320]
#EDN1 51-1 [320]
#EDN1 50-4 [320]
#MCD3 60-1 [480]
#CHC5 43-5 [480]
#PUR7 782-24 [480]
#GIG7 12-6 [480]
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#PHU3 71-7 [480]
#EDN1 51-1 [480]
#EDN1 50-4 [480]
#MCD3 60-1 [640]
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#PUR7 782-24 [640]
#GIG7 12-6 [640]
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#EDN1 51-1 [640]
#EDN1 50-4 [640]
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#GIG7 12-6 [800]
#PTA3 69-1 [800]
#PHU3 71-7 [800]
#EDN1 51-1 [800]
#EDN1 50-4 [800]
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#PTA3 69-1 [1095]
#PHU3 71-7 [1095]
#EDN1 51-1 [1095]
#EDN1 50-4 [1095]

Figure 3. Sequence alignment of 8 INF1-recognizing *ELRs*
Amino acid sequences of *ELR* and 7 INF1-recognizing orthologs were aligned by BioEdit. The LRR motif is LxxLxxLxxLDLSSNNLxGxIPxx (Jones et al. 1994) where the conserved L of the LRRs is often replaced by V, F, I, or M. The conserved motif LXXLXLSSN of 36 LRRs are highlighted in the dashed boxes. Sequence in the grey shade denotes the LRR(3-6) (from 121 aa to 226 aa) that is specific to INF1-recognizing ELR homologs, but absent in most amplified ELR variants that do not recognize INF1.

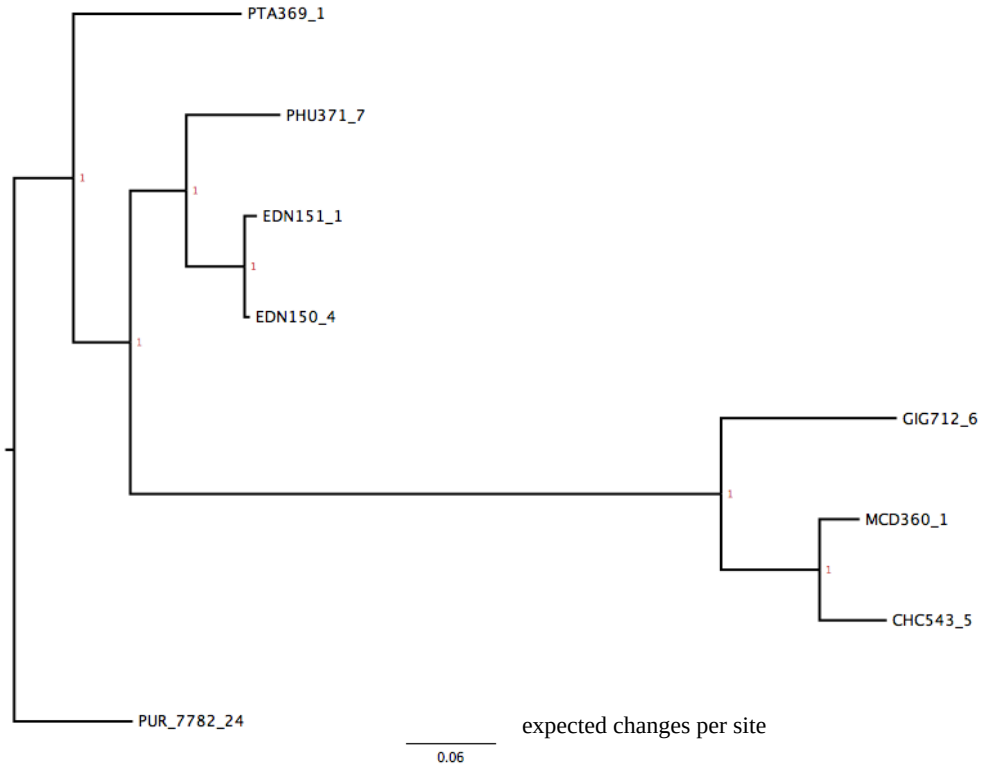

Figure 4. Phylogenetic relationship of INF1-recognizing ELRs.
A phylogenetic tree of INF1-recognizing ELRs from 8 different *Solanum* genotypes was created by MrBayes 3.2 with the homolog cloned from PUR7782-24 as an outgroup. The branch length represents expected changes per site and the number in each branch note is the prior probability.

ELR seems specific for *Solanaceae*

We performed Blast searches with the full ORF of the ELR amino acid sequence against diverse released genome databases of sequenced plants e.g. potato (*Solanaceae*) (Xu et al. 2011), *Nicotiana benthamiana* (*Solanaceae*) (Bombarely et al. 2012), tomato (*Solanaceae*) (Sato et al. 2012), *Brassica rapa* (*Cruciferae*) (Wang et al. 2011), *Arabidopsis* (*Cruciferae*) (Kaul et al. 2000), soybean (*Leguminosae*) (Schmutz et al. 2010), *Medicago truncatula* (*Leguminosae*) (Young et al. 2011), strawberry (*Rosaceae*) (Shulaev et al. 2011), rice (*Poaceae*) (Matsumoto et al. 2005), maize (*Poaceae*) (Schnable et al. 2009) and *Sorghum* (*Poaceae*) (Bedell et al. 2005) (Table 4). Since BLAST results revealed no apparent orthologs in other plant species, the presence of *ELR* seems to be restricted to potato and tomato. The most identical homolog found in potato genome was PGSC0003DMT400007484 (PGSC DM 1-3) and it was included for further analysis.

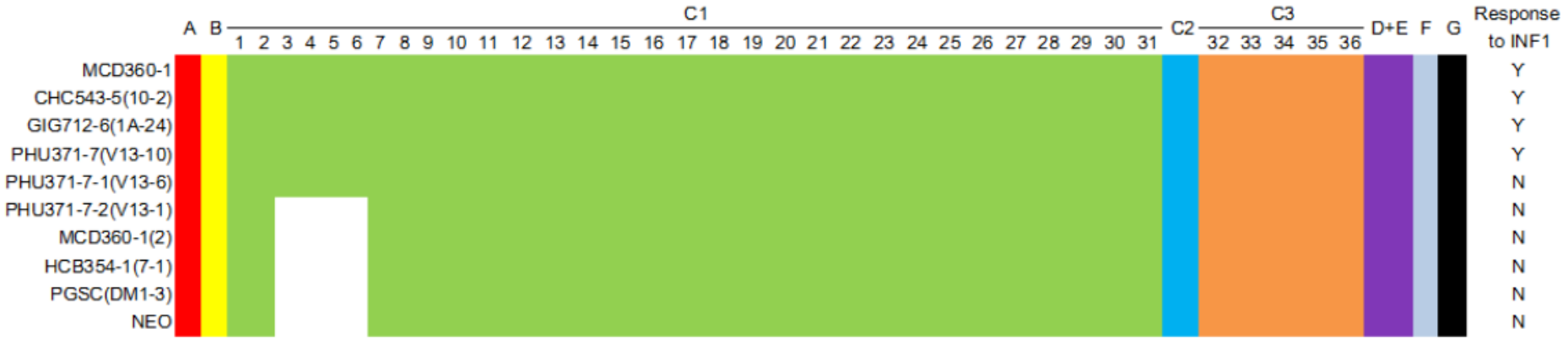

Figure 5. ELR homologs that lack LRR(3-6) do not recognize INF1.

Presented ELR homologs are from MCD360-1, CHC543-5, GIG712-6, PHU371-7, HCB354-1 (Table 2), *S. phureja* (PGSC DM 1-3) and *Solanum neorickii* (NEO). Some proteins show cell death with INF1 (Y), others do not (N). Sequences of LRR(3-6) are missing in the 5 lower genotypes. PGSC means the Potato Genome Sequencing Consortium.

Table 4. The identity of ELR to released plant genome.

Family	Species	References	Significant alignments	Identities (%)	E-value
Solanaceae	potato	(Xu et al. 2011)	PGSC0003DMT400007484	76	0
Solanaceae	tomato	(Sato et al. 2012)	Solyc12g009770.1.1	70	0
Solanaceae	*Nicotiana benthamiana*	(Bombarely et al. 2012)	NbS00014431g0006.1	46	1e-144
Cruciferae	*Arabidopsis*	(Kaul et al. 2000)	AT1G47890.1	35	1e-144
Cruciferae	*Brassica rapa*	(Wang et al. 2011)	Bra001992	32	1e-125
Leguminosae	soybean	(Schmutz et al. 2010)	Glyma16g28480.1	42	1e-180
Leguminosae	*Medicago truncatula*	(Young et al. 2011)	Medtr5g086530.1	44	0
Rosaceae	strawberry	(Shulaev et al. 2011)	gene18090	33	1e-125
Poaceae	rice	(Matsumoto et al. 2005)	LOC_Os01g06900.1	35	2.2e-121
Poaceae	maize	(Schnable et al. 2009)	18785.m000015	31	2.5e-80
Poaceae	*Sorghum*	(Bedell et al. 2005)	Sb03g005070.1	34	1e-117

Other *Solanaceae*, such as *Nicotiana* species are also known to recognize INF1, but a true homolog of *ELR* has remained undiscovered so far. The draft genome sequence of *N. benthamiana* has not reached sufficient quality yet to draw conclusions on this aspect at this moment, but preliminary data point to potential homologs (E. Domazakis, unpublished data). Outside *Solanaceae*, various radish cultivars are known to respond to INF1 (Keizer et al. 1998). However, no evident *ELR* homologs were detected by BLAST searches in *Cruciferae* (Table 4), and based on these data we cannot conclude whether ELR homologs that recognize elicitins occur in *Cruciferae*. The possibility that response to INF1 may be conferred by other receptors than *ELR* cannot be excluded either.

A domain of 4 LRRs is specific to elicitin-recognizing ELR homologs

To identify the essential domains to recognize INF1, we also sequenced the INF1-nonrecognizing *ELR* homologs from the wild *Solanum* species (Table 2). We aligned the amino acid sequences of ELR, 3 INF1-recognizing orthologs, 4 INF1-nonrecognizing homologs, $ELR^{PGSC\ DM\ 1\text{-}3}$ that is retrieved from the potato genome (Table 4) and ELR^{NEO} from *Solanum neorickii* (NEO) (Table S1), a wild tomato species (Faino et al. 2012). The most striking difference was the presence of four extra LRR domains in INF1-recognizing ELR orthologs, whereas this sequence is generally lacking in homologs that do not recognize INF1. This sequence strech reaches from aa121 to aa226 of ELR, and covers LRR 3 to LRR 6. In the alignment of 8 INF1-recognizing ELR orthologs, we found LRR(3-6) highly conserved and only noted 2-3 SNPs (Figure 3), which suggests that it is likely to be important for ELR function. In a few INF1-nonrecognizing ELR homologs like $ELR^{PHU371\text{-}7\text{-}1}$ the LRR(3-6) is also present (Figure 5), but those genes have multiple SNPs elsewhere throughout the gene, which may also affect activity.

To test the role of LRR(3-6), domain swap experiments were initiated. Chimeric ELR proteins were generated from the original INF1-recognizing ELR of MCD360-1 and two non-recognizing ELRs, namely, $ELR^{PHU371\text{-}7\text{-}1}$ and $ELR^{PHU371\text{-}7\text{-}2}$ (Figure 6A). $ELR^{PHU371\text{-}7\text{-}1}$ contains LRR(3-6) while $ELR^{PHU371\text{-}7\text{-}2}$ does not. Recombinant ELRs #1.4, #1.6, #3.2 and #5.2 were cloned into the expression vector pK7WG2 and transformed into *Agrobacterium* strain AGL1. Then they were tested by agro-co-infiltration with INF1 in *S. hjertingii* 349-3. Results show that only ELR#3.2 responds to INF1 while ELR#5.2 does not (Figure 6B). ELR#3.2 contains LRR(3-6) while ELR#5.2 does not. These data suggests that LRR(3-6) is likely to be essential for response to INF1. However, other amino acids that differ between $ELR^{PHU371\text{-}7\text{-}1}$ and $ELR^{PHU371\text{-}7\text{-}2}$, i.e. 17 polymorphic amino acids in domain B and C1-1, may also play an essential role in the INF1 recognition (Figure 7).

Recombinant ELR#1.6 did not recognize INF1. This suggests that differences in part 6 (C-terminal from domain C1-7, Figure 7) of $ELR^{PHU371\text{-}7\text{-}2}$ compromise INF1 response. Compared part 6 with the 8 INF1-recognizing ELR orthologs, 47 polymorphic aa could be excluded while a deletion in the C-domain between C3-32 and C3-34 and an amino acid mutation of L to Q in the C1 domain could be the

cause for the loss of INF1 response (Figure 7). Further studies are required to test whether such amino acids are essential for INF1 response. In addition, e.g. complementation of ELR#5.2 with LRR(3-6) of ELR could provide further information on the importance of these four LRRs for INF1 response. To sum up, although further experiments are required, we suspect that LRR(3-6) is likely to be important for the recognition of INF1 by ELR.

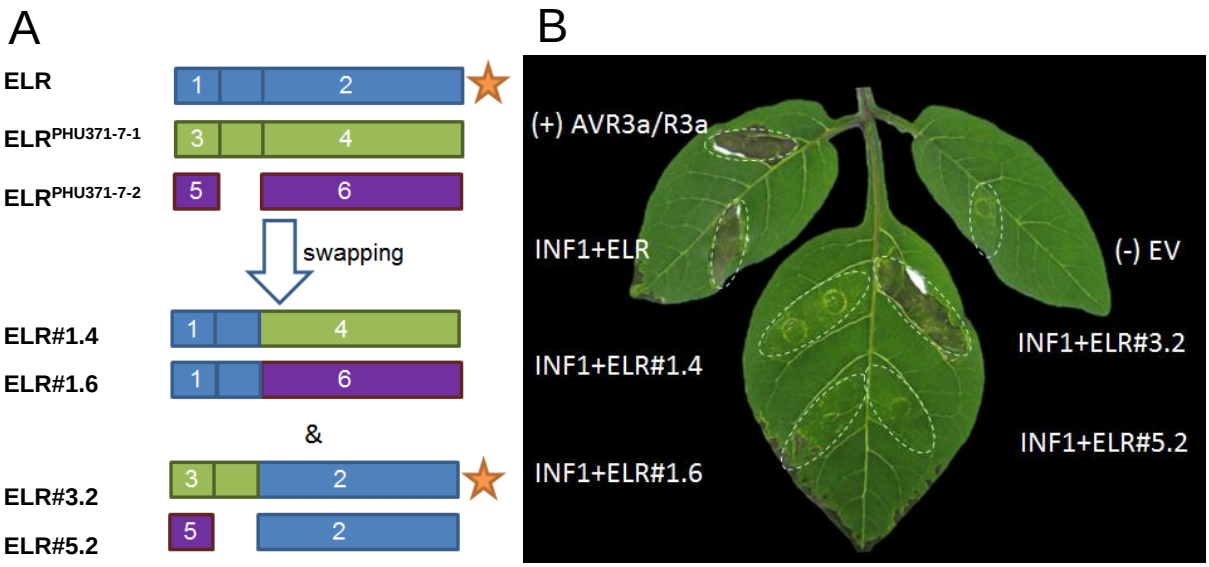

Figure 6. Domain swaps of extra LRRs.

(A) Illustration of domain swaps. ELR and two ELR homologs ELR$^{PHU371-7-1}$ and ELR$^{PHU371-7-2}$ that do not respond to INF1 were used. Chimeric ELR proteins were generated as indicated. The asterisk marks the ELR that show cell death when co-expressed with INF1. B) A photo of agro-co-infiltration of pCB302-3-INF1 and pK7WG2-chimeric ELRs in HJT349-3. 4-5-week-old plants were transiently co-infiltrated with the different recombinant *A. tumefaciens*. Confluent cell death showed in the leaf panel co-infiltrated with a mixture of pBINplus-R3a and pK7WG2-AVR3a (Armstrong et al. 2005, Huang et al. 2005) (positive control). No symptom showed to agroinfiltrated empty vector (EV) pK7WG2 (negative control). Cell death showed in the leaf panel co-infiltrated with a mixture of pCB302-3-INF1 and pK7WG2-ELR#3.2 by a 1:1 ratio. No symptom showed in the leaf panel co-infiltrated with a mixture of pCB302-3-INF1 and pK7WG2-ELR#1.4, pK7WG2-ELR#1.6 or pK7WG2-ELR#5.2. Photos were taken at 4 d after infiltrations.

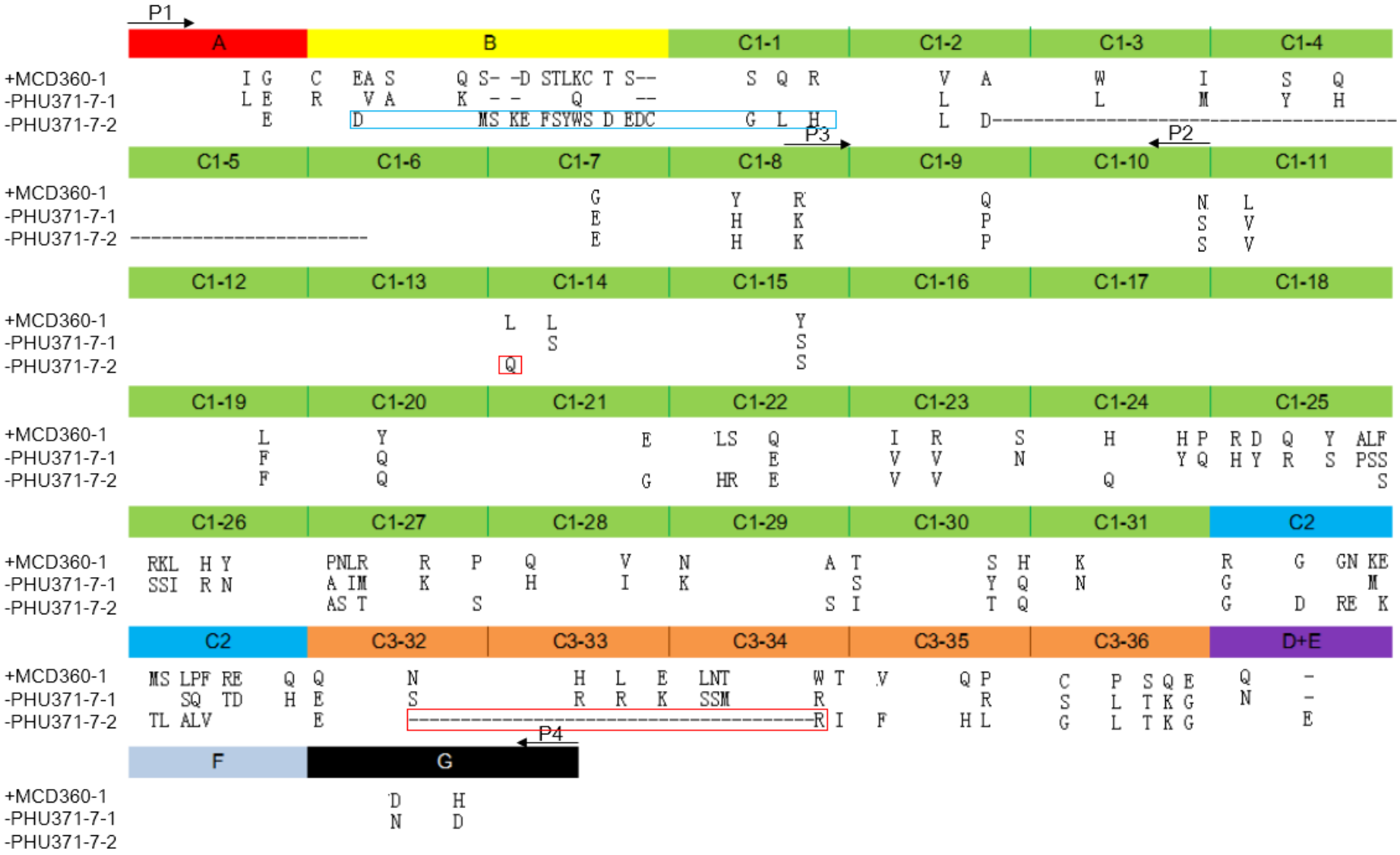
P1
A
B
C1-1
C1-2
C1-3
C1-4
+MCD360-1
-PHU371-7-1
-PHU371-7-2
P3
P2
C1-5
C1-6
C1-7
C1-8
C1-9
C1-10
C1-11
C1-12
C1-13
C1-14
C1-15
C1-16
C1-17
C1-18
C1-19
C1-20
C1-21
C1-22
C1-23
C1-24
C1-25
C1-26
C1-27
C1-28
C1-29
C1-30
C1-31
C2
C3-32
C3-33
C3-34
C3-35
C3-36
D+E
P4
F
G

Figure 7. Alignment of ELR sequences for domain swaps.

The protein is divided into eight domains from A to G as described in Wang et al. (2008) and Wulff et al. (2009) A: a putative signal peptide, B: a Cys-rich or mature N-terminus, C: LRR domain subdivided into three subdomains, where C1 and C3 are LRR region and C2 a non-LRR island, D: a spacer, E: an acidic domain, F: a transmembrane domain and G: a short cytoplasmic region. "+" and "-" before the abbreviations mean functional and non-functional individually. Primer 1 (P1) is from the start codon, P2 from 975 nt to 998 nt, P3 from 839 nt to 863 nt and P4 ends in stop codon. The sequence above the dashed line from C1-2 to C1-6 indicates the four extra LRRs. In the blue box shows 17 amino acids difference of $ELR^{PHU371-7-2}$ compared to the original ELR. In the red box shows a deletion between C3-32 and C3-34.

Elicitin response patterns are similar in *Nicotiana* and *Solanum* species

To study whether the seven newly identified ELR orthologs have altered recognition specificities compared to ELR, they were co-infiltrated with diverse elicitins of *Phytophthora* and *Pythium* species in HJT349-3. Besides, we also directly tested the elicitin responses in *Nicotiana* species, for which the putative ELR ortholog has not been identified. In total, 16 selected elicitins (**Chapter 4** Figure 2) were tested in *N. benthamiana* and *N. tabacum* cv. Xanthii by agroinfiltration. Results showed that most elicitins caused cell death in *Nicotiana* species except INF4, INL1, INL4A and PYU1, (Table 5, Figure 8A). Similar response patterns were found for ELR and the 7 orthologs. However, quantitative responses were found for INF2B in both *Nicotiana* species, also for SOJB and CRY2 by co-infiltration with ELR-PTA369-1. This may point to slightly altered recognition levels by respective ELR homologs. Typical illustrations of pK7WG2-INF1 and pK7WG2-ELR-orthologs are shown in Figure 8C. Our data suggest that elicitin-triggered defense response patterns are conserved in *Nicotiana* and *Solanum* species and that *Nicotiana* contains ELR homologs with similar recognition specificity. However, to exclude the possibility that lack of response to INF4, INF7, INL4A and PYU1 is due to e.g. loss of protein stability, additional experiments are required.

Table 5. Elicitins response patterns are conserved in *Solanum* and *Nicotiana* species.

Tests of 16 selected elicitins (**Chapter 4** Figure 2) show that elicitin-triggered defense responses are conserved between *Solanum* and *Nicotiana* species. Elicitins were directly agroinfiltrated in *Nicotiana speices* while agro-co-infiltrated with different ELR orthologs in HJT349-3. Typical photos of responses in red boxes are shown in Figure 8. +/- means that cell death was not confluent. RLP207 is the closest paralog of $ELR^{MCD360-1}$ and is included as a negative control, since it does not recognize INF1 (**Chapter 4** Figure 1). As expected, RLP207 could not recognize any of the tested elicitins.

Cell death	pK7WG2	INF1	INF2A	INF2B	INF4	INF5	INF6	INF7	PARA1	CRY1	RAM1	CIN	SOJB	CRY2	BRA1	INL4A	PYU1
N. benthamiana	-	+	+	+/-	-	+	+	-	+	+	+	+	+	+	+	-	+/-
***N. tobacum* cv. Xanthii**	-	+	+	+/-	-	+	+	-	+	+	+	+	+	+	+	-	+/-
ELR-MCD360-1	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	-
ELR-CHC543-5	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	-
ELR-PUR7782-24	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	-
ELR-GIG712-6	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	-
ELR-PTA369-1	-	+	+	-	-	+	+	-	+	+	+	+	+/-	+/-	+	-	-
ELR-PHU371-7	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	-
ELR-EDN151-1	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	-
ELR-EDN150-4	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	-
RLP207-MCD360-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

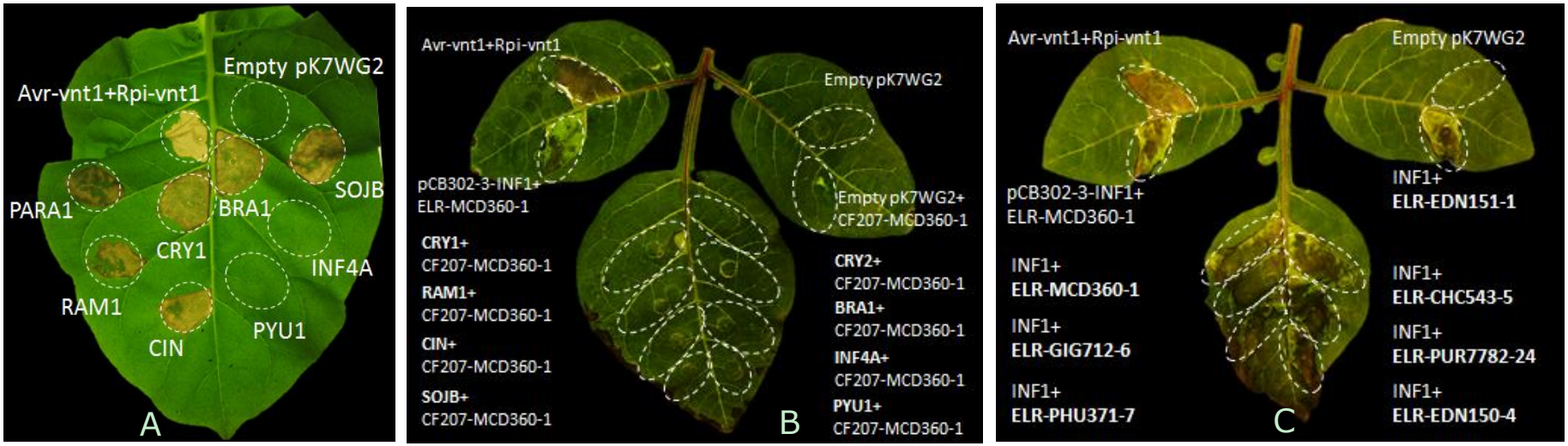

Figure 8. Typical photos of elicitin responses in Table 5.

Typical photos are shown for responses in red boxes of Table 5. In 4-5-week-old Xanthii plants, confluent cell death are found in the leaf panels infiltrated with pK7WG2-PARA1, pK7WG2-CRY1, pK7WG2-RAMa1, pK7WG2-CI16, pK7WG2-SOJ1B and pK7WG2-BRA1 but not with pK7WG2-INL4A, pK7WG2-PYU1 **(A)**. In 4-5-week-old HJT349-3 plants, no responses was found in the leaf panels co-infiltrated with a mixture of pK7WG2-RLP207 (a paralog of ELR cloned from MCD360-1) **(B)**. In contrast, confluent cell death was found in the leaf panels co-infiltrated with a mixture of pK7WG2-INF1 and 7 pK7WG2-ELR homologs **(C)**.

INF1-triggered cell death is suppressed by AVR3a^{KI} in potato

Previous research showed that INF1-mediated cell death is suppressed by AVR3a^{KI} in *N. benthamiana* (Bos et al. 2006). To test if the suppression also happens in potato, we agro-co-infiltrated a mixture of pK7WG2-Avr3a^{KI}, pK7WG2-ELR and pK7WG2-INF1 in the leaf panels of HJT349-3. Also CRY1, which is 90% identical at the aa level compared to INF1 (**Chapter 4** Figure S3E) was subjected to the same test. For both elicitins, the Avr3a-mediated suppression of cell death response was detected in potato, similar as in *Nicotiana* (Figure 9).

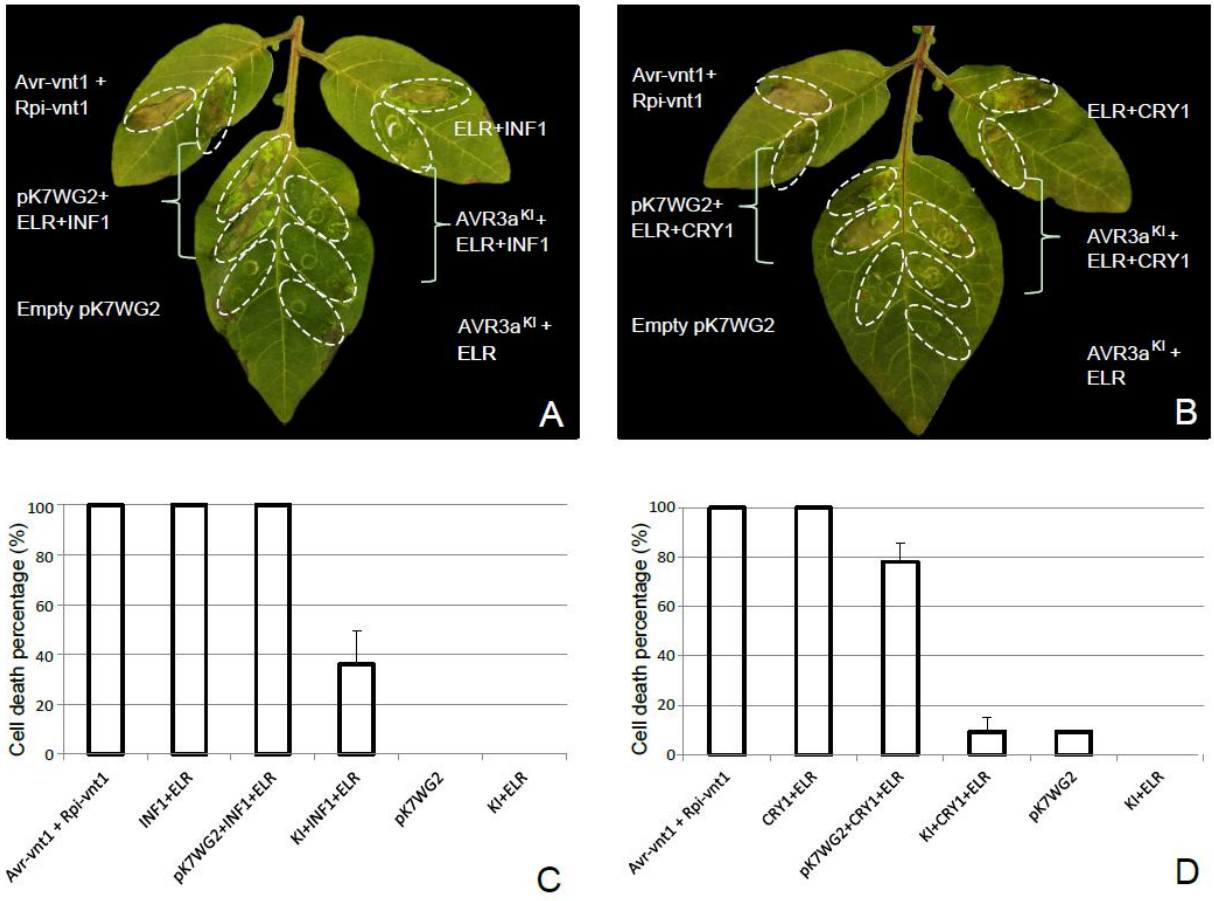

Figure 9 Hypersensitive response of INF1 or CRY1 and ELR is suppressed by AVR3a^{KI} on HJT349-3.

Agro-co-infiltration of pK7WG2-Avr3a^{KI} and pK7WG2-ELR with pK7WG2-INF1 or pK7WG2-CRY1 in HJT349-3 show reduced cell death. 4-5-week-old plants were transiently co-infiltrated with the different recombinant *A. tumefaciens*. Confluent cell death showed in the leaf panel co-infiltrated with a mixture of pGRAB-Rpi-vnt1 and pK7WG2-AVR-vnt1 (Foster et al. 2009, Pel et al. 2009, Pel 2010) (positive control). No symptom showed to agroinfiltrated empty pK7WG2 (negative control). Confluent cell death is found in the leaf panel co-infiltrated with a mixture of pK7WG2-ELR and pK7WG2-INF1 **(A)** or pK7WG2-CRY1 **(B)**, or with empty pK7WG2 by a 1:1 or 1:1:1 ratio. Cell death showed in the leaf panels co-infiltrated with a mixture of pK7WG2-Avr3a^{KI}, pK7WG2-ELR with pK7WG2-INF1 **(C)** or pK7WG2-CRY1 **(D)** decreased to around 40% and 10%, respectively. Photos were taken at 4 d after infiltrations.

Discussion

To explore evolutionary characteristics of ELR, the genetic variation of ELR in various plant species was exploited. In a screening test of 115 *Solanum* genotypes covering a broad range of *Solanum* section *Petota* (Jacobs et al. 2008, Vleeshouwers et al. 2011), 32 genotypes showed necrotic responses to INF1. They are found widely distributed in both Central and South America, and are located in different taxa of the phylogenetic tree of *Solanum* species (Rietman 2011). This suggests that ELR existed before the speciation of the tuber-bearing *Solanum* section *Petota*.

How ancient is *ELR*? The presence of *ELR* appears to be restricted to *Solanaceae* species, since BLAST results revealed no apparent orthologs in species out of this plant family e.g. *Brassica rapa* (*Cruciferae*) (Wang et al. 2011), *Arabidopsis* (*Cruciferae*) (Kaul et al. 2000), soybean (*Leguminosae*) (Schmutz et al. 2010), *Medicago truncatula* (*Leguminosae*) (Young et al. 2011), strawberry (*Rosaceae*) (Shulaev et al. 2011), rice (*Poaceae*) (Matsumoto et al. 2005), maize (*Poaceae*) (Schnable et al. 2009) and *Sorghum* (*Poaceae*) (Bedell et al. 2005). This finding is similar to the situation of ReMAX/RLP1, which appears to be restricted to species of *Brassicales* (Jehle et al. 2013). Besides, more *Solanum* RLPs like *Ve1*, *Cf-4*, *Cf-9* and *Eix2* found in tomato have no functional counterpart in *Arabidopsis* (Fradin et al. 2011). It seems that some RLPs with functions as PRRs have evolved and diversified by means of reduplication and shuffling of LRR subdomains (Wang et al. 2010). Similar events might have happened for LRR(3-6) reported in this chapter. However, elicitins also cause HR in some cultivars of *Brassica rapa* (rape) and *Raphanus sativus* (radish), and these could be conferred by a homolog of *ELR* that may have diversified or be absent in the model plant *Arabidopsis* (Kamoun et al. 1993, Keizer et al. 1998, Takemoto et al. 2005). Alternatively, the elicitin response in rape and radish might be caused by other defense mechanisms in *Brassicaceae* species.

In the structure-functional analysis of ELR, we found that INF1-recognizing ELRs normally contain a subdomain of four extra LRRs (3-6). This suggests that the LRR(3-6) domain is essential for INF1-mediated cell death. Domain swap results of INF1-recognizing $ELR^{MCD360-1}$ and INF1-nonrecognizing $ELR^{PHU371-7-1}$ that both contain LRR(3-6) indicate several potential essential domains for INF1 recognizing. Further domain swap and site-directed mutation experiments need to be performed to identify which domains or amino acids are essential for INF1 recognition.

The phenomenon that elicitin could cause HR in *Nicotiana* species was discovered much earlier than that was found in *Solanum* species (Ricci et al. 1989, Vleeshouwers et al. 2006). However, molecular cloning of elicitin receptors from *Nicotiana* species has been hampered because of little genetic variation in response to elicitin (Kamoun et al. 1993) and the difficulty of map-based cloning in *Nicotiana* species. In this study, we found that elicitin response patterns are conserved between *Nicotiana* and *Solanum* species, although the stability of elicitin proteins still needs to be tested. In

addition, we found that INF1-triggered cell death can also be suppressed by AVR3a^{KI} in a wild potato HJT349-3, similar as in *N. benthamiana* as previously reported (Bos et al. 2006). All these data suggest that a similar elicitin perception system is likely to be conserved between *Nicotiana* species and potato. Most likely, elicitins are recognized by an ELR ortholog of *Nicotiana* species. According to the BLAST searches against the *N. benthamiana* genome database, we found a homolog, of which partial sequences have high similarity to ELR. It needs further functional analysis whether this putative homolog is a functional ELR ortholog and elicitin receptor. As elicitins evoke stronger responses in *Nicotiana* than in *Solanum* (Vleeshouwers et al. 2006), potentially the ELR homolog in *Nicotiana* could enhance the resistance to *P. infestans* at higher levels.

Table S1. Sequence identity matrix of ELR homologs

Amino acid sequences were aligned by BioEdit. Data in the table denote sequence identities between each two ELR orthologs. ID means identical.

Sequence	ELR	EDN150-4(19)	EDN151-1(9-1)	PTA369-1(8-16)	7782-24(V3-8)	GIG712-6(1A-24)	PHU371-7(V13-10)	CHC543-5(10-2)	GIG712-6(1A-2)	GIG712-6(1A-16)	HCB354-1(7-1)	EDN151-1(9-11)	PHU371-7(V13-6)	PHU371-7(V13-1)	PHU371-7(V13-13)	MCD360-1(2)	MCD360-1(5)	RLP207	NEO	PGSC0003DMT400007484	Solyc12g009770.1.1
ELR	ID																				
EDN150-4(19)	94%	ID																			
EDN151-1(9-1)	94%	100%	ID																		
PTA369-1(8-16)	93%	97%	97%	ID																	
7782-24(V3-8)	94%	97%	98%	98%	ID																
GIG712-6(1A-24)	98%	94%	94%	93%	94%	ID															
PHU371-7(V13-10)	94%	99%	99%	97%	98%	94%	ID														
CHC543-5(10-2)	99%	94%	94%	93%	94%	98%	94%	ID													
GIG712-6(1A-2)	77%	75%	75%	75%	76%	78%	75%	77%	ID												
GIG712-6(1A-16)	92%	89%	90%	89%	89%	94%	90%	92%	79%	ID											
HCB354-1(7-1)	74%	73%	73%	72%	73%	74%	73%	74%	70%	72%	ID										
EDN151-1(9-11)	93%	93%	93%	92%	92%	94%	93%	93%	74%	91%	71%	ID									
PHU371-7(V13-6)	93%	93%	94%	92%	93%	93%	94%	93%	74%	90%	72%	94%	ID								
PHU371-7(V13-1)	80%	83%	83%	82%	82%	80%	83%	80%	72%	76%	75%	78%	79%	ID							
PHU371-7(V13-13)	88%	91%	92%	90%	91%	89%	92%	88%	72%	85%	71%	87%	87%	89%	ID						
MCD360-1(2)	73%	72%	72%	72%	72%	72%	72%	73%	69%	70%	84%	71%	71%	73%	68%	ID					
MCD360-1(5)	72%	71%	71%	70%	71%	71%	71%	72%	67%	70%	79%	70%	71%	72%	68%	77%	ID				
RLP207	80%	79%	79%	79%	79%	79%	79%	80%	70%	78%	76%	78%	79%	69%	75%	81%	70%	ID			
NEO	71%	70%	71%	70%	71%	71%	71%	71%	66%	70%	75%	70%	70%	71%	66%	74%	77%	69%	ID		
PGSC0003DMT400007484	70%	70%	70%	69%	69%	70%	70%	70%	66%	69%	75%	69%	69%	71%	66%	74%	75%	68%	72%	ID	
Solyc12g009770.1.1	75%	74%	75%	74%	74%	75%	75%	75%	66%	75%	62%	74%	74%	63%	70%	62%	61%	69%	63%	60%	ID

Materials and Methods

Plant manipulations

The growing and testing conditions of potato and *Nicotiana* species and plant agroinfiltration methods are according to **Chapter 3**.

Generation of expression constructs of elicitin and ELR homologs

ELR homologs were cloned using the Gateway technology (Invitrogen, SanDiego, CA, USA). Primers 85CF2.startATTB and 85CF2.stopATTB, previously used to amplify the full-length sequence of ELR (Table S2), were used to clone *ELR* homologs from different plant species, of which DNA was used as template for the PCR. The PCR program is as follows: 30 s at 98 ℃ and 33 cycles of 10 s at 98 ℃, 30 s at 58 ℃, 2 min at 72 ℃ before final elongation for 10 min at 72 ℃. The ORFs of the *ELR* homologs were first cloned into the gateway vector pDonr221 (INVITROGEN) using BP clonase (INVITROGEN). Then the ORFs of elicitins were transferred into destination vector pK7WG2 (Karimi et al. 2002) by LR-Reaction II (Untergasser 2006). Isolated plasmids for each construct were introduced by electroporation into *A. tumefaciens* strain AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG (pBBR1MCS-5.virGN54D) (van der Fits et al. 2000) for agroinfiltration and PVX agroinfection assays.

High resolution melting curve analysis

ELR homologs cloned from the INF1-recognizing *Solanum* genotypes were analysed with the LightScanner (Idaho Technology). They were first cloned into the gateway vector pDonr221 as described in the above method. Then 24 *E. coli* colonies of each genotype were selected for further analysis and used as PCR template. Primers 85CF2.start (ATGGTCATGAGTCTGTTTTTCTTTTAT) and lscan1-R (CACAAGTGACTCCATCCCAA) were used for the PCR amplification. The PCR was performed using the following cycle profile: an initial cycle at 98 ℃ for 1 min then 43 cycles of 98 ℃ for 10 s, 60 ℃ for 20 s, 72 ℃ for 30 s; followed by several final steps: 72 ℃ for 30 s, 94 ℃ for 30 s and 25 ℃ for 30 s. Then PCR products were visualized and analysed with the LightScanner (Idaho Technology).

Reconstruction of the phylogenetic relation of the INF1-recognizing species

A phylogenetic tree of 28 identified INF1-recognizing *Solanum* genotypes was constructed by MrBayes 3.2 using 200 AFLP markers (Jacobs et al. 2008, Ronquist et al. 2012). Mesquite was used for formatting data. MrBayes v3.2 was used to estimate the posterior distribution by Markov Chian Monteca Carlo (MCMC) methods. The output was diagnosed by Tracer v1.5 and the phylogenetic tree was visualized by Figuretree.

ELR sequence analysis

BLAST searches with the full ORF of ELR amino acid sequence were performed against different released plant genome databases e.g. potato (http://solanaceae.plantbiology.msu.edu/integrated_searches.shtml), *N. benthamiana*, tomato and *Arabidopsis* (http://solgenomics.net/tools/blast/index.pl), *Medicago truncatula* (http://www.medicagohapmap.org/tools/blastform), soybean (http://soybase.org/GlycineBlastPages/), strawberry (https://strawberry.plantandfood.co.nz/cgi-bin/nph-blast.cgi?Jform=0), *Brassica rapa* (http://brassicadb.org/brad/blastPage.php), rice (http://rice.plantbiology.msu.edu/analyses_search_blast.shtml), maize (http://blast.jcvi.org/er-blast/index.cgi?project=zma1), *Sorghum* (http://mips.helmholtz-muenchen.de/plant/sorghum/searchjsp/blast.jsp?organism=sorghum).

Domain swaps

The INF1-recognizing *ELR* and two INF1-non-recognizing *ELR*$^{PHU371-7-1}$ and *ELR*$^{PHU371-7-2}$ were used as templates for domain swaps. Two pairs of primers (P1 and P2) and (P3 and P4) (Table S2) were applied to amplify each gene. The N-terminal amplified PCR product of ELR$^{MCD360-1}$ was mixed with the C-terminal amplified PCR products of ELR$^{PHU371-7-1}$ and ELR$^{PHU371-7-2}$ individually as templates for further amplification with primers P1 and P4. Similarly, the C-terminal amplified PCR product of ELR$^{MCD360-1}$ was also mixed with the N-terminal amplified PCR products of ELR$^{PHU371-7-1}$ and ELR$^{PHU371-7-2}$ individually as templates for further amplification with primers P1 and P4. Then PCR products of four recombinant sequences were cloned into the expression vector pK7WG2 following the same way for cloning *ELR* homologs. Then four constructs were co-infiltrated with pCB302-3-INF1 on INF1-nonrecognizing potato HJT349-3 (Figure 6).

Acknowledgments

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Chapter 6

General discussion

General discussion

Potato late blight, caused by the oomycete pathogen *Phytophthora infestans*, is considered as one of the most devastating diseases in the world. To control this disease in an environmental-friendly way, potato breeders have been breeding resistant cultivars since the 1950s (Black et al. 1953, Mastenbroek 1953). The introgressed *R* genes all belong to the class of the intracellular NB-LRR receptors, which have shown to be quickly defeated. Research described in this thesis was performed to study another, first line of defense, namely apoplastic immunity. The rationale is based on a recent cloning of an apoplastic immune receptor - ELR (elicitin response) that can recognize *Phytophthora* elicitins. The identification of ELR was facilitated by effectoromics assays. Here we analysed the application aspects of effectoromics in potato resistance breeding (Chapter 2 and 3), functionally characterized ELR in potato (chapter 4) and studied the natural variation of ELR homologs in tuber-bearing *Solanum* species (chapter 5). Our work has: 1) resulted in useful suggestions for applying effectoromics in potato breeding, 2) provided robust and reproducible protocols for performing effectoromics assays, 3) proved that ELR can confer enhanced resistance to *P. infestans* in potato, 4) shown that ELR can recognize a broad-spectrum of elicitins from various *Phytophthora* species and induce defense responses, 5) shown that ELR associates with the immune co-receptor BAK1/SERK3, 6) discovered that ELR homologs are widely conserved in tuber-bearing *Solanum* species. These results increased our knowledge for further resistance breeding considerably. Perspectives for breeding of varieties with more durable resistance by molecular strategies are discussed.

Apoplastic PRRs-triggered immunity

To defend late blight disease, potato involved two layers to sense pathogens, namely, PRRs at the plant cell surface and NB-LRR *R* proteins inside the plant cell. However, in the past, PRR-triggered immunity was not known or used for potato resistance breeding, most likely because of its weaker resistance phenotype. Recently, it was recognized that apoplastic immunity triggered by pathogen-associated molecular patterns (PAMPs) could be more durable, because PAMPs are essential for a pathogen's life cycle and/or pathogenicity by definition (Jones and Dangl 2006, Segonzac and Zipfel 2011). Pathogens cannot avoid apoplastic immunity by losing essential PAMPs, and allelic variation of PAMPs is expected to be limited by evolutionary constraints on their structure (Bittel and Robatzek 2007, Boller and Felix 2009, McCann et al. 2012). Because of these reasons, we hypothesize that apoplastic immunity has a greater possibility for durable resistance breeding.

Response to elicitins provides another layer of defense to late blight

Studies with elicitins of diverse oomycetes revealed that the response to elicitins is widespread and conserved in *Solanum* species (**Chapter 5**). This is in line with the characteristics of PAMPs to be

widely recognized. Findings in this thesis support the hypothesis that apoplastic immunity can contribute to enhanced late blight resistance. By exploring *Solanum* germplasm using effectoromics, a wild potato genotype *Solanum microdontum* (MCD) 360-1 that shows field resistance (Figure 1) was identified to specifically respond to INF1 (Vleeshouwers et al. 2006). The INF1 receptor *ELR* was successfully isolated by map-based cloning. ELR encodes a RLP that contains an extracellular LRR, a transmembrane domain and a short cytoplasmic tail (Verzaux 2010) (**Chapter 4**). To test whether ELR confers late blight resistance, we expressed *ELR* in potato and inoculated leaves with *P. infestans*. *ELR*-transgenic potatoes indeed displayed higher levels of resistance (**Chapter 4**). Although the resistance level is not as strong as for most *R* genes that have been isolated so far, it will set the stage for future apoplastic immunity studies in the potato-*P. infestans* pathosystem.

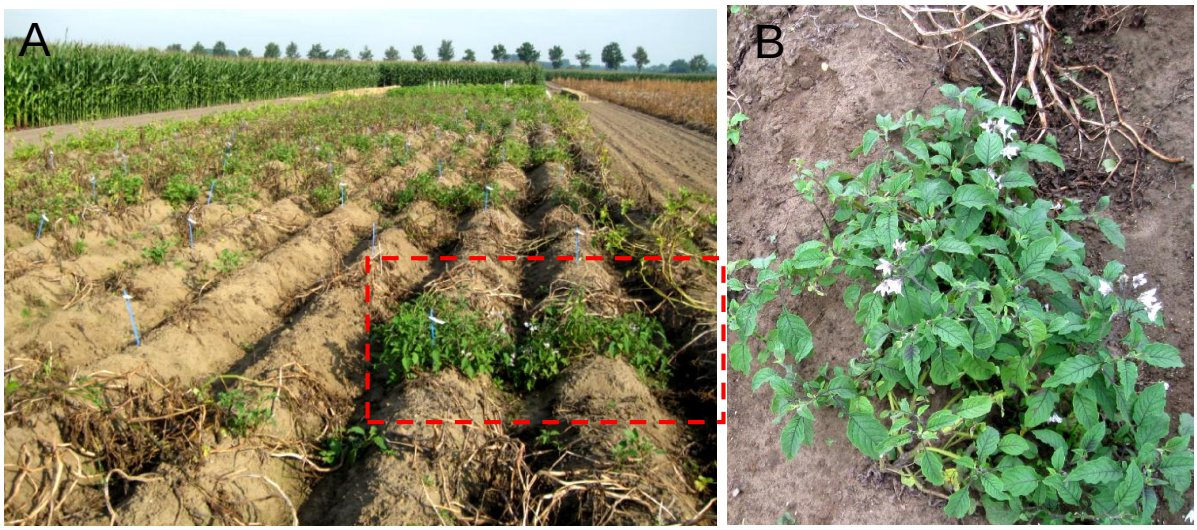

Figure 1. Potato field trial infected by *Phytophthora infestans*.
A) A potato field at 45 days after spraying of *P. infestans* inoculum. Plants in the red box are *Solanum microdontum* (MCD) 360-1 and **B)** a closer look at MCD 360-1 at 45 dpi.

Interfamily / genus transfer of ELR orthologs to potato

To explore the genetic variation of ELR in *Solanum*, we amplified *ELR* homologs from various INF1-rsponding *Solanum* genotypes. We obtained 36 ELR homologs and tested them by transient expression. For 7 ELR homologs, defense responses were induced when co-expressed with INF1 elicitin (**Chapter 5**). To test the response spectrum of ELR, elicitins from diverse oomycete species were tested by transient co-expression with ELR homologs. Surprisingly, the response patterns are quite conserved between all tested *Solanum* homologs, and even similar to the response patterns of two *Nicotiana* species (**Chapter 5**). This suggests that the recognition specificity of elicitin perception between *Nicotiana* and *Solanum* is likely to be conserved, and that the elicitin recognition system may be based on an ELR ortholog in *Nicotiana*. Since elicitins can also trigger defense response in *Brassicaceae* species, an ELR ortholog may even be conserved in *Brassicaceae*. Previous studies showed that

interfamily transfer of PRRs can retain their activities (Lacombe et al. 2010, Fradin et al. 2011). Studying transfer of ELR orthologs from *N. benthamiana* and perhaps *Brassicaceae* species into potato is a next step, and could potentially broaden the pool of immune receptors for enhancing resistance.

Enhancing immunity mediated by recognition of apoplastic effectors of Phytophthora infestans

Considering the weaker responses in apoplastic immunity compared to the typical *R* genes, we hypothesize that the combination of multiple PRRs may lead to more adequate levels of resistance than by over-expressing single surface receptors. Besides elicitins, other apoplastic effectors with putative PAMP characteristics are e.g. the extracellular protease inhibitor (EPI) (Tian et al. 2004), small cysteine-rich (SCR) proteins (Liu et al. 2005), cellulose binding elicitor lectin (CBEL) (Gaulin et al. 2006), Nep1-like protein (PiNPP) (Kanneganti et al. 2006), and the cystatin-like protease inhibitor EPIC of *P. infestans* (Tian et al. 2007). For example, EPIC2B binds and inhibits Rcr3, the tomato apoplastic cysteine protease that functions in fungal resistance (Rooney et al. 2005). Also SCR74, to which we found highly specific responses in *Solanum hougassi* (Liu et al. 2005, Rietman 2011), may be a good target for apoplastic immunity. In addition, the *P. infestans* genome sequence has provided us with a wide resource of predicted apoplastic effectors with PAMP characteristics.

Cytoplasmic *R* genes-triggered immunity

Intracellular immunity based on *R* genes has been extensively explored in potato resistance breeding. Till now, many *R* genes have been bred, identified and even cloned from tuber-bearing *Solanum* section *Petota*. For example *R1*, *R2*, *R3a* and *R3b* have been cloned from *S. demissum* (Ballvora et al. 2002, Huang et al. 2005, Lokossou et al. 2009, Li et al. 2011); *Rpi-blb1/RB* (Song et al. 2003, van der Vossen et al. 2003), *Rpi-blb2* (van der Vossen et al. 2005), and *Rpi-blb3* from *S. bulbocastanum* (Lokossou et al. 2009); *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* (Vleeshouwers et al. 2008); *Rpi-abpt*/*R2-like* from unknown species used in a pre-breeding program (Lokossou et al. 2009), and *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* from *S. venturii* (Foster et al. 2009, Pel et al. 2009). However, most cloned *R* genes have been defeated by fast evolving *P. infestans* effectors.

Encouragingly, there are many more wild potato species that could be exploited. The tuber-bearing *Solanum* section *Petota* consists of 189 species including the cultivated species *S. tuberosum* (Spooner and Salas 2006). Most of the resources in *Solanum* section *Petota* remain unexploited. More *Rpi*-genes are expected to be cloned in the future, which will develop more avenues to protect potatoes against late blight (Smilde et al. 2005, Rauscher et al. 2006, Tan et al. 2008, Hein et al. 2009, Jacobs et al. 2010).

Effectoromics assists in durable resistance breeding

With the effectoromics approach, the exploitation of novel immune receptors can be greatly accelerated. Briefly, effectoromics can contribute to breeding in four aspects, i.e. accelerating *R* gene identification, distinguishing functional redundancy, detecting recognition specificity, and assisting in *R* gene deployment (Vleeshouwers et al. 2011) (**Chapter 3**). Besides, in **Chapter 4**, we can see that the effectoromics approach also plays an essential role in identification of PRRs. Due to the clear responses to INF elicitins in *Solanum* germplasm, a map-based cloning of the ELR receptor was feasible (Verzaux 2010). This would never have been reached by phenotyping for resistant phenotypes to *P. infestans* isolates in segregating populations (Vleeshouwers et al. 2006). In **Chapter 5**, we also describe the great acceleration of the allele mining process of ELR orthologs using the apoplastic effector INF1. This enables us to perform efficient further analysis of the genetic variation of ELR.

The effectoromics approach has already made an important contribution (as outlined in the general introduction) to potato resistance breeding and will continue to assist it in the future. By implementing this approach, more *Rpi*-genes and PRRs are expected to be identified more efficiently than before from wild potato resources. Then they can be applied in current potato cultivars by traditional sexual crosses making new varieties or by modern techniques like cis- or transgenesis improving existing varieties. When the information of the *Rpi*-genes or PRRs is ready, one can also implement targeted mutagenesis (genome editing) by new technologies, such as zinc finger nuclease–based approaches (Shukla et al. 2009, Townsend et al. 2009), transcription activator-like (TAL) effectors that can be fused to DNA nucleases to target a precise site in a genome to create genetic variation, or CRISPR system that Cas9 nucleases can be directed by short RNAs to induce precise cleavage at endogenous genomic loci (Boch et al. 2009, Bogdanove and Voytas 2011, Cong et al. 2013)

Exploit key defense-responsive genes for resistance breeding

Plant defense is a very complex procedure. Immune receptors like *R* and *PRR* genes mediate the recognition of pathogens. The defense signals need to be transduced downstream to induce successful defense responses. Defense-responsive genes are those induced downstream of the recognition event and of which activation contributes directly to potential resistance mechanisms. Defense-responsive genes respond to a pathogen attack by altering expression or post-translationally modifying their encoding proteins (Eulgem 2005, Benschop et al. 2007). In several pathosystems, the overexpression of defense-responsive genes has led to an enhanced resistance level in transgenic dicots and monocots (Leckband and Lorz 1998, Christensen et al. 2004, Wu et al. 2009, Ni et al. 2010, Ni et al. 2010, Deng et al. 2012, Shi et al. 2012).

In the downstream signaling pathways mediated by *R* or *PRR* genes or even both, many defense-responsive genes are involved (Kou and Wang 2010, Win et al. 2012) (Figure 2). Some genes may have redundant function while some other genes are essential for plant resistance. Proper manipulation of those key defense-responsive genes has a possibility to achieve durable and broad-spectrum resistance.

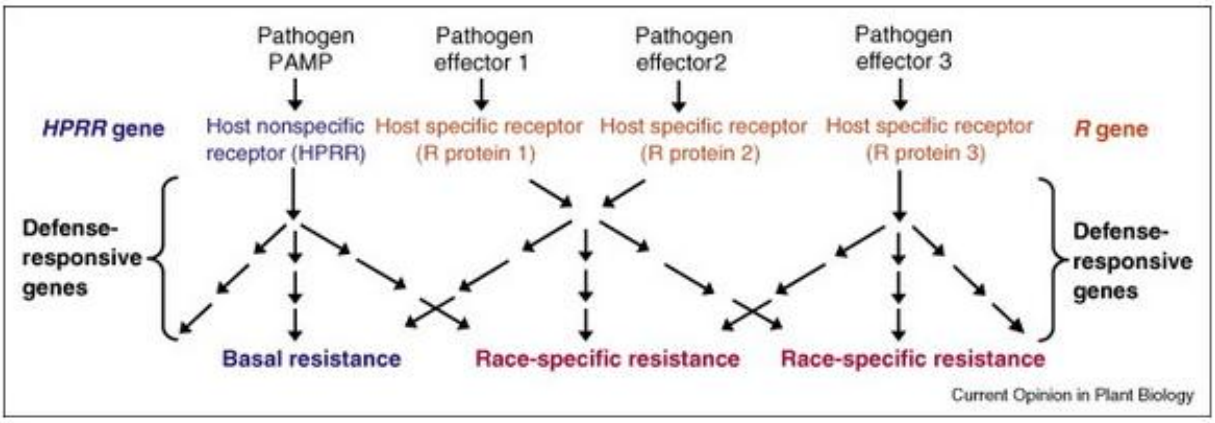

Figure 2. Cross-talks between basal and race-specific resistance pathways and between different race-specific resistance pathways. Published in (Kou and Wang 2010)

A typical example of the key defense-responsive genes is the LRR-RLK BAK1/SERK3 that belongs to a family of five related SERK proteins (Hecht et al. 2001). Actually, BAK1 was initially identified as an interactor and positive regulator of the brassinosteroid (BR) receptor BRI1 (Li et al. 2002, Nam and Li 2002), unexpectedly, it was later proved to also function as a master positive regulator of innate immunity. For instance, BAK1 is required for responses triggered by the bacterial PAMPs flg22, elf18, lipopolysaccharides (LPSs), peptidoglycans (PGNs), HrpZ, csp22 (derived from cold shock protein), the DAMP AtPep1, and the oomycete PAMP INF1 (Chinchilla et al. 2007, Heese et al. 2007, Shan et al. 2008, Postel et al. 2010, Chaparro-Garcia et al. 2011). In **chapter 4**, we also show that the INF1 receptor ELR associates with BAK1. Besides, BAK1 is also important for resistance to obligate biotrophic fungi *Verticillium* and oomycete *Hyaloperonospora arabidopsidis* (Hpa) and also to hemibiotrophic bacterium *Pseudomonas syringae* (Fradin et al. 2009, Fradin et al. 2011, Roux et al. 2011, Schwessinger et al. 2011, de Jonge et al. 2012). Moreover, BAK1 is recently found to contribute to resistance against diverse RNA viruses, namely, the tobamoviruses Tobacco mosaic virus (TMV) strain U1 and ORMV, and the tombusvirus Turnip crinkle virus (TCV) (Korner et al. 2013).

Previously, defense-responsive genes have been studied in potato in different ways, e.g. suppression subtractive hybridization (SSH) (Tian et al. 2003), microarray analysis (Wang et al. 2005), cDNA-AFLP (Li et al. 2009). According to the above analysis, many defense-responsive genes to *P. infestans* have been identified and most of them are related to metabolism, plant defense, signaling and transcription regulation, involving the whole process of plant defense response to pathogens (Tian et al. 2003, Wang et al. 2005, Li et al. 2009). Subsequently, 63 of those identified defense-responsive genes

were selected as candidate genes and screened by a transient assay, namely virus-induced gene silencing (VIGS) (Du et al. 2013). And this is followed by detached leaf disease test on both *Nicotiana benthamiana* and potato to identify the important genes (Du et al. 2013). Results led to identification of two genes, i.e. a *lipoxygenase* and a *suberization-associated anionic peroxidase* (Du et al. 2013). They may play a role in the resistance to late blight, although further complementation tests needs to be performed to verify their function. Overexpression of the key defense-responsive genes may raise the resistance level of plants, and provide an additional tool for breeders to control the late blight disease.

Exploit susceptibility genes in potato

The barley recessive *mlo* gene is famous in the induction of strong resistance which is lasting for more than 30 years in European agriculture (Lyngkjaer et al. 2000). *Mlo* is nowadays also called *S* (susceptibility)-gene (Pavan et al. 2009). In Arabidopsis a number of *S*-genes have been found against different pathogens (Pavan et al. 2009). Such recessive susceptible genes should also exist or can be induced in potato. Till now, no researches about this kind of mutated genes have been reported in potato. A bottle neck is that potato is autotetraploid, so not easy to get homozygous recessive for a particular gene. But it does not mean that it is not a good way to try, especially if RNAi is applied which turns loss of gene function from recessive into a dominant trait.

Conclusion

Plant resistance is a complicated system. A single *R* gene is not reliable to fight against *P. infestans*. More effective molecular strategies need to be explored to achieve durable and broad-spectrum resistance in potato breeding.

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Summary

Potato (*Solanum tuberosum* L.), which originates from the Andes in South America, is the third most important food crop in the world. Potato plays an important role for both human consumption and industrial purposes. However, it suffers from various diseases, which cause huge economic losses every year. One of the most devastating diseases is late blight caused by the Irish potato famine agent *Phytophthora infestans.* During the last 10 years, many resistance (*R*) genes have been cloned and some of them have been introgressed into potato cultivars by sexual crossing. Yet it was found that virulent races of *P. infestans* quickly emerge and evade recognition by single introgressed *R* genes. Sometimes, the new cultivar was defeated by the fast evolving *P. infestans* even without going into the commercial pipelines. So it is clear that we cannot rely on single *R* genes and it is urgent to exploit additional effective ways to provide durable resistance. One way of providing more durable resistance could be stacking of multiple *R* genes.

Although most cloned *R* genes have been defeated by *P. infestans*, the virulent isolates are not everywhere. Proper use of known *R* genes and rapid cloning of new *Rpi*-genes could still make contributions to resistance breeding, as such immune receptors typically provide high levels of resistance. Traditional resistance breeding is slow and inefficient, and therefore the evolution speed of *P. infestans* is hard to beat. Encouragingly, effectoromics has recently been proven a successful strategy to accelerate to achieve resistance to late blight in agriculture. In **Chapter 2**, we analysed the application aspects of effectoromics in the form of "*Do's* and *Don'ts*". Briefly, we summarized seven advantages in the *Do's* and three practical notes in the *Don'ts*. This should allow other researchers in potato but also other pathosystems to set up similar approaches as well.

Agroinfiltration and PVX agroinfection are two efficient transient expression assays routinely used for effectoromics. In **Chapter 3**, we presented our protocols of the two assays in both potato and *Nicotiana benthamiana*. The protocols themselves are not complicated, but the robustness and reproducibility will ensure whether results are reliable. Furthermore, we give tips on how to gain more experience and how to analyze the results. We share our long-term experience for scoring and also discussed about the advantages and disadvantages of both assays in effectoromics application.

Generally, plants rely on two layers of immunity to defend against pathogens. Despite the defeated intracellular *R* genes, the other layer of immunity occurs at the cell surface. Since surface receptors can recognize conserved pathogen associated molecular patterns (PAMPs), we hypothesize that immunity conferred by pattern recognition receptors (PRR) may be more durable. For the potato – *P. infestans* pathosystem, this apoplastic layer of immunity has not yet been explored, mainly because the quantitative resistance phenotype conferred by PAMP-triggered immunity is hampering map-based

cloning approaches of surface receptors. Taking advantage of effectoromics assays, clear phenotypes could be obtained for recognition of elicitins, a family of proteins of *P. infestans* that are recognized as oomycete PAMPs. The receptor-like protein *ELR* (elicitin response) was successfully cloned from the wild potato species *Solanum microdontum*. Based on ELR, we studied the first layer of immunity in potato. In **Chapter 4**, we performed repeated disease tests on an *ELR*-expressing transgenic potato cultivar (the normally susceptible cv. Désirée). Promising results showed that overexpression of *ELR* could enhance the resistance of potato to late blight. Furthermore, we also found that the transgenic Désirée expressing *ELR* recognize a broad spectrum of *Phytophthora* elicitins and induce defense responses.

In **Chapter 5**, we further explored the genetic variation of ELR in various *Solanum* species. INF1-responsive wild potato species are distributed in both Central and South America. From those species, we successfully cloned 7 ELR orthologs, which show high levels of amino acid sequence identity with ELR. By functionally testing these ELR orthologs for response to various elicitins, we found that patterns of elicitin-triggered defense are conserved in all tested *Solanum* spp., and even match the response pattern in *Nicotiana* species. Moreover, we proved that INF1-triggered cell death mediated by ELR can be suppressed by $AVR3a^{KI}$ in a wild potato species (*Solanum hjertingii* 349-3), similar as has been shown in *N. benthamiana*.

Taken together, our study has 1) led to useful suggestions for applying effectoromics, 2) proven that the newly cloned INF1 receptor ELR can enhance resistance to *P. infestans* and induce broad-spectrum defense responses to different *Phytophthora* elicitins, and 3) discovered that ELR is widely conserved in *Solanum* species. Our results implicate that pyramiding cell surface pattern recognition receptors with intracellular immune receptors maximizes the potential of generating a broader and presumably more durable resistance to the devastating late blight pathogen.

Samenvatting

Aardappel (*Solanum tuberosum* L.) is wereldwijd het derde grootste voedselgewas en is belangrijk voor zowel menselijke consumptie als industriële doeleinden. Echter, de aardappelplant is vatbaar voor diverse ziekten, die elk jaar weer grote economische verliezen veroorzaken. Vooral de 'aardappelziekte', veroorzaakt door *Phytophthora infestans*, is sinds de Ierse hongersnood in 1845 nog steeds berucht en een grote bedreiging voor het aardappelgewas. Om de aardappelplant te beschermen tegen Phytophthora, gebruikt de plant diverse strategieën waarvoor plantenveredelaars een aantal resistentiegenen (*R* genen) hebben geïdentificeerd. Deze *R* genen, die coderen voor resistentie eiwitten (R eiwitten), zijn met behulp van kruisingen in aardappelrassen geïntroduceerd. Helaas bleek *P. infestans* in staat zich snel aan te passen en herkenning door de R eiwitten te ontwijken. Soms werd een nieuw aardappelras zelfs al voor de commercialisering verslagen door de snel evoluerende *P. infestans*. Het is duidelijk dat we niet kunnen vertrouwen op introductie van één enkel *R* gen, maar dat er meer nodig is om een duurzame resistentie tegen Phytophthora te bereiken. Een van de strategieën is het stapelen van meerdere *R* genen.

Hoewel Phytophthora zich reeds aangepast heeft aan de meest voorkomende en (ten dele) gekloneerde *R* genen, zijn de virulente isolaten niet overal. Correct gebruik van de bekende *R* genen en snelle klonering van nieuwe *R* genen kan nog steeds bijdragen aan de resistentieveredeling, aangezien de nieuwe *R* genen doorgaans geselecteerd worden om een hoog niveau van resistentie geven. Maar aangezien de traditionele resistentieveredeling traag en inefficiënt is, en dus de evolutie snelheid van *P. infestans* moeilijk bij te houden is, is een versnelling van *R* gen identificatie noodzakelijk. De 'effectoromics' benadering, die gebruik maakt van *Phytophthora* eiwitten (effectors) om *R* genen te identificeren, heeft onlangs bewezen de identificatie en klonering van *R* genen tegen Phytophthora aanzienlijk te versnellen. In hoofdstuk 2 analyseren we de toepassing van effectoromics in de vorm van "*Do's en Don'ts*". We vatten zeven voordelen samen in de "*Do's*" en geven drie praktische notities in de "*Don'ts*". Dit moet het ook voor andere onderzoekers mogelijk maken om een soortgelijke aanpak op te zetten, zowel in aardappel als andere gewassen.

Agroinfiltratie en PVX agroinfectie zijn twee efficiënte transiënte expressiemethoden die routinematig gebruikt worden voor effectoromics. In hoofdstuk 3 presenteren we de protocollen van deze twee methoden, voor zowel aardappel als de modelsoort *Nicotiana benthamiana*. De protocollen zelf zijn vrij eenvoudig, maar aandacht voor de robuustheid en reproduceerbaarheid is essentieel om te beoordelen hoe betrouwbaar de resultaten zijn. We delen onze ervaringen met fenotypering en geven tips voor resultaten analyse. Ook bespreken we de voordelen en nadelen van beide testmethoden in effectoromics toepassingen.

Volgens het gangbare model, verdedigen planten zich tegen ziekteverwekkers in twee fasen. Vóór de R eiwit-gebaseerde afweer (intracellulair), bestaat er een eerste afweer op het celoppervlak (apoplastisch) die geactiveerd wordt door patroonherkenning receptoren (PRR). Deze apoplastische PRRs kunnen pathogeen-geassocieerde moleculaire patronen (PAMPs) herkennen, en vervolgens afweerreacties aanschakelen. Aangezien de PAMPs geconserveerde eiwitten zijn, veronderstellen we dat PRR-gebaseerde resistentie duurzamer kan zijn. Voor het aardappel - *P. infestans* pathosystem is deze 'apoplastische afweer' nog niet onderzocht, met name omdat positionele klonering belemmerd wordt door het kwantitatieve fenotype van PRR-gebaseerde resistentie. Echter, met behulp van effectoromics assays is het wel mogelijk duidelijke fenotypes te verkrijgen. Elicitines, een familie van eiwitten die erkend worden als PAMPs van *Phytophthora*, kunnen herkend worden door een aantal wilde aardappelsoorten. Het ELR gen (ELicitin Response), dat verantwoordelijk is voor de herkenning van elicitines, is met behulp van effectoromics assays gekloneerd uit de wilde aardappel soort *Solanum microdontum*. *ELR* codeert voor een Receptor-Like Protein (RLP). Op basis van de ELR-elicitine interactie, hebben we de 'apoplastische afweer' tegen Phytophthora in aardappel onderzocht. In hoofdstuk 4 hebben we ziekteproeven uitgevoerd op transgene Désirée-ELR aardappel. De veelbelovende resultaten toonden aan dat overexpressie van ELR de resistentie tegen de aardappelziekte zou kunnen verbeteren. Daarnaast vonden we dat de Désirée-ELR aardappeltransformanten een breed spectrum van elicitines, zowel van *P. infestans* en andere *Phytophthora* soorten, kunnen herkennen en afweerreacties induceren.

In hoofdstuk 5 hebben we de genetische variatie van het ELR gen in verschillende *Solanum* soorten verder onderzocht. Wilde aardappelsoorten die INF1 elicitine herkennen, komen zowel in Midden- als Zuid-Amerika voor. Van deze *Solanum* soorten hebben we 7 ELR orthologen gekloneerd, die op aminozuurniveau in hoge mate overeenkomen met ELR. Functionele toetsen van de ELR orthologen met diverse elicitines wijzen erop dat het patroon van elicitine-geïnduceerde afweer geconserveerd is in alle geteste *Solanum* soorten, en zelfs overeen komt in *Nicotiana* soorten. Daarnaast hebben we aangetoond dat de elicitine-geïnduceerde reactie door ELR kan worden onderdrukt door AVR3aKI in wilde aardappel (*Solanum hjertingii* 349-3), vergelijkbaar zoals bekend is in *N. benthamiana*.

Samengevat heeft onze studie 1) geleid tot nuttige suggesties voor het toepassen van effectoromics in de plantenveredeling, 2) aangetoond dat ELR de resistentie tegen *P. infestans* kan verbeteren en afweerreacties induceert tegen een breed spectrum van elicitines van diverse *Phytophthora* soorten, en 3) aangetoond dat ELR wijd geconserveerd is in *Solanum* soorten. Onze resultaten impliceren dat het stapelen van PRR met R genen de mogelijkheden maximaliseert om een bredere, en vermoedelijk meer duurzame, resistentie te genereren tegen de gevreesde aardappelziekte.

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Juan Du
杜鹃

About the author

Juan Du was born on April 13th, 1985 in Wuhan, China. In September 2003, she was enrolled in Huazhong Agricultural University (Wuhan) to study Horticulture. She obtained her bachelor degree in June 2007. In the meantime, based on her good results during four years study, she was directly selected to follow a combined master and PhD program in the Key Laboratory of Horticultural Plant Biology (Huazhong Agricultural University). Her project was to study quantitative resistance to potato late blight under the supervision of Prof. Dr. Conghua Xie. When she entered the first year of her PhD phase in 2010, she successfully gained a fellowship for a PhD program from China Scholarship Council (CSC). She then spent the rest of her PhD time studying at Wageningen UR Plant Breeding, Wageningen University and Research Centre (the Netherlands). There she worked on a new layer of resistance – apoplastic immunity to potato late blight under the supervision of Dr. Vivianne Vleeshouwers and her two promoters Prof. Dr. Richard Visser and Prof. Dr. Evert Jacobsen.

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List of Publications

Du J, Tian ZD, Liu J, Vleeshouwers VGAA, Shi XL, Xie CH. Functional analysis of potato genes involved in quantitative resistance to *Phytophthora infestans*. *Molecular Biology Reports*, 2013, 40: 957-967

Du J, Rietman H, Vleeshouwers VGAA. Agroinfiltration and PVX agroinfection in potato and *Nicotiana benthamiana Journal of Visualized Experiments*, 2014, 83: e50971

Du J, Vleeshouwers VGAA. The *Do's* and *Don'ts* of effectoromics. In: Methods in Molecular Biology, 2014, 1127: 257-268

Du J, Verzaux E, Chaparro-Garcia A, Bijsterbosch G, Keizer LCP, Zhou J, Liebrand TWH, Xie CH, Govers F, van der Vossen EAG, Jacobsen E, Visser RGF, Kamoun S, Vleeshouwers VGAA. Elicitin recognition confers enhanced resistance to the Irish potato famine pathogen. (in preperation)

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Juan Du**
Date: **16 June 2014**
Group: **Plant Breeding, Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Natural variation and functional characterization of elicitin-mediated apoplastic defense in potato	Apr 18, 2011
► **Writing or rewriting a project proposal**	
Identification and characterization of durable resistance genes to potato late blight	Dec 2011
► **Writing a review or book chapter**	
The Do's and Don'ts of effectoromics, Paul Birch et al (eds), Plant-Pathogen Interactions: Methods and Protocols, Methods in Molecular Biology, Vol 1127, 2014. Springer Science New York	2013
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student days, University of Amstterdam, NL	Nov 30, 2012
EPS PhD student days, Leiden University, NL	Nov 29, 2013
► **EPS theme symposia**	
EPS Theme 2 and the Willie Commelin Scholten Day, Wageningen University, NL	Feb 10, 2012
EPS Theme 2 and the Willie Commelin Scholten Day, Utrecht University, NL	Jan 24, 2013
► **NWO Lunteren days and other National Platforms**	
ALW Meeting Experimental Plant Sciences, Lunteren, NL	Apr 04, 2011
ALW Meeting Experimental Plant Sciences, Lunteren, NL	Apr 02-03, 2012
ALW Meeting Experimental Plant Sciences, Lunteren, NL	Apr 22-23, 2013
► **Seminars (series), workshops and symposia**	
Plant Breeding research day 2011	Mar 07, 2011
Symposia: How to write a world-class paper	Apr 19, 2011
Invited seminar "The genome of Dothistroma septosporum, a close relative of Cladosporium fulvum; what have we learnt so far?" Rosie Bradshaw	Aug 04, 2011
Invited seminar "Mobile RNA silencing in plants" Prof. David Baulcombe	Sep 27, 2011
EPS - Mini-symposium 'Plant Breeding in the genomics era'	Nov 25, 2011
Plant Breeding research day 2012	Feb 28, 2012
Invited seminar "Arabidopsis thaliana as a model system for the study of evolutionary questions", Detlef Weigel	Feb 27, 2013
Invited seminar "Metabolomics-based functional genomics - from Arabidopsis to crops and medicinal plants", Kazuki Saito	Apr 08, 2013
Invited seminar "Using the Nicotiana-TMV system to study resistance gene evolution and plant genome stability", Hanhui Kuang	Sep 11, 2013
► **Seminar plus**	
► **International symposia and congresses**	
3rd European Retreat for PhD students in Plant Sciences, Orsay, France	Jul 05-08, 2011
Mini-Symposium on New Horizons in Plant-Pathogen Interactions, UK	Apr 30-May 03, 2012
Oomycete Molecular Genetic Meeting, Nanjing, China	May 26-28, 2012
XV International Congress on Molecular Plant-Microbe Interactions (IS-MPMI), Kyoto, Japan	Jul 29-Aug 02, 2012
4th European Retreat for PhD students in Plant Sciences, Norwich, UK	Aug 14-17, 2012
Next Generation Plant Breeding Conference, Ede, the Netherlands	Nov 11-14, 2012
10th Solanaceae Conference (SOL 2013), Beijing, China	Oct 13-17, 2013
► **Presentations**	
Poster: 3rd European PhD retreat, Orsay, France	Jul 05-08, 2011
Oral: EPS Autumn School 2011 'Host-Microbe Interactomics'	Nov 01-03, 2011
Oral: in Sainsbury Lab, Norwich	Nov 14-17, 2011
Oral: Mini-Symposium on New Horizons in Plant-Pathogen Interactions, UK	Apr 30-May 03, 2012
Oral: EPS Summer School 2012 'Natural variation of plants'	Aug 21-24, 2012
Poster: 10th Solanaceae Conference (SOL 2013), Beijing, China	Oct 13-17, 2013
► **IAB interview**	
Meeting with a member of the International Advisory Board	Nov 15, 2012
► **Excursions**	
Subtotal Scientific Exposure	*19.7 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Molecular Phylogenies: Reconstruction & Interpretation	Oct 18-22, 2010
EPS Autumn School 2011 'Host-Microbe Interactomics'	Nov 01-03, 2011
EPS Summer School 2012 'Natural variation of plants'	Aug 21-24, 2012
► **Journal club**	
literature discussions, Plant Breeding, Wageningen / Key Laboratory of Horticultural Plant Biology (Huazhong Agricultural University), China	Sep 2010 - Sep 2013
► **Individual research training**	
project training in Sainsbury Lab, Norwich, UK	Nov 14-17, 2011
Subtotal In-Depth Studies	*7.9 credits**

4) Personal development	*date*
► **Skill training courses**	
Workshop for Phytopathology: Search the digital library efficiently	Jan 25, 2011
Techniques for Writing and Presenting Scientific Papers	Sep 06-09, 2011
Advanced course Guide to Scientific Artwork	May 07-08, 2012
Mobilising your - Scientific - Network	Sep 18 and 25, 2012
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**44.1**
Herewith the Graduate School declares that the PhD candidate has complied with the educational	

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

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