**RxLR and CRN effectors of
the spinach downy mildew
Peronospora effusa and their
interacting plant proteins**

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RxLR and CRN effectors of the spinach downy mildew *Peronospora effusa* and their interacting plant proteins

RxLR en CRN effectoren van de valse meeldauw van spinazie (*Peronospora effusa*) en de planteneiwitten waarmee ze interacteren.

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1 **General introduction**

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General introduction

Plants are confronted with many different microbial pathogens, ranging from viruses to bacteria, fungi and oomycetes. Each in their own way, pathogens interact with the plant to acquire nutrients for growth and reproduction. When a pathogen attack is sensed by a plant it can induce a strong immune response to fight of the pathogen. This ever-changing battleground is a fascinating setting for the study of plant-pathogen interactions, co-evolution and the molecular signaling involved in plant resistance and susceptibility.

The focus in this thesis is on the molecular interaction between the obligate biotrophic oomycete *Peronospora effusa* (previously known as *P. farinosa forma specialis spinaciae*, and here referred to as *Pfs*) and its spinach host [1]. Here, I provide a background into the biology and taxonomy of phytopathogenic oomycetes, the lifecycle of *Pfs* and an overview of the molecular interactions that occur between oomycetes and plants.

Biology and taxonomy of phytopathogenic oomycetes

Oomycetes are eukaryotic microorganisms that have a filamentous appearance, which resembles the mycelial growth of true fungi. This resemblance is the result of convergent evolution rather than evolutionary kinship because oomycetes and fungi species are very distantly related within the eukaryotes [2, 3]. As opposed to fungi, oomycetes have aseptate hyphae, cell walls composed mainly of cellulose rather than chitin and typically motile biflagellate zoospores (that have been lost in many species) [4]. Oomycetes belong to the Straminipila phylum that includes diatoms and brown algae and hosts species that occupy diverse habitats and have an extraordinary array of lifestyles [5]. In fact, even within the oomycete group, lifestyles and habitats range from free-living/saprotrophic marine and freshwater species, to human pathogens like *Pythium insidiosum* and a large number plant pathogenic species (phytopathogens) [6].

The ability to be pathogenic on plants evolved multiple times in the evolutionary history of oomycetes. Phytopathogenic species can be found in five different taxa; the *Aphanomyces* genus, the white blister rusts (*Albugo*), *Pythium*, *Phytophthora* and downy mildew species [7]. The species in each clade have a different lifestyle and infection strategy. Lifestyles vary from necrotrophic (killing cells and feeding on the contents) in *Aphanomyces* and *Pythium* species to obligate biotrophic in downy mildew and *Albugo* species that strictly require living host tissue to grow and fulfill their life cycle. *Phytophthora* species are hemi-biotrophs that transition from an initial biotrophic to a necrotrophic phase when the infection progresses [8].

Phytopathogenic species of the early diverging *Aphanomyces* genus are necrotrophic pathogens of seedlings and plant roots [9] that spread by producing mobile zoospores in the soil. They have a sexual and asexual reproduction cycle and are found on a wide range of plants including crop and ornamental species like *Iris hollandica*, *Spinacia oleracea* and legume species, e.g. on *Medicago* [10]. White blister rusts in the Albuginales order are a group of obligate biotrophic species that infect angiosperms. They are phylogenetically distant from the Peronosporales species and likely evolved obligate biotrophy independently of those species [7]. They primarily reproduce asexually via motile flagellated zoospores that are released from zoosporangia. The zoospores settle in stomata and form a germ tube and subsequently hyphae that grow intercellularly and penetrate plant cells [11]. To fulfill their life cycle they produce zoosporangia that rupture the plant epidermis resulting in characteristic white blisters [12]. Sexual reproduction, that enables recombination providing increased genetic diversity, results in the formation of oospores that can survive harsh conditions [13]. *Albugo* species differ in host range; some species like *Albugo candida* occur on a wide range of *Brassicaceae* species while others like *Albugo laibachii* are found on *Arabidopsis thaliana* only [14]. *Albugo* species were shown to exhibit remarkable defense suppression capacity [15]. Plants infected with *Albugo* are more susceptible to co-infection and to infection with

incompatible pathogens, including avirulent strains of the downy mildew *Hyaloperonospora arabidopsidis*. The molecular mechanisms behind *Albugo* mediated suppression of non-host resistance against *Phytophthora infestans* may involve manipulation of tryptophan metabolism resulting in an decrease of tryptophan-derived microbial compounds [16]. The other groups of phytopathogenic oomycetes all occur within the large and diverse order of Peronosporales and include saprotrophic, necrotrophic, hemi-biotrophic and obligate biotrophic genera [17].

Within the Peronosporales order the Pythiaceae form the basal lineage to the Peronosporaceae that encompass the *Phytophthora* and downy mildew species [18]. One has to note that other authors classify the Pythiaceae in a separate order, Pythiales, rather than as a family within the Peronosporales order [19]. Species in the genus *Pythium* have a diversity of host-ranges, varying from pathogens of marine red algae to pathogens of animals. Phytopathogenic *Pythium* species are generalist facultative necrotrophic phytopathogens that affect all parts of the plant, including seeds and sprouts [18, 20]. Phytopathogenic *Pythium* species grow filamentously and do not develop haustoria, feeding structures that are found in *Phytophthora* and downy mildew species. Like *Aphanomyces*, *Pythium* primarily spreads through zoospores in the soil but unlike *Aphanomyces* also in water [8]. No differential responses of host cultivars to *Pythium* infection have been observed, suggesting that gene-for-gene interactions do not take place in contrast to what has been often found for *Phytophthora* and downy mildew species [18].

By far the highest number of phytopathogenic oomycete species belong to the Peronosporaceae, the other Peronosporales group. This large family includes the remorseless *Phytophthora* species and several groups of downy mildew species. One of the most infamous members is *P. infestans*, the causal agent of potato late blight that led to the death of around one million people during the Great Irish famine around 1845 [21]. The downy mildew genera also cause diseases that are of great economic importance, like *Plasmopara viticola* on grapevine and *Pfs* on spinach [22, 23].

Until recently, *Phytophthora* and the downy mildew species were considered sister groups based on morphological and biological traits [6, 24]. Biologically, downy mildew species are obligate biotrophs that fully depend on living tissue for their survival and are usually host species or genus specific. *Phytophthora* species are hemi-biotrophs that after an initial biotrophic phase kill the host and are able to grow off death tissue. Most *Phytophthora* species have a broader host range than downy mildews. Morphologically, hyphae of downy mildew species are terminated when forming the spore bearing sporangiophores whereas *Phytophthora* hyphae continue to grow [25]. However, with the growing number of species studied, it became clear that these characteristics were not so strictly separated between the two phyla as species with intermediate characteristics were identified [25, 26].

These initial observations of morphological traits that are not strictly separated between *Phytophthora* and downy mildew species were later supported by genetic evidence. Studies using multiple genes or full genome comparisons suggested that the downy mildews not only reside within, but also have multiple independent origins in the larger *Phytophthora* clade [19, 22, 27]. This means that the obligate biotrophic lifestyle emerged at least two times independently within the *Peronosporales* order. One downy mildew clade encompasses the majority of downy mildew species. It includes graminicolous grass-infecting downy mildews, the downy mildews with colored conidia and the brassiculous downy mildews [27]. The downy mildews with colored conidia encompass the largest downy mildew genus *Peronospora* and the genus *Pseudoperonospora* that both have purple to black sporangia [17]. The second clade contains downy mildew species with pyriform haustoria that has recently been shown to be more closely related to *Phytophthora* clade 1 than to the other downy mildew species [22]. Downy mildews with pyriform haustoria includes eight genera, which are almost exclusively infecting *Asteraceae*, including crops like lettuce (*Bremia lactucae*) [17, 28]. The exception is the genus *Plasmopara* that infects other species like grapevine (*P. viticola*) and sunflower (*P. halstedii*) [22, 29].

Pfs, the causal agent of downy mildew on spinach, belongs to the *Peronospora* family. Downy mildew is a major disease in commercial spinach fields worldwide and restrains both crop quality and quantity [30]. *Pfs* was first reported in 1824 [31] and, following the introduction of resistant spinach cultivars, new isolates evolved rapidly over the last two decades [23, 32].

Life cycle of *Peronospora effusa*, the downy mildew of spinach

Pfs has a sexual as well as an asexual life cycle. The asexual sporangiospores germinate when they have landed on plant tissue. The emerging germ tubes develop to penetrate into plant tissue and initiate the infection [30]. Hyphae continue to grow in the tissue between the plant cells. Specialized hyphae form haustoria that are thought to be the site of interaction between oomycete and the plant cell (discussed in the following section). The hyphae form terminal sporangiophores, branched spore-bearing structures that grow out of the stomata and contain the sporangia that are visible on infected leaves as a grey furry layer [25] (**Fig. 1**). The sporangia travel aerially and through rain splash and facilitate rapid spread of *Pfs* [30].

During sexual reproduction in gametangia thick-walled oospores are produced that genetically represent a combination of both parental genomes. *Pfs* is heterothallic, i.e. parental lines (P1 and P2) have to be of a different mating type for sexual reproduction to occur [33, 34]. Sexual recombination can generate extensive genetic variation in a pathogen population. As opposed to sporangia, the microscopic oospores do not occur on the leaf surface but inside plant tissue, below the epidermal layer [30]. Oospores have also been observed in roots and seeds and it was recently shown that *Pfs* can be transmitted through seeds [35]. Further research on survival and germination of oospores in seed lots is required to understand its contribution to spread of *Pfs* [30].

Figure 1. Spinach leaves infected with *Peronospora effusa*.

The visible symptoms include irregular chlorotic spots and gray to brown sporangial masses, primarily on the underside of the leaf. The symptoms develop between seven to ten days post infection [30]. Picture taken by Pim Goossens.

Molecular interaction with the plant

Peronosporales species interact with plant cells at specialized feeding structures called haustoria. ‘Haustorium’ literally means ‘drawer of water’ which is illustrative of the presumed function of the structure in acquisition of water and nutrients from the plant [36]. To establish an haustorium, the cell wall of the plant cell is locally degraded so that the plasma membrane can be invaginated by the haustorium [37]. The oomycete remains separated from the host cell by the extrahaustorial matrix (EHMx) and the extrahaustorial membrane that is continuous with the plasma membrane. The extrahaustorial matrix contains material from both plant and pathogen origin [38].

The oomycete derived materials in the EHMx are both actively secreted and derivatives of the oomycete cell wall and cell membrane [39]. Many pathogenesis-related proteins of the plant host are hydrolytic enzymes (e.g. glucanases and proteases) that break down cell walls and membrane components that then release into the EHMx [40]. These

components can serve as pathogen-associated molecular patterns (PAMPs); potent triggers of basal plant immune responses. PAMPs are recognized by membrane-bound pattern recognition receptors (PRRs) that then activate a series of anti-microbial defenses like callose-deposition, production of reactive oxygen species (ROS), release of antimicrobial compounds, alteration of hormone signaling and changes in gene expression. These collectively contribute to pattern-triggered immunity (PTI) [41, 42, 43].

Cell wall derived β -glucan oligomers are an example of PAMPs in the recognition of both fungal and oomycete pathogens in many plant species, although the mechanisms are not fully understood [44, 45]. β -glucan oligomers are insoluble polysaccharides that have a structural role in cell walls [46]. They are the most abundant cell wall components in oomycetes [45]. Unlike the elicitor response triggered by chitin, β -glucan elicitation of defense is not conserved between plants and different species respond differently. This observation is possibly explained by the large diversity of β -glucan structure between species of oomycetes and fungi [47]. Other examples of oomycete cell wall and membrane derived PAMPs are transglutaminases and fatty acids [39].

Apoplastic effectors

Oomycetes secrete proteins into the EMHx during infection [44]. Pathogen-secreted proteins that help to establish and maintain infection are called effectors. An important function of effector proteins is to avoid or suppress pathogen recognition and subsequent immune responses of the plant. Effector proteins can either function in the apoplast or inside host cells. Apoplastic effectors include protease- and glucanase-inhibitors, elicitors and necrosis and ethylene-inducing peptide 1 like proteins (NLPs), among others. Protease and glucanase inhibitors function to protect the oomycete against damage induced by hydrolytic enzymes of the plant, thereby also reducing the release of PAMPs [40]. An example of apoplastic effectors with protease inhibitor function are the EpiC effectors of *P. infestans* that inhibit plant papain-like cysteine proteases [48].

Elicitins are small secreted proteins that induce necrosis and defense hormone signaling in plant species from a wide range of plant families [49, 50]. They are possibly involved in acquiring plant sterols, compounds that *Phytophthora* and downy mildew species are unable to synthesize themselves [49].

NLPs are present in the secretomes of plant-associated bacteria, fungi and oomycetes [51]. Plant-infecting oomycetes have large expansions of *NLP* genes, suggesting they play an important role in the phytopathogenic lifestyle [52, 53]. Their name is derived from their ability to induce necrosis in eudicots as a result of their cytotoxicity. However, in many species also non-cytotoxic NLPs have been identified. The numbers of cytotoxic NLPs are highly expanded within *Phytophthora* species but not in the obligate biotrophic *H. arabidopsidis*. In fact, the genome of *H. arabidopsidis* encodes 10 NLPs that are all non-cytotoxic [52]. As opposed to cytotoxic NLPs, the non-cytotoxic ones are expressed during the early stages of infection, suggesting a role in penetration or early colonization of the host [39, 52, 54].

Host-translocated effectors

In addition to effectors that function in the apoplast, oomycetes produce proteins that are known to be translocated across the plant cell membrane into plant cells. Two types of oomycete host-translocated effectors are currently known, the RxLR and CRN effectors [55, 56]. RxLR and CRN effectors manipulate plant processes, like immune signaling, and protein transport, to the benefit of the oomycete [57]. CRN effectors are thought to be ancient effectors because they are found in all phytopathogenic oomycete species, including the basal root pathogen *A. euteiches*, of which protein AeCRN5 was shown to possess a domain that is able to facilitate host translocation [56]. RxLR effector proteins were originally identified in *H. arabidopsidis* and *Phytophthora* genomes [55]. Especially *Phytophthora* species have largely expanded sets, adding up to hundreds of secreted RxLR effectors per species [58]. Large repertoires of 100 to 200 RxLRs were also identified in other downy mildew species [29, 59,

60, 61]. One notable exception is the downy mildew of basil (*Peronospora belbahrii*) that only has 22 RxLR like proteins [62]. Smaller numbers of RxLR effector proteins have been reported from *Pythium* and *Albugo* but not from other oomycete species. The contribution of RxLR effectors to virulence in *Albugo* and *Pythium* is not clear although some preliminary hints for a role of *Pythium* and *Albugo* RxLR-like effectors in virulence have been found. For example, it was demonstrated that *P. syringae* pv. *tomato DC3000* harboring *Albugo laibachii* RxLR effector 1 and 2 were more virulent on plants compared to wildtype *Pst DC3000* and RxLR effectors of *Pythium* species can induce cell death when expressed in *N. benthamiana*, pointing at recognition of the effectors by the plant immune system [63, 64].

RxLR and CRN effectors are modular proteins that are characterized by the presence of a signal peptide at the N-terminus, a presumed host-translocation domain and a variable C-terminal part, called the effector domain, which executes the function of the effector in the plant cell. The RxLR translocation domain is characterized by a RxLR-EER (arginine, any amino acid, leucine arginine) domain that is thought to be required for translocation of RxLR effectors [65, 66, 67]. Mutation of the RxLR-EER motif of *P. infestans* effector Avr3a was shown to prevent translocation of the protein into the host cell [65]. The means by which the RxLR effectors are translocated are not yet fully understood. Unlike bacterial phytopathogens, oomycetes (and fungi) have no known translocation system like the type III secretion system that injects bacterial proteins into the host cell. Recently, it was demonstrated that delivery of RxLR effector Pi04314 was independent of the conventional ER-to Golgi secretion system. Infiltration with brefeldin A, a fungal compound that interferes with Golgi-vesicle formation, did not inhibit translocation of Pi4314 into *N. benthamiana* cells [68, 69]. Golgi-independent translocation was also observed for effectors of the fungal pathogen *Magnaporthe oryzae* [70]. The unconventionally secreted signal peptide-containing proteins are thought to be transported from the ER directly to the plasma membrane, without passing through the Golgi [69, 71].

Ultrastructural studies revealed the presence of exosomes/vesicles in the EHMx and multivesicular bodies within fungal and plant cells [72, 73]. Potentially, unconventional secretion of oomycete and fungal effectors may be mediated by extracellular vesicles that are released upon fusion of multivesicular bodies with the oomycete plasma membrane [74]. The extracellular vesicles may then deliver their cargo into plant cells by fusing to the host cell-or extrahaustorial membrane [71].

In CRN effectors, the signal peptide is followed by a LxFLAK motif, DWL domain and HVLVXXP motif [58, 75]. Protein fusions between N-termini of CRN2, CRN16, CRN8 and the effector domain of *P. infestans* RxLR effector Avr3a, transgenically expressed in *P. capsici* were shown to translocate into *N. benthamiana* cells, triggering a defense response. This suggests that the N-termini, including the LFLAK domain are important for translocation [56]. The means by which the CRN effectors are translocated are not yet understood.

The C-terminal effector domains are responsible for the virulence function of the host-translocated effectors and usually lack known functional domains and show little sequence conservation between species. However, about 44% of the *Phytophthora* RxLR effectors do share a conserved tertiary structure, an α -helical fold, termed the WY-fold (Trp (W) and Tyr (Y)). WY-folds were found to occur as individual units, duplications, dimers and tandem repeats in different RxLR proteins [76]. Due to the α -helical orientation functionally important and polymorphic residues are presented at the surface of the proteins [77]. It is hypothesized that the WY-folds provide molecular stability to the proteins while maintaining functional variability of the effector domain. The structure may allow the evolution of effectors to maintain their function while evading recognition by the plant immune system [77]. Effectors with WY-folds were thus far implicated in various processes such as RNA silencing suppression (*Phytophthora* suppressor of RNA silencing 2 (PSR2, *P. sojae*) and manipulation of vesicular trafficking (PexRD54, *P. infestans*) [78, 79].

Plants have evolved intracellular receptors that recognize effectors or their activity. Recognition is mediated by nucleotide-binding domain containing leucine-rich repeat receptors (NB-LRR) that directly or indirectly interact with the effector proteins and trigger an immune response termed effector-triggered immunity [80]. As opposed to PAMPs, effectors are variable and dispensable resulting in co-evolution and large diversification of both effectors and intracellular receptors. The cellular responses triggered by ETI are similar to those triggered by PTI, but stronger and faster and are often associated with localized cell death (the hypersensitive response (HR)) [80].

Plant processes manipulated by oomycete effectors

Host-translocated RxLR and CRN effectors are potent manipulators of a plethora of plant cell functions to enable infection of the host. Although the molecular function of the majority of effectors remains unknown, efforts in recent years have led to the identification of the molecular function of a growing number of oomycete effectors (**Table 1**). The host-translocated effectors manipulate processes as broad as cellular programming (through immune-and hormone signaling and direct manipulation of transcription/translation), PAMP-induced production of ROS, and vesicular trafficking (**Fig. 2**). In addition, effectors can suppress ETI, e.g. by the *P. sojae* CRN effector PsCRN70 that suppresses PsAvh421, PsCRN63 and Avr3a-induced cell death. However, the molecular mechanism is not yet understood [81]. Examples of manipulation of each of the above mentioned processes by an oomycete effector are detailed in the following paragraphs.

Table 1. RxLR and CRN effectors and their role in manipulation of host cell processes.

Species	Effector	Affected process	Molecular mechanism	Reference
<i>P. infestans</i>	PexRD2	Immune signalling	Suppression of MAPKKKε activity	King et al. 2014 [82]
<i>P. infestans</i>	SFI 3 ^a	Immune signalling	Interacts with a positive regulator of PTI (UBK)	He et al. 2019 [83]
<i>P. infestans</i>	Pi02860	Immune signalling	Indirectly manipulates SWAP70, positive regulator of cell death	He et al. 2018 [84]
<i>P. capsici</i>	Avr3a12	Immune signalling	Suppresses a PPIase, that is required for ER stress-mediated immunity	Fan et al. 2018 [85]
<i>P. infestans</i>	Pi03192	Immune signalling	Prevents re-localization of NACs from ER to nucleus, hence suppressing PTI.	McLellan et al. 2013 [86]
<i>P. sojae</i>	Avh262	Immune signalling	Stabilizes BiPs ^b , that act as negative regulators ER stress-mediated immunity	Jing et al. 2016 [87]
<i>P. infestans</i>	IPI-O	Immune signalling	Binds lectin receptor kinase that has a structural and signalling role in the cell wall and plasma membrane.	Bouwmeester et al. 2011 [88]
<i>P. sojae</i>	Avr3b	Immune signalling	Acts as Nudix hydrolase, a negative regulator of immunity.	Dong et al. 2011 [89]
<i>P. infestans</i>	Pi04314/ PexRD24	Immune signalling	Interacts with phosphatase 1 catalytic (PP1c) isoforms, susceptibility factors that promote disease.	Boevink et al. 2016 [90]
<i>H. arabidopsisidis</i>	HaRxL44	Hormone signalling	Enhanced jasmonic acid signaling	Caillaud et al. 2013 [91]
<i>P. infestans</i>	Avr2	Hormone signalling	Upregulation of bHLH TF to suppress immunity	Turnbull et al. 2017 [92]
<i>H. arabidopsisidis</i>	HaRxL10	Hormone signalling	Interacts with JAZ3, a transcriptional repressor involved in jasmonic acid signaling	Anderson et al. 2019 BioRxiv [93]
<i>P. sojae</i>	PSAVH238	Hormone signalling	Destabilizes Type2 ACSs ^c and suppresses ethylene biosynthesis, thereby facilitating infection.	Yang et al. 2019 [94]
<i>P. sojae</i>	PSR1/2 ^d	Translational regulation	Suppressor of RNA silencing	Zhang et al. 2019 [95]

Species (continued)	Effector	Affected process	Molecular mechanism	Reference
<i>H. arabidopsidis</i>	HaRxL21	Transcriptional regulation ^e	Interacts with TPL and TPR4, repressors of defense-related genes	Harvey et al. 2020, BioRxiv [96]
<i>P. sojae</i>	CRN108	Transcriptional regulation	Reprograms expression of HSP proteins by targeting their promoters.	Song et al. 2015 [97]
<i>P. sojae</i>	Avh23	Transcriptional regulation	Interferes with histone acetylation, reduces defense gene induction	Kong et al. 2017 [98]
<i>P. infestans</i>	PI04089	Translational regulation	Increases the abundance of an RNA-binding protein (KRBP1), that is a susceptibility factor to promote disease.	Wang et al. 2015 [99]
<i>P. sojae</i>	Avr3c	Translational regulation	Stabilizes serine/lysine/arginine-rich proteins, negative regulators of plant immunity involved in alternative splicing.	Huang et al. 2017 [100]
<i>P. sojae</i>	CRN63	ROS production	Interacts with catalases to enhance ROS	Zhang et al. 2015 and others [101, 102, 103]
<i>P. sojae</i>	CRN115	ROS production	Suppresses PsCRN65-mediated accumulation of H ₂ O ₂	Zhang et al. 2015 [103]
<i>P. infestans</i>	AVRblb2	Vesicular trafficking	Inhibits secretion of C14 protease into apoplast	Bozkurt et al. 2011 [104]
<i>P. infestans</i>	Avr3a	Vesicular trafficking /immune signalling	Reduction of FLS2 endocytosis, cell death suppression	Chaparro-Garcia et al. 2015 [105]
<i>P. infestans</i>	PexRD54	Vesicular trafficking	Binds autophagy protein ATG8CL to stimulate autophagosome formation to counteract host defense.	Dagdas et al. 2016 [106]
<i>P. infestans</i>	AVR1	Vesicular trafficking	Stabilizes Sec5 thereby potentially disturbing vesicle trafficking involved in defense.	Du et al. 2012 [107]

^a Alternative names: Pi06087, PexRD16

^b ER-luminal binding immunoglobulin proteins (BiPs)

^c 1-aminocyclopropane-1-carboxylate synthase (ACS)

^d Orthologs of PSR1 from *P. parasitica* (PpPSR1L) and *P. capsici* (PcPSR1L) possess the same function

^e Hypothesized function, further research is needed. TPL = Topless, TPR4

Figure 2. Plant processes manipulated by oomycete effectors

Phytophthora and downy mildew pathogens interact with plant cells at specialized feeding structures called haustoria. Two types of effectors, RxLR and CRN effectors, are translocated into plant cells. Oomycete effectors have been shown to manipulate vesicular trafficking, ROS production and cellular programming (*italics*). Manipulation of cellular programming occurs by interfering with immune and defense hormone signaling and transcription and translation. The anticipated effector-mediated suppression of ETI is not indicated because no molecular mechanism of manipulation by an oomycete effector has been described to our knowledge.

Cellular programming: Immune signaling

P. infestans effector Pi02860 was found to repress immune signaling through indirect manipulation of SWAP70, a positive regulator of infestatin 1 (INF1)-triggered cell death [84, 108]. The effector was found to interact with StNRL1 (NPH3/RPT2-like), a predicted substrate adaptor of a CULLIN3-associated ubiquitin E3 ligase [109]. Silencing of the *N. benthamiana* ortholog of *NLR1* abolished the ability of Pi02860 to suppress cell death and led to reduced plant colonization by *P. infestans*. These observations suggest that *NLR1* is a susceptibility factor that negatively regulates immunity. *NLR1* homodimers were found to bind SWAP70, a guanine exchange factor that positively regulates cell death, and target it for proteasome-mediated degradation. By interacting with *NLR1* *P. infestans* effector Pi02860 enhances the turnover of SWAP70, thereby suppressing the cell death response [84].

Cellular programming: Hormone signaling

The tight and accurate regulation of plant immune responses is regulated by defense hormones. Generally, salicylic acid is induced upon infection with biotrophic pathogens while jasmonic acid and ethylene are upregulated when a plant is attacked by a necrotrophic pathogen or herbivore. These opposite hormonal signaling networks are known to antagonize each other to optimally arm the plant to fight off the current attacker. The crosstalk between the hormone pathways is exploited by a RxLR effector of *H. arabidopsidis*. Effector HaRxL10 was shown to interact with JAZ3, a transcriptional repressor involved in jasmonic acid signaling, and to target it for proteasome-mediated degradation. Degradation of JAZ3 led to enhanced jasmonic acid signaling and eventually reduced SA-signaling mediated by the release of the JAZ3-guarded MYC2 transcription factor. Arabidopsis plants expressing HaRxL10 also showed enhanced susceptibility to the biotrophic bacterium *Pseudomonas syringae* pv. DC3000 compared to Col-0 [93].

Cellular programming: transcriptional and posttranscriptional manipulation

Cellular reprogramming by oomycete effectors can occur through gene regulation [86] and post transcriptionally through alternative splicing [100] and interference with RNA silencing [110]. *P. sojae* RxLR effector PsAvh23 was found to reduce histone acetylation levels thereby suppressing the activation of defense genes [98]. DNA in cells is organized around nucleosomes, that consist of an octamer of basic proteins called histones. Acetylation of the tails of these proteins by histone acetyltransferases (HATs) is associated with enhanced gene transcription whereas histone deacetylases (HDACs) reverse the modification and repress gene transcription [111]. In plants, the HAT General Control Non-depressive 5 (GCN5) is part of the prototypical nucleosome-acetylating modification complex. GCN5 is modulated by Alteration/Deficiency IN Activation 2 (ADA2) that directs the complex to the histone H3-tail. PsAvh23 competitively binds to ADA2 leading to reduced HAT activity of GCN5 hence impairing the activation of defense-related genes. Possibly, the known ability to suppress cell death is a result of this manipulation [98].

ROS production

Two highly similar *P. sojae* CRN effectors, PsCRN65 and PsCRN115 were, surprisingly, found to induce opposite responses in *N. benthamiana* when expressed in planta. Both effectors interact with catalases, proteins that degrade H_2O_2 which is important for activation of cell death [112]. PsCRN65 promoted H_2O_2 accumulation and hence cell death whereas PsCRN115 suppresses the PsCRN65-mediated accumulation and cell death [103]. Expression of the individual CRN effectors with NbCAT1 induced subcellular relocalization of the catalase to the nucleus. Both CRN proteins were found to be required for full virulence [101].

Vesicle trafficking

1

Vesicle trafficking is an important process in the plant immune response [113]. The plant secretory system (exocytic) pathway is required for delivery of antimicrobial proteins and compounds involved in the first layer of defense. It is not surprising that this important defense process is targeted by oomycete effectors. *P. infestans* effector AVR1 binds to and stabilizes Sec5, a subunit of the plant exocyst complex. Expression of AVR1 in *N. benthamiana* reduced callose deposition and enhanced susceptibility to *P. infestans*, a phenotype that can be mimicked by the silencing of Sec5 [107]. In addition, endocytosis is implicated in the regulation of PAMP-triggered and effector-triggered immunity. The Arabidopsis receptor-like kinase FLAGELLIN SENSITIVE 2 (FLS2) recognizes and binds the flagellin-derived peptide flg22 [114]. Upon binding a MAP kinase cascade is activated that induces defense gene transcription. At the same time the flg22-bound (activated) FLS2 receptors are internalized in endosomal compartments and likely targeted for lysosomal degradation [115]. *P. infestans* effector Avr3a was found reduce internalization of activated FLS2 and suppress flg22-triggered immunity. AVR3a associates with a plant GTPase (Dynamin-Related protein 2 (DRP2) that is required for ligand-induced FLS2 internalization [105].

Over the past ten years many oomycete genomes have been sequenced and assembled. These genomes are a valuable source of information that can be used in understanding the biology of plant-pathogen interactions. The molecular mechanisms by which effectors manipulate host plant processes are now beginning to be revealed. The positioning of host-translocated effector proteins at the interface between pathogens and plants provides a unique opportunity to study both pathogen infection strategies and the plant immune system.

Thesis Outline

The aim of my research described in this thesis was to identify and characterize host-translocated RxLR and CRN effector proteins of *P. effusa* (*Pfs*), the downy mildew that infects spinach plants. RxLR and CRN effectors are translocated into plant cells, where they manipulate plant processes to establish and maintain a successful infection. Manipulation occurs by binding of effectors to plant proteins, thereby altering their function or affecting their stability. In the introductory chapter above I discussed the biology of oomycete plant pathogens and their molecular interaction with host plants (**Chapter 1**).

Identification the effector repertoire of *Pfs* starts with the sequencing and assembly of its genome. Due to the obligate biotrophic nature of this pathogen, DNA can only be isolated from infected spinach leaves. DNA from bacteria and fungi that live on those leaves ends up in the isolated DNA, complicating the assembly of the genome. I describe the usage of the CAT tool, originally developed for filtering bacterial genomes, to assign taxonomy to each open reading frame on an assembled contig, aiding the selection of *Pfs*-derived contigs. The assembled genome was used to predict the subset of genes that encode secreted proteins that are in direct contact with the plant cell during infection. The secretome of *Pfs* was compared to that of other plant pathogenic oomycete species to study differences and similarities in functionality of secretomes of species that have different lifestyles and taxonomy (**Chapter 2**).

The identified candidate RxLR and CRN effectors were studied in more detail in relation to their occurrence and evolution. RxLR and CRN effectors have a modular organization, characterized by a relatively conserved N-terminus that has a signal peptide and translocation domain and a diverse C-terminal effector domain. The C-terminal effector domain is responsible for the virulence function and usually lacks known protein domains or considerable conservation with other proteins. The lack of conservation is thought to help avoid recognition of the effectors by the plant immune system. Some examples of evolutionary conserved effectors are known, and it is hypothesized that these conserved effectors

are especially important for oomycete virulence. Evolutionary conserved effectors from different species have similar functions, probably as a result of interaction with conserved plant proteins. Hence, evolutionary conserved effectors provide an opportunity to study plant processes commonly targeted by effectors. In chapter 3 I characterize the *Pfs* RxLR and CRN effector repertoire by assessing their evolutionary conservation in other species. We found that of the 99 candidate RxLR and 14 CRN effectors, 39 candidate effectors are specific to *Pfs*, 10 are conserved between downy mildew species, 23 are conserved in downy mildew and *Phytophthora* and 40 are more broadly conserved. Note that these numbers total 112, not 113 candidate effectors because one protein has both a canonical RxLR and LFLAK motif and hence qualifies as RxLR as well as CRN candidate effector. A selection of *Pfs* effectors was used for yeast two-hybrid assays to identify interacting plant proteins. This resulted in the identification of the first 40 potential plant targets of *Pfs* effectors (**Chapter 3**).

Nine high-confidence effectors and their fourteen plant interactors were subsequently selected for functional characterization in plants. The proteins were fused to fluorescent proteins and expressed in *N. benthamiana* to visualize their subcellular localization. We found that the majority of the plant interactors and corresponding effectors localized to the same subcellular compartment when co-expressed, a prerequisite for interaction. Additionally, the subcellular localization of six plant interactors and two effector proteins was altered upon co-expression of the effector and interactor proteins and seven plant proteins showed a more stable signal upon co-expression with the effector (**Chapter 4**). Relocalization and stabilization may result from the interaction with the effector protein. Finally, the evolutionary genomics of oomycete pathogens, specifically downy mildew species, is discussed with emphasis on the evolution of pathogenicity and genome size. Furthermore, the acquired knowledge on the *Pfs* RxLR and CRN effectors was assessed and used to provide a future outlook on the study of *Pfs* virulence on spinach (**Chapter 5**).

2

Genome reconstruction of the non-culturable spinach downy mildew *Peronospora effusa* by metagenome filtering

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Abstract

Peronospora effusa (previously known as *P. farinosa* f. sp. *spinaciae*, and here referred to as *Pfs*) is an obligate biotrophic oomycete that causes downy mildew on spinach (*Spinacia oleracea*). To combat this destructive many disease resistant cultivars have been bred and used. However, new *Pfs* races rapidly break the employed resistance genes. To get insight into the gene repertoire of *Pfs* and identify infection-related genes, the genome of the first reference race, *Pfs1*, was sequenced, assembled, and annotated. Due to the obligate biotrophic nature of this pathogen, material for DNA isolation can only be collected from infected spinach leaves that, however, also contain many other microorganisms. The obtained sequences can, therefore, be considered a metagenome. To filter and obtain *Pfs* sequences we utilized the CAT tool to taxonomically annotate ORFs residing on long sequences of a genome pre-assembly. This study is the first to show that CAT filtering performs well on eukaryotic contigs. Based on the taxonomy, determined on multiple ORFs, contaminating long sequences and corresponding reads were removed from the metagenome. Filtered reads were re-assembled to provide a clean and improved *Pfs* genome sequence of 32.4 Mbp consisting of 8,635 scaffolds. Transcript sequencing of a range of infection time points aided the prediction of a total of 13,277 gene models, including 99 RxLR(-like) effector, and 14 putative Crinkler genes. Comparative analysis identified common features in the predicted secretomes of different obligate biotrophic oomycetes, regardless of their phylogenetic distance. Their secretomes are generally smaller, compared to hemi-biotrophic and necrotrophic oomycete species. We observe a reduction in proteins involved in cell wall degradation, in Nep1-like proteins (NLPs), proteins with PAN/apple domains, and host translocated effectors. The genome of *Pfs1* will be instrumental in studying downy mildew virulence and for understanding the molecular adaptations by which new isolates break spinach resistance.

Introduction

Phytopathogenic oomycetes are eukaryotic microbes that infect a large range of plant species. Due to their hyphal infection structures they appear fungal-like, however, taxonomically they belong to the Stramenopiles [2]. The most devastating phytopathogenic oomycetes are found within the orders of *Albuginales*, *Peronosporales* and *Pythiales* (*Pythiaceae*).

The highly radiated *Peronosporales* order contains species with different lifestyles. The most infamous species of this order are in the hemi-biotrophic *Phytophthora* genus. Other species within the *Peronosporales* are the obligate biotrophic downy mildews that cause disease while keeping the plant alive. The relationships between downy mildews and *Phytophthora* species have long been unresolved [27]. Until recently, downy mildew species were underrepresented in studies addressing oomycete phylogeny. This is mainly because the obligate biotrophic nature of the species makes them hard to work with and they are, therefore, under-sampled compared to other oomycete phytopathogens.

The first phylogenetic trees based on morphological traits and single gene comparisons [6, 24] classified the downy mildews as a sister clade to the *Phytophthora* species within the order of *Peronosporales*. Recently published studies using multiple gene and full genome comparisons, including a number of downy mildew species, suggest that the downy mildews have multiple independent origins within the *Phytophthora* genus [19, 22, 27].

The downy mildew *Peronospora effusa* (previously known as *P. farinosa forma specialis spinaciae*, and here referred to as *Pfs*), is the most important pathogen of spinach. *Pfs* affects the leaves, severely damaging the harvestable parts of the spinach crop. Under favorable environmental conditions, *Pfs* infection can progress rapidly resulting in abundant sporulation within a week post inoculation that is visible as a thick grey ‘furry layer’ of sporangiophores producing abundant asexual spores [30]. Preventing spread of this pathogen is difficult, since only a few fungicides are effective in chemical control [116]. As a result, the disease can cause severe losses in this popular crop, and infected fields

often completely lose their market value.

During infection, hyphae of *Pfs* grow intercellularly through the tissue and locally breach through cell walls to allow the formation of haustoria [117]. These invaginating feeding structures form a platform for the intimate interaction between plant and pathogen cells, and function as a site for the exchange of nutrients, signals and proteins. Oomycetes deliver proteins into plant cells to alter host immunity [118], thereby escaping and suppressing plant immune responses [119]. These and other molecules are secreted by pathogens to promote the establishment and maintenance of a successful infection in the host are called effectors. Effector proteins can either be functional outside the plant cells (apoplastic effectors) or inside plant cells (host-translocated effectors). Two types of host translocated are known in oomycetes; the RXLR and crinkler (CRN) effectors. They are characterized by the presence of a signal peptide, a conserved domain at the N-terminus and a variable C-terminal part which is responsible for the function of the effector in the cell [56, 120, 121].

Here we describe the sequencing of genomic DNA obtained from *Pfs* spores collected from infected spinach plants using a combination of Illumina and PacBio sequencing. Sequencing of obligate biotrophic species is complicated as the spore washes of infected plant leaves contain many other microorganisms. Bioinformatic filtering on taxonomy using the recently developed Contig Annotation Tool CAT [122] was deployed to remove the majority of contaminating sequences. The obtained assembly of race *Pfs1* was used to predict genes and compare its proteome, in particular its secretome, with that of other oomycete taxa. We show that the secretomes of obligate biotrophic oomycetes are functionally more similar to each other than to that of more closely related species with a different lifestyle.

Materials and Method

Downy mildew infection

Peronospora effusa race 1 (*Pfs1*) was provided by the Dutch breeding company Rijk Zwaan Breeding BV in 2014. As *Pfs1* is an obligate biotrophic maintenance was done on *Spinacia oleracea* Viroflay plants. Seeds were sown on soil, stratified for two days at 4°C and grown under long day condition for two weeks (16h light, 70% humidity, 21°C). sporangiophores were washed off infected plant material in 50 ml Falcon tubes. The solution was filtered through miracloth and the spore concentration was checked under the microscope. Four-day-old *Spinacia oleracea* Viroflay plants were infected with *Pfs* by spraying a spore solution (70 spores/ul) in tap water. Seven days post inoculation, *Pfs* sporangiospores were collected from heavily-infected spinach leaves with tap water, using a soft brush to prevent plant and soil contamination and used for DNA isolation and genome sequencing.

DNA isolation and genome sequencing

The sporangiospores were freeze-dried, ground and dissolved in CTAB (Cetyltrimethyl ammonium bromide) extraction buffer, lysed for 30 minutes at 65 °C, followed by a phenol-chloroform/isoamyl-alcohol, and chloroform/isoamyl-alcohol extraction. DNA was precipitated from the aqueous phase with NaOAc and ice-cold isopropanol. The precipitate was collected by centrifugation, and the resulting pellet washed with ice cold 70% ethanol. DNA was further purified using a QIAGEN Genomic-tip 20/G, following the standard protocol provided by the manufacturer. DNA was quantified using a Qubit HS dsDNA assay (Thermo Fisher Scientific) and sheared using the Covaris S220 ultrasonicator set to 550 bp. The sequencing library was constructed with the Illumina TruSeq DNA PCR-Free kit. Fragment size distribution in the library was determined before and after the library preparation using the Agilent Bioanalyzer 2100 with HS-DNA chip (Agilent Technologies). The library was sequenced on an Illumina Nextseq machine in high output mode with a 550 bp genomic insert paired end 150 bp reads. Illumina reads with low quality ends were trimmed (Q<36) using prinseq-lite [123].

For PacBio sequencing the input DNA was amplified by WGA (Whole Genome Amplification) using the Illustra GenomiPhi V2 DNA Amplification (GE Healthcare). The sequencing library for PacBio was constructed according to the manufacturer protocol. The resulting library was sequenced on 24 SMRT cells (P6 polymerase and C4 chemistry) using the RSII sequencer (KeyGene N.V., Wageningen). The obtained PacBio reads were error-corrected using the FALCON pipeline [124] with the standard settings using the SMRT Portal that is part of the SMRT analysis software package version 2.3.0 from PacBio [125]. The analysis software package was installed according to the installation instructions on an Amazon WebService (AWS) cloud-based computer and operated via its build in GUI.

Taxonomic classification of long reads

The taxonomic origin of each error corrected PacBio read was determined using the CAT (Contig Annotation Tool) pipeline version 1.0 with default parameters [122]. To do this, CAT first identifies open reading frames (ORFs) on the long sequences or contigs using Prodigal [126] and queries them against the NCBI non-redundant (nr) protein database (retrieved November 2016) using DIAMOND [127]. A benchmarked weighting scheme is then applied that allows the contig to be classified with high precision [122].

Genome assembly and identification of repeats

A pre-assembly was made using taxonomically filtered and corrected PacBio sequences and 60% of the Illumina reads using SPAdes version 3.5.0 [128]. The error-corrected PacBio reads were used as long reads in the assembly, SPAdes was set to use *k*-mer lengths of: 21, 33, 55, 77, 99, 127 for the assembly and the --careful option was used to minimize the number of mismatches in the final contigs. The contigs derived from the pre-assembly were filtered using the CAT tool (see above), and sequences that were designated as bacterial or non-stramenopile eukaryotes were

collected. The entire set of Illumina sequencing reads were aligned to the collection of removed sequences (annotated as bacterial and non-stramenopile) with Bowtie version 2.2.7 using default settings [129]. Illumina reads that aligned to these sequences were removed from the Illumina data set. The remaining Illumina reads (Illumina filtered), and PacBio sequences were re-assembled with SPAdes (same settings as the preassembly), which resulted in a final *Pfs1* genome assembly. A custom repeat library for the *Pfs1* genome assembly was generated with RepeatModeler [130]. Repeat regions in the assembled *Pfs1* genome were predicted using RepeatMasker 4.0.7 [130].

Quality evaluation of the assembly

K-mers of length 21 in the filtered Illumina data set were counted with Jellyfish count version 2.0 [131] with settings -C -m 21 -s 1000000000 followed by Jellyfish histo. The histogram was plotted with GenomeScope [132] to produce a graphical output and an estimate of the genome size. The coverage of the genome by PacBio sequences was determined by aligning the unfiltered error-corrected PacBio reads to the *Pfs1* genome assembly using BWA-mem [133] and selected -x pacbio option. The BBmap pileup [134] script was used to determine the percentage covered bases by PacBio reads in the final assembly of the *Pfs1*.

The GC-content per contig larger than 1kb was calculated using a Perl script [135][83]. GC density plots were generated in Rstudio version 1.0.143 using GGplot version 3.1 [136]. For comparison, the same analysis was done on a selection of other publicly available oomycete assemblies; *Hyaloperonospora (H.) arabidopsidis* [59], *Peronospora (P.) belbahrii* [137], *Phytophthora (Ph.) infestans* [58], *Bremia (B.) lactucae* [61], *Phytophthora parasitica* [138], *Phytophthora ramorum* (Pr102) [138], *Phytophthora sojae* [138], *Peronospora tabacina* (968-S26) [60] and *Plasmopara (Pl.) viticola* [22].

Kaiju [139] was used to analyze the taxonomic origin by mapping reads to the NCBI nr nucleotide database (November 2017). The input for Kaiju was generated using ART [140] set at 20x coverage with 150

bp Illumina to create artificial sequencing reads from the various FASTA assembly files of the genomes of different oomycetes.

Genome completeness and gene duplications were analyzed with BUSCO version 3 [141] with default settings using the protists Ensembl database (May 2018).

RNA sequencing and gene model prediction

RNA of *Pfs1* at different stages during the infection was isolated and sequenced to aid gene model prediction. Infected leaves and cotyledons were harvested every day from three days post infection (dpi) until sporulation (7 dpi). Besides these infected leaves, spores were harvested, and a subset of these spores were placed in a petri dish with water and incubated overnight at 16 ° C to allow them to germinate. RNA was isolated using the RNeasy Plant Mini Kit from Qiagen, and the RNA was analyzed using the Agilent 2100 bioanalyzer to determine the RNA quality and integrity. The RNA-sequencing libraries were made with the Illumina TruSeq Stranded mRNA LT kit. Paired-end 150 bp reads were obtained from the different samples with the Illumina Nextseq 500 machine on high output mode. RNA-seq reads from all the samples were pooled, aligned to the *Pfs1* assembly using Tophat [142], and used as input for gene model prediction using BRAKER1 [143]. The obtained gene models for the *Pfs1* genome together with the RNA-seq alignment result, the repeat models, and results obtained from a BLASTp search to the nr NCBI database (January 2017), were loaded into a locally-installed WebApollo [144] instance. Gene models on the 100 largest contigs of the genome were manually curated and all gene models were exported from WebApollo for further use.

Gene annotation and the identification of functional domains

Bedtools intersect version 2.27 was used to determine the overlap between *Pfs1* gene models and annotated repeat elements in the genome. Gene models that had more than 20% overlap with a region marked as a repeat-

containing gene. ANNIE [145] was used to annotate proteins on the *Pfs1* genome based on Pfam domains [146] and homologous sequences in the NCBI-Swissprot database (accessed Augustus 2017). Sequences that were annotated as transposons by ANNIE were removed from the gene set. SignalP 4.1 [147] was used to predict the presence and location of a signal peptide, the D-cutoff for noTM and TM networks were set at 0.34 to increase sensitivity [148]. TMHMM version 2 [149] was used to predict the presence of transmembrane helices in the proteins of *Pfs1*. To identify proteins that possess one or more WY domains an HMM model made by Win *et al.* [150] was used. Protein sequences that possessed a WY domain were extracted and realigned. This alignment was used to construct a new HMM model using HMMER version 3.2.1 [151] and queried again against all protein models in the *Pfs* genome to obtain the full set of WY domains containing proteins.

Effector identification

Putative effectors residing on the genome of *Pfs1* were identified with a custom- made pipeline [152] constructed using the Perl [153] scripting language. Secreted proteins were screened for the occurrence of known translocation domains within the first 100 amino acids after the signal peptide. Proteins with a canonical RxLR, or a degenerative RxLR (xxLR or RxLx) combined with either an EER-like or a WY domain or both were considered putative RxLR effectors. A degenerative EER domain was allowed to vary from the canonical EER by at most one position.

Proteins with a canonical LFLAK motif or a degenerative LFLAK and HVL motif in the first 100 amino acids of the protein sequence. A HMMer profile was constructed based on the LFLAK or HVL containing proteins. This HMMer profile was used to identify Crinkler effector candidates lacking the LFLAK or HVL motif.

Proteins with an additional transmembrane domain or a C-terminal ER retention signal (H/KDEL) were removed. WY domains were identified using hmmsearch version 3.1b2 [154] with the published *Phytophthora* HMM model (see above) [77]. *Pfs* WY-motif containing

protein sequences were realigned and used to construct a *Pfs* specific WY HMM model using hmmbuild version 3.1b2 [154]. Based on the *Pfs* specific HMM model WY-motif containing *Pfs* proteins were determined.

The effector prediction for the comparative analysis was done in a similar fashion, except the published *Phytophthora* HMM model for RxLR prediction and a published model for CRN prediction was used [155]. The prediction of effectors using the same model in each species enabled the comparison.

2

Comparative gene distance analysis

Based on the gene locations encoded in the GFF file the 3' and 5' intergenic distances between genes on contigs were calculated as a measure of local gene density. When a gene is located next to beginning or end of a contig, the distance was taken from the start or end of the gene to the end of the contig. Putative high confidence RxLR effector sequences that encode for proteins with either an exact canonical RxLR motif or an RxLR-like motif in combination with one or more WY-motifs were selected for the comparison (66 in total). Distances were visualized using a heat map constructed with the GGPlot geom_hex function [136]. Statistical significance was determined using the Wilcoxon signed-rank test [156].

Comparative secretomics

The predicted proteomes of eighteen plant pathogenic oomycetes were obtained from Ensembl and NCBI (**Table S1**). Proteins in the collected proteomes that have a predicted secretion signal [147] (SignalP v.4.1, D-cutoff for SignalP-noTM and TM networks = 0.34 [148]), no additional transmembrane domain (TMHMM 2.0 [149]) or C-terminal K/HDEL domain were considered secreted. Functional annotations of the secreted proteins were predicted using InterProScan [157] and the CAZymes database [158] using the dbCAN2 meta server [159].

Phylogenetic analysis

The phylogenetic relationships between the proteomes of the studied species were inferred using Orthofinder [160]. Orthofinder first identifies 'orthogroups' of proteins that descended from a single ancestral protein. Next it determines pairwise orthologs between each pair of species. Orthogroups with only one protein of each species were used to make gene trees using MAFFT [161]. The species tree was inferred from the gene trees using the distance algorithms of FastMe [162] and visualized using EvolView v2 [163].

Principal Component Analysis

The total number of InterPro and CAZymes domain per species was summarized in a counts table. For each domain the number was divided by the total number of domains for that species. The normalized matrix has been loaded into Phyloseq version 1.22.3 [164] with R version 3.4.4 [165] in RStudio [166]. A PCA plot has been made with the Phyloseq ordinate function on euclidean distance. The PCA plot has been made with the GGPlot R package [136]. The biplot has been generated with the standard prcomp function in R with the same normalized matrix. Figures were optimized using Adobe Photoshop 2017.01.1.

Permutational analysis of variance (PERMANOVA)

A PERMANOVA using distance matrices was used to statistically test whether there is a difference between the clades based on their CAZymes and InterPro domains. PERMANOVA is a non-parametric method for multivariate analysis of variance using permutations. The data has been double root transformed with the vegdist function from the R-package vegan version 2.5-3 [167]. After the transformation the PERMANOVA has been calculated with the adonis function from the Vegan package. A total number of 999 permutations have been made to retrieve a representative permutation result.

Enrichment analysis

A chi-square test with Bonferroni correction was used to identify under- and over-represented Pfam domains in each group (*Hyaloperonospora/Peronospora*, *Plasmopara*, *Albugo*) compared to *Phytophthora*. The actual range was the sum of the proteins that have a given domain. The expected range was the fraction of proteins with a given domain that is expected to belong to a species cluster giving the overall ratio of Pfam domains between species clusters.

2

Results

An early race 1 isolate, *Pfs1*, of *Peronospora effusa* was used to create a reference genome as it predates resistance breeding in spinach and its infection is effectively stopped by all spinach resistance genes known to date. Race 1 was first identified in 1824 [168]. Since downy mildews cannot be grown axenically we isolated asexual sporangiospores by carefully washing highly-infected leaves of the universally susceptible cultivar Viroflay. Genomic DNA was isolated from freeze-dried spores and used to construct libraries for PacBio and Illumina sequencing, resulting in 1.09 million PacBio reads with a N50 of 9,253 bp, and 535 million Illumina reads of 150 bp. The paired-end Illumina reads were used for a trial assembly using Velvet. Inspection of the draft assembly showed that many contigs were of bacterial instead of oomycete origin. This is likely caused by contamination of the isolated *Pfs* spores with other microorganisms that reside on infected leaves and that are collected in the wash-offs. We, therefore, decided to treat the sequences as a metagenome and bioinformatically filter the sequences and corresponding reads.

Taxonomic filtering

To filter out the sequences that could be classified as contaminants we deployed CAT [122] on long reads and contigs derived from assemblies. Details on the CAT method are described in the materials and methods section. In short, CAT utilizes the combined taxonomic annotations of multiple individual ORFs found on each sequence to determine its likely

taxonomic origin. This allows for a robust taxon classification that is based on multiple hits, rather than a single best hit. An example of the CAT taxonomic classification for two of our sequences (contigs) is visualized in **Fig.1**.

Figure 1. Taxonomic classification by CAT.

Two contigs are depicted and per ORF a single top hit is shown. (A) Contig from the pre-assembly assigned by the CAT tool as bacterial, ORFs of bacterial origin are colored green, and ORF with no hits to the database are colored white. On this contig most ORFs had a highest blast hit with *Rhodococcus* species. The ΣB_{max} for this contig is 10982, and the highest ΣB_{taxon} is for the *Rhodococcus* genus at 9660, which is well above the cutoff of 5491 ($\Sigma B_{max} * 0.5$). The taxonomic origin of this contig was therefore assigned to the genus *Rhodococcus*, and as a consequence this contig was regarded as non-*Pfs* and removed. (B) Contig from the pre-assembly assigned by the CAT tool as an oomycete contig. On this contig all ORFs have a best hit to an oomycete species, and the ΣB_{max} is 2328. In fact, most ORFs have a best hit to species in the *Phytophthora* genus (ΣB_{taxon} :1184), or the Peronosporales family (ΣB_{taxon} :184). The ΣB_{taxon} for the *Phytophthora* genus is above the cutoff at 1164 ($\Sigma B_{max} * 0.5$) thus assigning this contig to the *Phytophthora* genus, and consequently this contig is maintained for the *Pfs* genome assembly.

CAT was first used on the long PacBio reads. As these reads contain about 15% base call errors on average, they were first error-corrected using the FALCON pipeline. The FALCON pipeline fixes long PacBio reads by mapping short reads obtained in the same runs. The resulting 466,225 PacBio reads had a total length of 1,003 Mb with a N50 of 3,325 bp and were subsequently assigned a taxonomic classification using CAT. PacBio reads that were classified as prokaryotic, or non-stramenopile eukaryotic (e.g. Fungi) were removed, whereas reads with the assigned taxonomy “stramenopiles” or “unknown” were retained. This resulted in a cleaned set of 232,846 PacBio reads with a total length of 522 Mb with a N50 of 3,458 bp that was used for a hybrid pre-assembly. In order to evaluate the effectiveness of the CAT tool in removing contaminating genomic sequences we analyzed the GC-content of the reads. The corrected PacBio reads showed two distinct peaks (**Fig. 2a**), whereas oomycete genomes have a GC band-width around 50%, as shown in Fig. S1a for the contigs of the *Phytophthora infestans* genome [58]. After CAT filtering a single peak remained with a narrow GC-content distribution around ~48%, demonstrating that the tool, that does not take into account GC-content but uses a weighting scheme based on protein sequence similarity, was effective in removing contaminating sequences (**Fig. 2b**).

Hybrid assembly

A hybrid pre-assembly was generated using the genome assembler SPAdes that can combine long PacBio with short Illumina reads. The input consisted of all corrected and filtered PacBio reads together with 60% randomly extracted Illumina reads (321 Million read, 96.3 Gb, to decrease assembly run time and memory requirements). The pre-assembly consisted of 170,143 contigs with a total length of 176 Mb and an N50 of 6,446 bp, of which only 21,690 contigs were larger than 1 kb. CAT filtering was applied to the contigs of the pre-assembly, CAT marked 16,518 contigs consisting of 91.5 Mb (52% of total assembled bases) as contaminant sequences. Next, Illumina reads were aligned to these and Illumina read-pairs of which at least one end aligned were removed from

the data set. A final assembly was generated with the CAT-filtered PacBio and remaining 77.6 million Illumina reads, resulting in 8,635 scaffolds with a total length of 32.4 Mb. The assembly size corresponds with the estimate genome size of 36,2 Mb that was determined based on *k*-mer count frequency (**Table 1**) in the filtered Illumina reads.

Filtering results

The effect of filtering with CAT on the pre-assembly is well visualized by plotting the GC-content of the contigs (**Fig. 2c**), similar as for the PacBio reads. In the pre-assembly many contigs with a GC-percentage deviating from the 40-55% range are present, indicating that it contains many contaminating sequences. After filtering, the final assembly shows one major peak of the expected GC-content at ~48%, with a minor shoulder of slightly higher GC-content (**Fig. 2d**).

Figure 2. Density plot of the GC values of PacBio reads and assembly before and after CAT filtering of sequences.

The yellow bar indicates the region between 40 and 55% GC, based on reads >1 kb. (A) PacBio reads before CAT-filtering show a bimodal distribution with a presumed peak of contaminating sequences with a GC content of ~40%. (B) PacBio reads after CAT-filtering show a distribution consisting of a single peak with a GC content around ~46%. (C). GC content of the *Pfs1* contigs from the pre-assembly before filtering shows additional peaks at around 30 and 60 GC%, indicating that there are many contaminant contigs. (D) GC content of the *Pfs1* contigs after filtering of the reads with the CAT tool shows that the additional peaks are no longer present and have thus been successfully filtered out.

To assess the effectiveness of the taxonomic filtering we used Kaiju [139] as a complementary tool. Kaiju is typically used for taxonomic classification of sequencing reads in metagenome analysis but here we used it to determine the effect of taxonomic filtering by CAT. For this, genome assemblies of *Pfs1* and other oomycetes were divided into artificial short reads. The taxonomic distributions generated by Kaiju provide a clear picture of the removal of contaminating sequences from the *Pfs1* genome data (**Fig. 3**). Whereas the pre-assembly mostly contained artificial reads with an assigned bacterial taxonomy, this was reduced to 14% in the final assembly. The percentage of >80% of oomycete-assigned reads in the *Pfs1* final assembly is similar to what we observe for the high-quality

genome assemblies of *P. infestans* and *P. sojae*, pathogens that can be grown axenically, i.e. free of contaminating other microbes (**Fig. 3**).

Figure 3. Taxonomic classification of reads in assemblies of different oomycetes.

Kaiju bar plot showing the percentage of reads assigned to three taxonomical classes; Oomycetes, Fungi and Bacteria and other non-oomycetes. In error corrected PacBio reads 42.64% are assigned to oomycetes, after filtering with CAT 88.09% of the reads are assigned to oomycetes. For the pre-assembly (96.3 Gb), only 5% of the artificial reads is assigned to oomycetes. For the *Pfs1* final assembly (32.4 Mb), 88.6 % of the reads are assigned to oomycetes. This is comparable to other oomycetes that can be axenically grown on plates, indicating that the remaining non-oomycete-assigned sequences are most likely a result of an incorrect classification in the database.

Genome statistics

To assess the quality of the assembly we re-aligned the Illumina reads to the contigs and found a large variation in coverage between the contigs smaller than 1 kb and the larger contigs, suggesting that these small contigs contain a high number of repeats or assembly errors. In addition, the CAT pipeline depends on classification of individual ORFs on contigs, so it's accuracy may be expected to improve with contig length. Therefore, several small contigs could possibly be derived from microbes other than *Pfs*. Removing contigs smaller than 1 kb (5027 contigs) resulted in a small

reduction of 1.9 Mb in genome length, slightly reducing the assembly size to 30.5 Mb, but resulting in a 58% reduction in the number of contigs. The remaining 3608 contigs, larger than 1 kb, had an N50 of 36,273 bp. The statistics of the size-filtered assembly are further detailed in Table 1.

Table 1. Summary of statistics for the hybrid assembly of the *Pfs1* genome.

	<i>Pfs1</i> final	<i>Pfs1</i> size-filtered
Assembly size	32.40 Mb	30.48 Mb
GC content	47.75%	47.80%
Longest scaffold	310.10 kb	310.10 kb
Repeat size	6.93 Mb	6.38 Mb
# Contigs	8,635	3,608
N50	32,837 bp	36,273 bp
# Gene models	13,227	12,630

	k-mer estimation
Assembly size	36.18 Mb
Repeat size	8.76 Mb
Read Error Rate	1.04%

Data is provided for the final assembly (*Pfs1* final) and size-filtered assembly omitting the contigs smaller than 1 kb (*Pfs1* filtered). In addition, genome information based on *k*-mer counting of the Illumina reads is provided, giving an estimate for the predicted genome size and repeat content.

To assess the gene space completeness of our assembly in comparison to other oomycete genomes we used BUSCO that identifies single core orthologs that are conserved in a certain lineage. Here, we used the protist Ensembl database as the protist lineage encompasses the oomycetes and other Stramenopila. According to the BUSCO analysis the gene space in our final assembly is 88.9% complete with only 0.5% fragmented genes and 0.5% duplicates. This gene space completeness score is similar to that of other downy mildew genomes, but slightly lower than of genomes of *Phytophthora* species (Table S2). Furthermore, the low number of duplicates suggests that there is a low incidence of

erroneous assembly of haplotypes, suggesting that the obtained *Pfs* assembly represents most of the single-copy gene space of the *Pfs* genome [141].

Repeat content

In addition to a genome size estimate, the *k*-mer analysis estimated a repeat content of ~8.8 Mb. This is slightly higher than the observed repeat content in the final assembly of ~6.9 Mb (~6.4 Mb in the size-filtered assembly) (**Table 1**). The difference between the estimated repeat size and the repeat content in the assembly (1.87 Mb) is most likely caused by long repetitive elements that are hard to assemble. Repeatmasker [130] identified a total of 13,089 repeat elements of which most are part of the Gypsy and Copia superfamily. We also identified 562 LINEs (Long interspersed nuclear elements) and only 16 SINE (short interspersed nuclear elements), which belong to the class I transposon (retrotransposons). Other repeat elements consisted of 2297 simple repeats, 298 Low complexity regions, 391 different types of DNA transposons (**Table 2**), and several (278) other minor repeat types; full details can be found in Table S3.

Table 2. Total number and size of major repeat types identified in the *Pfs1* genome assembly.

Repeat type	Count	% of total count	Total length (bp)
LTR	9247	70,65	6532069
LINE	562	4,29	201127
Simple repeat	2297	17,55	97983
DNA repeats/TE	391	2,99	46677
Rolling Circle TE	97	0,74	26123

The percentage of total count is based on the total number of repeat types identified in the assembly which can be found in Table S3.

When we compare the genome assembly size of *Pfs* (30.5 Mb) to other sequenced oomycete genomes such as those of *Ph. infestans* (240 Mb), *H. arabidopsidis* (100 Mb), *Pl. halstedii* (75.3 Mb) or the relatively small genome of *P. tabacina* (63.1 Mb), *Pfs* has a strikingly compact genome (**Table S4**). The repeat content (21%) is also low compared to that of other oomycetes, e.g. *Ph. infestans* (74%), *H. arabidopsidis* (43%), *Pl. halstedii* (39% Mbp) and more comparable to *P. tabacina* (24%).

2

***Pfs* gene prediction RNA sequencing**

Gene prediction is greatly aided by transcript sequence information. We, therefore, isolated and sequenced mRNA from *Pfs* spores and *Pfs*-infected spinach leaves at several time points during the infection. For this, leaves were harvested daily starting from 3 days post inoculation (dpi) until 7 dpi when sporulation was observed. In addition, mRNA was also isolated from sporangiospores and germlings grown from spores that were incubated in water overnight. The 7 different samples ensure a broad sampling of transcripts to facilitate gene identification. Illumina transcript sequences (659 million) were aligned to the assembled *Pfs* genome which resulted in ~100 million aligned read pairs. Most of the other reads map to the spinach genome but were not further analyzed.

Predicted proteins

The aligned transcript read pairs served as input for the BRAKER1 [143] pipeline to generate a *Pfs* specific training set for gene model prediction. This was then used to predict 13227 gene models on the final assembly. The corresponding protein models were annotated using ANNIE [145] and provided putative annotations for 7297 *Pfs* proteins (**Table S5**). We found that 12630 protein models reside on contigs larger than 1 kb and are thus contained in the size-filtered assembly. In addition, we found that 2983 gene models had 20% or more overlap with a repeat that was identified by RepeatMasker [130], another 952 protein models were annotated by ANNIE as transposable elements. When analyzing protein

models that reside on small contigs (<1 kb) we observe that most of them (61%) have a significant overlap with a repeat region and are marked by ANNIE as transposons. The number of gene models found in the assembly of *Pfs1* is strikingly low in comparison to that in *Ph. infestans* (17,792), *H. arabidopsidis* (14,321), *Pl. halstedii* (15,469) and more similar to *P. tabacina* (11,310).

Secretome and host-translocated effectors

For the identification of the *Pfs* secretome as well as of candidate host-translocated RxLR and Crinkler effectors we choose to start with the proteins encoded by the initial 13,227 gene set. This reduced the risk of missing effectors that are encoded on smaller contigs (< 1 kb). SignalP [147] prediction identified 783 proteins with a N-terminal signal peptide. Of these, 231 were found to have an additional transmembrane domain (as determined by TMHMM [149] analysis) leaving 557 proteins. In addition, five of these carried a C-terminal H/KDEL motif that functions as an ER retention signal. The resulting set of 552 secreted proteins, ~ 4% of the *Pfs1* proteome, was used for secretome comparison.

Previous research showed that some effectors of the lettuce downy mildew *Bremia lactucae* have a single transmembrane domain in addition to the signal peptide [169]. Therefore, we chose to predict the host-translocated effectors not only from the secretome but also from the set of proteins with a signal peptide and an additional transmembrane domain. A total of 99 putative RxLR or RXLR-like proteins and 14 putative Crinkler effectors were identified (**Table S6**). Ten putative RxLR effector proteins were found to have a single transmembrane domain. Also, five putative RxLR effectors were found on contigs smaller than 1 kb (**Table S6**). Of the 99 RxLR effectors, 64 had a canonical RxLR domain, while 35 had a degenerative RxLR domain combined with an EER-like and/or WY domain [170]. The number of host-translocated effectors in *Pfs* is significantly smaller compared to that of *Phytophthora* species (eg. 563 RxLR and 385 effector genes in the genomes of *P. infestans* [58] and *P. sojae* [171] respectively). Crinkler effectors are characterized by

the N-terminal five amino acid “LFLAK” domain [56] Five of the identified putative Crinkler effectors had a canonical LFLAK domain. The others had a degenerative LFLAK combined with an HVL domain or were identified using the custom made Crinkler HMM.

Genomic distribution of effectors

It was previously described for the potato late blight pathogen *P. infestans* that effectors often reside in genomic regions with a relatively large repeat content compared the rest of genome [172]. To test this in *Pfs*, the distance between neighboring genes was measured to estimate the genomic context of the 13277 *Pfs1* genes in general and for 66 selected RxLR effector (canonical RxLR and degenerative RxLR with WY-motifs) genes specifically. To get a good overview of the intergenic distances we plotted the 3' and 5' values for all the genes in the *Pfs1* genome on a log10 scaled heat map (**Fig. 4**).

Figure 4. Genome spacing of predicted genes of *Pfs1*.

The distance between neighbouring genes was depicted by plotting the 5' and 3' intergenic distances (on a log₁₀ scale) for each of the 13,227 predicted genes. The scale bar represents the number of genes in each bin, shown as a color-coded hexagonal heat map in which red indicates a gene dense and blue a gene-poor region. The locations of putative *Pfs* effector genes are indicated with white dots.

The genome of *Pfs1* is highly gene dense and effectors show a modest but significant (Wilcoxon rank sum test, $p = 1.914e^{-11}$) enrichment in the gene-spare regions of the genome (**Fig. 4**). The median 3' and 5' combined spacing for all genes is 925 bp, while for the selected effector genes it is 2976 bp. However, the difference in gene density between the effectors and core genes is not as strong as in the *P. infestans* two-speed genome [58].

Comparative analysis of orthologs

Eighteen phytopathogenic oomycete species, that represent a diverse taxonomic range and different lifestyles, were chosen for a comparative analysis with *Pfs* (**Table 3**). The objective of the comparison is to see whether the biotrophic lifestyle of downy mildew species, like *Pfs*, is reflected in the secretome. For the analysis, the secretome of *Pfs* was compared to that of closely related *Phytophthora* (hemibiotrophic), *Plasmopara* (biotrophic) and more distantly related *Pythium* (necrotrophic) and *Albugo* (biotrophic) species. First, the predicted proteins of each species were used to create a multigene phylogenetic tree to infer their taxonomic relationships using Orthofinder. In total, 86.9% (267,813) of all proteins were assigned to 14,484 orthogroups. Of those, 2383 had proteins from all species in the dataset of which 152 groups contained proteins corresponding to single copy proteins in each species. These single-copy orthologous proteins of each species were used to infer a Maximum-likelihood species tree (**Fig. 5**).

Figure 5. Maximum likelihood tree of 18 plant infecting oomycete species based on core orthologous proteins. The tree was inferred from 152 single copy ortholog groups in which all species in the comparison were represented. Branch numbers represent bootstrap values of N=12171 trees. Five taxonomic clusters were defined for further analysis; *Hyaloperonospora/Peronospora* (green), *Plasmopara* (red), *Phytophthora* (blue), *Pythium* (grey) and *Albugo* (green). The obligate biotrophic clades are highlighted using green circle. The fish infecting species *Saprolegnia parasitica*, was used as an outgroup.

The resulting tree shows that *Pfs* clusters with *H. arabidopsidis* (*Hpa*), *P. tabacina* (*Pta*) and *P. belbahrii* (*Pbe*). The closest relative of *Pfs* in this study, based on single-copy orthologs is the downy mildew of tobacco *Pta*, followed by the basil-infecting *Pbe*. Based on the tree, *Hpa* is more divergent from the former three downy mildew species within the *Hyaloperonospora/Peronospora* clade. The *Plasmopara* downy mildew species are in a different clade that is more closely related to the *Phytophthora* species used in this study. The separation between the *Peronospora* lineage and the *Phytophthora/Plasmopara* lineages is well supported with a bootstrap value of 0.75. This clustering pattern is in line with the recent studies that suggest that the downy mildew species are not monophyletic within the Peronosporales [27, 173]. The *Phytophthora* species, although belonging to three different *Phytophthora* clades, are more closely related to each other than to the other species in this study. *Phytophthora vexans* appears as a sister group to the *Phytophthora/Peronospora* lineage, which is in line with a recently published multi gene phylogeny [20]. The other five species of *Pythium* form two clusters, as previously observed [20]. The two *Albugo* species form a cluster that is separated from the other clades with maximum bootstrap support.

Based on the core ortholog protein tree, we grouped the species into five phylogenetically-related clades; *Hyaloperonospora/Peronospora*, *Plasmopara*, *Phytophthora*, *Pythium* and *Albugo* for further analysis of the secretomes. Three of these clades only have obligate biotrophic species (*Hyaloperonospora/Peronospora*, *Plasmopara* and *Albugo*), whereas the *Phytophthora* cluster consists of hemi-biotrophs and *Pythium* cluster of necrotrophic species. *(Phyto)Pythium vexans* was included in the *Pythium* cluster. The fish-infecting oomycete *Saprolegnia parasitica* served as an outgroup for the phylogenetic tree and is not used for further comparison.

Secretome comparison

For each species, the total number of proteins and the subset that is predicted to be secreted (signal peptide, no additional transmembrane domains, no ER retention signal) is shown in Table 3. *Phytophthora* species generally have a larger proteome than downy mildew species and secrete a larger percentage of the predicted proteins. The *Phytophthora* species in this study are predicted to secrete 1976 proteins on average, whereas the *Plasmopara* and *Peronospora* species secrete an average of 1461 and 703 proteins, respectively.

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Table 3. Predicted secretomes of 18 oomycete species used in this study.

	Predicted proteins	Secretome	% secreted
<i>P. effusa</i>	13227	552	4,2
<i>P. belbahrii</i>	9049	494	4,7
<i>H. arabidopsidis</i>	14321	999	7
<i>P. tabacina</i>	18447	798	4,3
<i>Pl. halstedii</i>	15498	1071	6,9
<i>Pl. viticola</i>	12201	1850	15,2
<i>Ph. infestans</i>	18138	1885	10,4
<i>Ph. parasitica</i>	27942	2250	8,1
<i>Ph. sojae</i>	26584	2337	8,8
<i>Ph. capsici</i>	19805	1433	7,2
<i>Py. arrhenomanes</i>	13805	913	6,6
<i>Py. aphanidermatum</i>	12312	928	7,5
<i>Py. irregulare</i>	13805	961	7
<i>Py. iawyamai</i>	15249	1067	7
<i>Py. vexans</i>	11958	863	7,2
<i>Py. ultimum</i>	15322	1071	7
<i>A. candida</i>	13310	888	6,8
<i>A. laibachii</i>	13804	679	4,9

The total number of predicted proteins, those with a signal peptide (SP), proteins with SP but without additional transmembrane domains (TM), and the number of proteins with SP, no TM, and no C-terminal KDEL sequence are shown. In the final column the percentage of the proteome that is predicted to be secreted is highlighted.

Carbohydrate active enzymes and Pfam domains

The secretome content was compared between species by looking at the carbohydrate-active enzymes (CAZymes) and Pfam domains. CAZymes are, amongst others, involved in degrading and modifying plant cell walls, which is an important part of the infection process. The Pfam domain database represents a broad collection of protein families, including RxLR effectors, with diverse functions.

A total of 95 different CAZyme domains were found in the combined secretomes of the 18 oomycete species. The total number of CAZymes per species ranges from 35 in *A. laibachii* to 336 in *P. sojae*, and was lower in obligate biotrophic species (35 - 193) compared to *Phytophthora* species (197 - 336) (**Table S7**). A total of 1354 different Pfam domains were found in the combined secretomes of the oomycetes analyzed. The number of domains identified ranged from 304 in *Al. candida* to 1710 in *Ph. parasitica*. The total number as well as the relative number of Pfam domains in secretomes of obligate biotrophic species was lower in obligate biotrophic species compared to *Phytophthora* and *Pythium* (**Table S8**).

The presence and numbers of CAZyme and Pfam domains were compared between species using a Principal Component Analysis (PCA), a statistical reduction technique that determines what variables contribute most to the variation observed in a data set. We report the relative abundance of each CAZyme/Pfam domain to the total number of secreted Pfam/CAZyme domains per species, to account for the large variation in absolute numbers of proteins between the species (**Fig. 6**). PCAs based on the absolute numbers can be found in Fig. S2 and S3, which shows a similar pattern. The species clusters as depicted in Fig. 6 were confirmed using a PERMANOVA ($p < 0.001$).

Figure 6. Principal component analysis (PCA) of variation in the relative abundance of secreted CAZymes and Pfam domains.

The variation in secreted CAZyme (AB) and Pfam (CD) domains along PC1 and PC2 is depicted in the figure. The PCAs include all of the 18 species (AC) or the *Peronospora*, *Plasmopara* and *Phytophthora* species only (BD). The PERMANOVA test shows that the grouping based on the CAZyme and Pfam domains is significant ($P < 0.001$). Species are grouped by color based on the classes that were defined in the phylogenetic tree (Fig. 5). *Phytophthora* (blue), *Peronospora* (yellow), *Plasmopara* (red), *Albugo* (green) and *Pythium* (grey). Abbr. PFS; *Peronospora* (*P.*) *effusa*, PBE; *P. belbahrii*, PTA; *P. tabacina*, HPA, *Hyaloperonospora arabidopsidis*, PHA; *Plasmopara* (*Pl.*) *halstedii*, PVI; *Pl. viticola*, PIN; *Phytophthora* (*Ph.*) *infestans*, PSO; *Ph. sojae*, PCA; *Ph. capsici*, PPA; *Ph. parasitica*, ACA; *Albugo* (*A.*) *candida*, ALA; *A. laibachii*, PUL; *Pythium* (*Py.*) *ultimum*, PAR; *Py. arrhenomanes*, PAP; *Py. aphanidermatum*, PIR; *Py. irregulare*, PIW; *Py. lawyamae*, PVE; *Phytophthora vexans*

The CAZymes-based PCA supports the separate clusters of *Albugo*, *Phytophthora* and *Pythium* species as found in the core ortholog tree (**Fig. 5**). Remarkably, neither the *Hyaloperonospora/Peronospora* nor the *Plasmopara* species form a clear cluster, although the clustering is significant (PERMANOVA $p < 0.001$). The variation along PC1 (*Hyaloperonospora/Peronospora*) and PC2 (*Plasmopara*) indicates that the secreted CAZyme domains vary largely between the species in these groups, despite their close phylogenetic relationship and same lifestyle. The secreted CAZymes of the two *Plasmopara* species appear more similar to those of the *Hyaloperonospora/Peronospora* species than to the *Phytophthora* species, which is different from the results of the core ortholog protein comparison as shown in the phylogenetic tree (**Fig. 5**). To exclude the effect of the more distantly-related species on the separation between the downy mildew and *Phytophthora* species, the PCA was performed on the set without the *Pythium* and *Albugo* species (**Fig. 6b**). The pattern, as observed in the total set, is maintained when the more distantly related species are excluded from the analysis.

To look further into the properties of the secreted CAZymes we highlighted literature-curated domains of phytopathogenic oomycetes that are known to modify the main plant cell wall components; lignin, cellulose and hemicellulose [69] (**Fig. S4**). We found that the secretomes differ more in terms of the absolute number of plant cell wall-degrading enzymes than in the relative occurrence of the different corresponding CAZyme catalytic activities per species. Secretomes of obligate biotrophic and hemibiotrophic/necrotrophic oomycetes have secreted proteins with similar functions (like breakdown of cellulose, pectin, hemicellulose etc.) but the numbers and diversity of those proteins in obligate biotrophic species are reduced.

The Pfam-based PCA shows a clear separation between lifestyles (**Fig. 6cd**). The *Phytophthora* species cluster together and separate from all other species along PC1 (25,3%). The *Pythium* species form a cluster that separates clearly from the other species along PC2 (20,2%). All biotrophic species, including both groups of downy mildews

and the *Albugo* species, cluster together. Within the obligate biotrophic cluster the phylogenetic groups (*Hyaloperonospora/Peronospora*, *Plasmopara*, *Albugo*) as found in the core ortholog tree are still present but the differences are minor. To exclude the effect of the more distantly related species on the separation between the obligate biotrophs, the PCA was also performed without *Pythium* and *Albugo* species (**Fig. 7b**). The pattern observed in Fig. 6C is maintained when the more distantly related species are excluded from the analysis (**Fig. 6d**).

The repertoires of Pfam domains in the different groups of obligate biotrophs (*Hyaloperonospora/Peronospora*, *Plasmopara* and *Albugo*) are more similar than would be expected based on their taxonomic relationship. This could be the result of convergent evolution towards the obligate biotrophic lifestyle. *Plasmopara* and *Hyaloperonospora/Peronospora* CAZyme repertoires are similar as well, but the *Albugo* species have a different CAZyme profile.

We conclude that a different composition and abundance in secreted Pfam domains is clearly associated with obligate biotrophy, suggesting it is the result of convergent evolution towards an obligate lifestyle.

To look further into the properties of the secreted CAZymes we highlighted literature-curated domains of phytopathogenic oomycetes that are known to modify the main plant cell wall components; lignin, cellulose and hemicellulose [174]. We found that the secretomes differ more in terms of the absolute number of plant cell wall-degrading enzymes than in the relative occurrence of the different corresponding CAZyme catalytic activities per species. Secretomes of obligate biotrophic and hemibiotrophic/necrotrophic oomycetes have secreted proteins with similar functions (like breakdown of cellulose, pectin, hemicellulose etc.) but the numbers and diversity of those proteins in obligate biotrophic species are reduced.

Seven Pfam domains contribute largely to the difference between obligate biotrophs and others.

The Pfam domains that contribute to the variance in PC1 and PC2 were (Fig. 6 cd) identified using a biplot. In a biplot, the variables are presented as vectors, with their length reflecting their contribution. Many of the domains contribute to the differences between the biological groups, but seven of them stand out (Fig. 7. and Table 4).

Figure 7. Pfam domains that strongly contribute to the variation in the relative abundance between species.

Although many domains contribute to the variation, PF16810, PF05630, PF08238, PF14295, PF00090, PF00254 and PF00082 are the domains that contribute most, as evidenced by the length of their vectors in the biplot.

Two Pfam domains that have a higher relative abundance in *Phytophthora* contribute strongly to the separation between *Phytophthora* and the other species. The first, PF16810, represents a RxLR protein family with a conserved core α -helical fold (WY-fold). Some of the proteins that this domain was based on have a known avirulence activity [77], i.e. they are recognized by plant resistance proteins. On average, 82 PF16810 domains were identified in *Phytophthora* species compared to 1.3 in *Peronospora*, 1.0 in *Plasmopara* and none in *Albugo* species. Using HMMer searches, many more WY-fold proteins can be identified in *Plasmopara* and *Hyaloperonospora/Peronospora* downy mildew species. However, these proteins do not match to the PF16810 Pfam domain that is based on a larger protein sequence as the HMM.

	Hpa/Peronospora					Plasmopara			Phytophthora				Pythium					Albugo		
	Pfs	Pta	Pbe	Hpa	Pha	Pvi	Pin	Ppa	Pso	Pca	Par	Pap	Pir	Piw	Pve	Pul	Aca	Ala		
PF16810 RxLR	3	0	0	2	2	0	92	90	104	41	0	0	0	0	0	0	0	0		
PF05630 NPP1	7	18	2	10	15	10	31	54	59	42	4	3	3	2	5	7	0	0		
PF08238 Sel1 repeat	16	39	14	7	10	23	14	20	22	16	27	30	27	24	10	25	6	13		
PF14295 PAN/Apple	1	1	2	0	3	0	39	36	31	22	64	60	35	33	21	33	1	5		
PF00082 Subtilase	1	1	1	1	5	20	5	4	2	0	10	21	19	17	9	26	2	2		
PF00090 Thrombosp.	0	0	0	0	0	12	14	11	40	12	0	26	21	22	12	23	0	0		
PF00254 FKBP	1	13	1	1	1	2	1	1	1	1	2	1	0	2	1	1	0	1		

Table 4. Pfam domains that contribute most to the variation between species in the PCA.

Numbers represent the number of domains per secretome per species. Domains that are relatively less abundant are blue, domains that occur in relatively high numbers are yellow.

The second, PF05630, is a necrosis-inducing protein domain (NPP1) that is based on a protein of *Ph. parasitica* [175]. This domain is conserved in proteins belonging to the family of Nep1-like proteins (NLPs) that occur in bacteria, fungi and oomycetes [176]. Infiltration of cytotoxic NLPs in eudicot plant species results in cytolysis and cell death, visible as necrosis [177]. *Phytophthora* species are known to have high numbers of recently expanded NLP genes in their genomes, encoding both cytotoxic and non-cytotoxic NLPs [176]. *H. arabidopsidis* and other obligate biotrophs tend to have lower numbers and only encode non-cytotoxic NLPs [52, 176].

Domain PF08238 contributes to the distance between the *Phytophthora* and obligate biotrophic species and is relatively more abundant in the biotrophs (PC1). PF08238 is a Sel1 repeat domain that is found in bacterial as well as eukaryotic species. Proteins with Sel1 repeats are suggested to be involved in protein or carbohydrate recognition and ER-associated protein degradation in eukaryotes [178]. No function of proteins with a PF08238 domain is known for oomycete or fungal pathogens.

The distance between *Pythium* and the obligate biotrophic species along PC2 is largely caused by differences in four domains that are commonly reported in oomycete secretomes [173]. The first, PF14295, a PAN/Apple domain, is known to be associated with carbohydrate-binding module (CBM)-containing proteins that recognize and bind saccharide ligands in *Ph. parasitica*. Loss of these genes, as in the biotrophs, may facilitate the evasion of host recognition as some CBM proteins are known to induce plant defense [179]. Second, PF00082, is a subtilase domain, which is found in a family of serine proteases. Secreted serine proteases are ubiquitous in secretomes of plant pathogens [180]. Secreted proteases from fungal species have been shown to enhance infection success by degrading plant derived antimicrobial proteins [181]. A third is PF00090, a Thrombospondin type 1 domain that is present in large numbers in the secretome of *Phytophthora* and *Pythium* species but is absent from the secretomes of *Hyaloperonospora/Peronospora* species and *Plasmopara halstedii*. The function of proteins with this domain in oomycetes or plants

is unknown. Finally, PF00254 contributes to the separation along PC1, which seems mainly caused by 13 occurrences of the domain in the secretome of *P. tabacina* versus 2 or less in the secretomes of the other oomycete species.

Over and under-representation of Pfam domains in obligate biotrophic species

Statistical analysis of enrichment of Pfam domains, to identify under- and over-represented domains in each group (*Hyaloperonospora/Peronospora*, *Plasmopara*, *Albugo*) compared to *Phytophthora*, confirmed the pattern that was shown in the biplot. In total, 60 Pfam domains were found to be differentially abundant in obligate biotrophic species clusters compared to *Phytophthora* (**Table 5**). All of the seven Pfam domains that contributed most to the separation between phylogenetic groups in the PCA (**Fig. 7** and **Table 4**) were also found to be differentially abundant in at least one obligate biotrophic cluster compared to *Phytophthora* in the enrichment analysis.

Previous studies identified Pfam domains that are associated with virulence in other phytopathogenic oomycete species like *Pythium*, *Plasmopara*, *Peronospora* and *Phytophthora* [182]. The occurrence of these known virulence-associated domains in the *Pfs* proteome is summarized in Fig. S5. We found that obligate biotrophic species have a lower total, as well as relative, number of secreted proteins with virulence-associated domains compared to the other oomycete species.

Table 5. Over and under-representation of Pfam domains in the secretomes of *Hyaloperonospora/Peronospora* (HP), *Plasmopara* (PI) and *Albugo* (AI) compared to *Phytophthora* species.

Pfam	Name	Interpro#	HP	PI	AI
PF16810	RxLR protein, Avirulence activity	IPR031825	8,30e ⁻²⁴	2,70e ⁻¹⁶	3,90e ⁻⁰⁷
PF14295	PAN domain	IPR003609	1,80e ⁻⁰⁷	1,80e ⁻⁰⁴	16,913
PF00090	Thrombospondin type 1	IPR000884	4,50e ⁻⁰⁵	77,4987	1,98225
PF05630	Necrosis inducing protein (NPP1)	IPR008701	1,60e ⁻⁰¹	1,36788	2,13e ⁻⁰³
PF08238	Sel1 repeat	IPR006597	1,70e ⁻⁰⁷	4,61559	1,07208
PF00254	FKBP-type cis-trans isomerase	IPR001179	1,80e ⁻⁰⁴		
PF00050	Kazal-type serine protease inhibitor	IPR002350	2,05e ⁻⁰³		
PF07974	EGF-like domain	IPR013111	1,23e ⁻⁰²		
PF13456	Reverse transcriptase-like	IPR002156	2,60e ⁻¹⁰		
PF00300	Histidine phosphatase superfamily	IPR013078	7,74e ⁻⁰³		
PF00665	Integrase core domain	IPR001584	1,07e ⁻⁰²		
PF00571	CBS domain	IPR000644	1,66e ⁻⁰²		
PF00089	Trypsin	IPR001254		1,10e ⁻¹²	
PF01833	IPT/TIG domain	IPR002909		8,00e ⁻¹²	
PF00082	Subtilase family	IPR000209		3,10e ⁻¹⁰	
PF01341	Glycosyl hydrolases family 6	IPR016288		3,30e ⁻⁰⁵	
PF00182	Chitinase class I	IPR000726		2,60e ⁻⁰⁴	
PF01670	Glycosyl hydrolase family 12	IPR002594		1,09e ⁻⁰³	
PF03184	DDE superfamily endonuclease	IPR004875			2,40e ⁻⁰⁶
PF09818	Predicted ATPase of the ABC class	IPR019195			4,60e ⁻⁰⁶
PF00169	PH domain	IPR001849			8,10e ⁻⁰⁶
PF01764	Lipase (class 3)	IPR002921			1,30e ⁻⁰⁴

Table 5. continued

Pfam	Name	Interpro [#]	HP	PI	AI
PF00026	Eukaryotic aspartyl protease	IPR033121			2,30e ⁻⁰⁴
PF13405	EF-hand domain	IPR002048			3,70e ⁻⁰⁴
PF15924	ALG11 mannosyltransferase	IPR031814			3,70e ⁻⁰⁴
PF01546	Peptidase family M20/ M25/M40	IPR002933			3,70e ⁻⁰⁴
PF07687	Peptidase dimerisation domain	IPR011650			3,70e ⁻⁰⁴
PF03870	RNA polymerase Rpb8	IPR005570			3,70e ⁻⁰⁴
PF13041	PPR repeat family	IPR002885			3,70e ⁻⁰⁴
PF00443	Ubiquitin carboxyl- terminal hydrolase	IPR001394			1,38e ⁻⁰³
PF10152	Subunit CCDC53 of WASH complex	IPR019309			1,63e ⁻⁰³
PF00041	Fibronectin type III domain	IPR003961			2,29e ⁻⁰³
PF07727	Reverse transcriptase	IPR013103			6,09e ⁻⁰³
PF04130	Spc97 / Spc98 family	IPR007259			2,15e ⁻⁰²
PF01753	MYND finger	IPR002893			2,15e ⁻⁰²
PF03577	Peptidase family C69	IPR005322			2,15e ⁻⁰²
PF03388	Legume-like lectin family	IPR005052			3,02e ⁻⁰²
PF03133	Tubulin-tyrosine ligase family	IPR004344			3,02e ⁻⁰²
PF13181	Tetratricopeptide repeat	IPR019734			3,02e ⁻⁰²
PF01156	Nucleoside hydrolase	IPR001910			3,02e ⁻⁰²
PF06367	Diaphanous FH3 Domain	IPR010472			3,02e ⁻⁰²
PF04910	Transcriptional repressor TCF25	IPR006994			3,02e ⁻⁰²

Table 5. continued

Pfam	Name	Interpro [#]	HP	PI	AI
PF00044	Glyceraldehyde 3-ph. dehydrogenase	IPR020828			3,02e ⁻⁰²
	Glyceraldehyde 3-ph. dehydrogenase				3,02e ⁻⁰²
PF02800	dehydrogenase	IPR020829			
PF01428	AN1-like Zinc finger	IPR000058			3,02e ⁻⁰²
	Electron transfer FAD-binding domain				3,02e ⁻⁰²
PF00766	Electron transfer flavoprotein domain	IPR014731			3,02e ⁻⁰²
PF01012	UPF0160	IPR014730			3,02e ⁻⁰²
PF03690	(uncharacterized) Helicase C-terminal domain	IPR003226			3,02e ⁻⁰²
PF13307	Microtubule-binding	IPR006555			3,02e ⁻⁰²
PF08683	calmodulin-reg	IPR014797			
PF01846	FF domain	IPR002713			3,02e ⁻⁰²
PF13418	Galactose oxidase				3,02e ⁻⁰²
PF03776	MinE	IPR005527			3,02e ⁻⁰²
	Iguana/Dzip1-like				3,02e ⁻⁰²
PF13815	DAZ-interacting	IPR032714			
	Type III restriction enzyme				3,02e ⁻⁰²
PF04851	enzyme	IPR006935			
PF13831	PHD-finger				3,02e ⁻⁰²
	Arp2/3 complex, p34-				3,02e ⁻⁰²
PF04045	Arc	IPR007188			
PF08144	CPL (NUC119) domain	IPR012959			3,02e ⁻⁰²
	POLO box duplicated				3,02e ⁻⁰²
PF00659	region	IPR000959			
	SMP-30/ Gluconolactonase/				4,76E ⁻⁰²
PF08450	LRE-like	IPR013658			

Over (green) and under (blue)-representation was tested relative to the expected distribution of each Pfam domain. The abundance of each domain was compared between the species clusters using a Chi-square test with Bonferroni correction. Bonferroni corrected p-values are shown in the table. [#]The InterPro domain code corresponding to each Pfam domain is provided.

Host-translocated effectors

The RxLR effector models in the Pfam database (PF16810 and PF16829) mentioned above cover only a small fraction of the predicted RxLR effectors in secretomes of phytopathogenic oomycetes. We predicted the total number of host-translocated effectors for each secretome using a Perl regex script and HMM searches (see methods), including RxLR effectors without WY domains and CRN effectors (**Fig. 8**). RxLR effector proteins were more abundant in *Phytophthora* compared to the obligate biotrophic species. On average 399 RxLR effector proteins were found in *Phytophthora* whereas *Plasmopara* and *Hyaloperonospora/Peronospora* had 79 and 90. The same pattern is evident for CRN effectors. The average number of CRN proteins in *Hyaloperonospora/Peronospora* is 11, while *Plasmopara* has 12 and *Phytophthora* 56. We conclude that downy mildew species (*Hyaloperonospora/Peronospora* and *Plasmopara*) have fewer host-translocated effectors compared to *Phytophthora* species.

Figure 8. Predicted (a) RxLR and (b) CRN effectors in the secretome of *Hyaloperonospora/Peronospora*, *Plasmopara* and *Phytophthora* species.

The predicted effectors are classified into four (RxLR) or five (CRN) categories, based on the additional domains they possess. Please note that the number of *Pfs* effectors is slightly different from the numbers reported before (**Fig. S3**). For this comparison we used HMM models that were previously published rather than the models trained for *Pfs* (**Fig. S3**).

Discussion

Taxonomic filtering

2 The ability to sequence full genomes at high pace and relatively low cost has aided research in phytopathology dramatically. Over the past few years, the genomes of many phytopathogenic oomycetes have been sequenced and their genomes revealed an arsenal of protein coding genes with a putative virulence role. However, technical difficulties restricted the sequencing and assembly of genomes of obligate biotrophic oomycetes that cannot be cultured axenically. Obligate biotrophic species can only grow on living host tissue so when collecting spores for DNA isolation DNA of other microbes and the host plant will inevitably contaminate the sample, which complicates the genome assembly. In this paper we use a metagenome filtering method resulting in the assembly of a relatively clean genome sequence of the obligate biotrophic downy mildew of spinach, *Peronospora effusa*.

To get a clean assembly, sequence that are derived from different species were filtered out and removed. Several methods were considered to identify and filter contigs or reads that were likely contaminants in our data. Initially we considered to filter contigs or reads based on their GC content, since this differs between genomes of oomycetes [183] and many other microbes [184]. In addition, the GC content is not constant over the genome, so filtering based on this could potentially remove valuable parts of the genome.

Alternatively, reads of non-oomycete origin could be identified by mapping them to databases with sequences of known taxonomy. For example, a database containing only oomycete or bacterial genomes. This is not ideal as the databases are incomplete and are likely to contain annotation errors. In addition, it could lead to the removal of novel parts of the downy mildew genome that are not present in other oomycetes, and which would hamper the study of valuable species-specific parts of your genome.

The filtering we applied with the CAT tool does not classify a contig based on a single hit. Instead it determines the taxonomic origin of each ORF on an assembled contig or corrected PacBio read, providing a robust classification [122]. In our *Pfs* study, after filtering with the CAT-pipeline of the error-corrected PacBio reads, 50% remained, and were used in the assembly. Of the sequenced (unfiltered) Illumina reads, 56% could be aligned to the final assembly. This indicates that roughly half of our sequencing reads originated from other sources besides *Pfs*. Notably, while the classifications in the original CAT paper were only benchmarked on prokaryotic sequences [122], our study shows that the tool also performs well for classifying eukaryotic contigs. Thus, CAT may also be promising for classification of eukaryotes including oomycetes in metagenomic datasets, provided that long contigs, or corrected PacBio or Nanopore sequencing reads are available.

It should be noted that sequences of unknown taxonomy were maintained for the assembly, making it possible that these are still contaminants. When we compare the taxonomic distributions generated by Kaiju of the pre-assembly and final assembly, we see a dramatic reduction of sequences of bacterial origin (**Fig. 3**). The oomycete content according to Kaiju and the overall GC content of the final assembly is similar to that of genome assemblies of axenically-grown oomycetes. We can therefore conclude that the CAT filtering method, allowed the successful removal of sequences of non-oomycete origin.

Hybrid assembly

Most oomycete genomes sequenced to date were found to contain long repeat regions [185] that cannot be resolved using only a short-read technology such as Illumina. Long reads can potentially sequence over long repeats, and contribute to the contiguity of a genome assembly [186]. Therefore, our Illumina data was complemented with long read PacBio sequences in an attempt to close gaps between contigs. Although the inclusion of PacBio reads in our assembly improved the contiguity, the final result still consists of a large number of contigs, indicating that

our PacBio reads were unable to span many repeat regions. Besides biological reasons for the large number of contigs, there could also be a technical reason. Prior to PacBio sequencing whole genome amplification (WGA) with random primers was performed as the initial sequencing attempt with non-amplified DNA barely yielded sequencing reads. WGA might create a bias, where some parts of the oomycete genome may be under-represented in the PacBio data.

2

The genome of *Pfs1*

The assembled *Pfs1* genome size is 32.4 Mb divided over of 8,635 contigs. The genome is highly gene dense and contains in total 13,227 genes. Overall, the BUSCO analysis showed that this assembly contains most of the gene-space. Many of the 8,635 contigs were smaller than 1 kb. However, the CAT filtering method performs best on relatively large contigs containing multiple ORFs. Therefore, small contigs could still contain sequences derived from other organisms. The removal of these small contigs results in only a small genome size reduction (1.9 Mb) and loss of gene models (597), but significantly reduces the number of contigs (by 5,027). When we also account for genes that have a significant overlap (>20%) with repeats in the genome (3983 gene models), or that were annotated as transposable elements (36 gene models that did not had an overlap with a repeat region) we come to 8,976 high-confidence gene models.

The genomes of *Pfs* race 13 and 14 have recently been published [187, 188], with a similar genome size (32.1 Mb, and 30.8 Mb respectively) and gene content (~8000 gene models) compared to our *Pfs1* genome assembly. Contrary to our assembly method, the input data for those genome assemblies were filtered by alignment to an oomycete and bacterial database to discard reads that do not belong to the oomycete genus. This filtering method could potentially lead to the incorporation of bacterial sequences that are not in the public databases. Besides, the positive filtering for oomycete scaffolds against NCBI nt database could have resulted in the loss of *Pfs* specific genome sequences. In addition,

by filtering reads based on a database containing bacterial and fungal sequences, part of the *Pfs* genome yielded by horizontal gene transfer (HGT) may be discarded [189]. The CAT-tool overcomes this issue by determining the overall taxonomy of larger contigs based on multiple genes.

***Peronospora* species have reduced genomes**

Recent sequencing of *Peronospora* species shows that they have remarkably small and compact genomes (32.3 – 63.1 Mb) compared to *Phytophthora* (82- 240 Mb) species [5, 58, 60, 187]. The *k*-mer analysis predicts the *Pfs1* genome to be 36.2 Mb containing 8.8 Mb of repeats (24%). The predicted genome size of *Pfs R13* and *R14* based on *k*-mer analysis is 44.1 - 41.2 mb (repeats; 24 – 22%) [187]. The increased genome size of *Phytophthora* is attributed to an ancestral whole genome duplication in the lineage leading to *Phytophthora* and to an increase in the proportion of repetitive non-coding DNA [58, 190]. The duplication event has been proposed to have taken place after the speciation of *H. arabidopsidis* [191]. However new multigene phylogenies show that the *Peronospora* lineage has speciated after the divergence of *Phytophthora* clade 7 from clade 1 and 2. Notably, these three clades all contain species with duplicated genomes [19, 22, 27, 192]. This would suggest that an ancestral whole genome duplication before this speciation point would also apply to *Peronospora*, and would mean that duplication cannot account for the difference in genome size. The availability now of genomes of three *Peronospora* species for comparisons asks for a reevaluation of the timing of the duplication and subsequent speciation events.

Biologically, the question of how *Peronospora* species can be host-specific and obligate biotrophic while maintaining only a small and compact genome is interesting. It is argued that the trend in filamentous phytopathogens is towards large genomes with repetitive stretches to enhance genome plasticity [190]. Plasticity may enable host jumps and

adaptations that favor the species for survival over species with small, less flexible genomes [190]. The reduced genomes of *Peronospora* species show an opposing trend that cannot be attributed to their obligate biotrophic lifestyle alone, as it is not evident in *Plasmopara* species (75 Mb – 92 Mb) [22, 193]. Sequencing of multiple isolates of the same *Peronospora* species may shed light on genome plasticity at the species level.

2 Secretome reflects biotrophic lifestyle Evolving biotrophy

The biotrophic lifestyle has emerged on several independent occasions in the evolution of filamentous plant pathogens, in several branches of the tree of life. Convergent evolution is thought to be the main driving factor behind the development of biotrophy in such distantly related organisms [194]. However, it was shown that horizontal gene transfer can also occur between fungi and oomycetes, resulting in 21 fungal proteins in the secretome of *H. arabidopsidis*. Out of these 21 proteins, 13 were predicted to be secreted, indicating that horizontal gene transfer may affect a species' pathogenicity and interaction with the host [195, 196].

It was proposed that the critical step for adopting biotrophy in filamentous phytopathogens is the ability to create and maintain functional haustoria [63]. To do so, a species needs to be able to avoid host recognition or suppress the host defense response. A proposed mechanism for avoidance of host recognition is the loss of proteins involved in cell wall degradation, as evidenced by the reduction of cell wall degrading enzymes in mutualistic species compared to biotrophs [197]. In this and other studies, we find a reduction of the number of cell wall degrading enzymes in obligate biotrophic species compared to hemibiotrophic *Phytophthora* species (Fig. S3) [59]. This is true for all three obligate biotrophic groups in this study (*Hyaloperonospora*/*Peronospora*, *Plasmopara* and *Albugo*) although the difference is less clear in *Plasmopara*. Possibly this reduction is the result of a similar selection pressure to reduce recognition by the host plant in the biotrophic species,

where the hemi-biotrophic nature of the interaction between host and *Phytophthora* allows for slightly less caution in recognition avoidance.

The other mechanism of establishing a strong interaction is suppression or avoidance of the host defense response. Biotrophic infections are often accompanied by co-infection of species that are unable to infect the plant in the absence of the biotroph, indicating efficient defense suppression [15, 63]. We found enhanced numbers of secreted serine proteases (PF00082) (suppression) and reduced numbers of proteins with PAN/Apple domains that are known to be recognized by the plant immune system.

While the expansion of host translocated RxLR effectors is evident in both hemi-biotrophic and biotrophic species, their numbers are smaller in secretomes of obligate biotrophs. CRN effectors are especially reduced in secretomes of biotrophic species. As opposed to RxLR effectors, CRNs are an ancient class of effectors that are known to induce cell death. Obligate biotrophic species presumably lost them as they are not beneficial for their survival.

In this study we first showed that the CAT tool performs well for taxonomic filtering of eukaryotic contigs. We provided a clean reference genome of a race 1 isolate of the spinach infecting downy mildew, *Pfs1*. In a comparative approach, we found that the secretomes of the obligate biotrophic oomycetes are more similar to each other than to more closely related hemi-biotrophic species when comparing the presence and absence of functional domains, including the host translocated effectors. We conclude that adaptation to biotrophy is reflected in the secretome of oomycete species.

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Supplemental Tables

S1 Table. Species used for comparative secretomics.
Table S1 is available online:

<https://doi.org/10.17026/dans-zey-8aec>

S3 Table. Repeat elements in the *Pfs1* genome. Repeat elements identified in the *Pfs1* genome, for each repeat type the total numbers and percentage are shown. In addition, also a detailed annotation for each repeat element is provided.

Table S3 is available online:

<https://doi.org/10.17026/dans-zey-8aec>

S2 Table. Comparison of conserved eukaryotic genes for different oomycetes and the *Pfs1* assembly using BUSCO.

Genus	Species	Complete	Single	Duplicated	Fragmented	Missing	#predicted genes
<i>Phytophthora</i>	<i>sojae</i>	99,5%	95,8%	3,7%	0,5%	0,0%	28.142
<i>Phytophthora</i>	<i>ramorum</i>	98,1%	94,4%	3,7%	0,5%	1,4%	15.955
<i>Hyaloperonospora</i>	<i>arabidopsidis</i>	96,8%	92,1%	4,7%	0,5%	2,7%	14.606
<i>Phytophthora</i>	<i>infestans</i>	95,9%	92,6%	3,3%	0,9%	3,2%	25.638
<i>Plasmopara</i>	<i>halstedii</i>	95,8%	95,8%	0,0%	0,5%	3,7%	15.469
<i>Pseudoperonospora</i>	<i>cubensis</i>	93,1%	91,2%	1,9%	1,4%	5,5%	27.591
<i>Peronospora</i>	<i>tabacina</i>	91,6%	62,8%	28,8%	1,9%	6,5%	18.000
<i>Peronospora</i>	<i>effusa</i>	88,9%	88,4%	0,5%	0,5%	10,6%	13.223
<i>Saprolegnia</i>	<i>parasitica</i>	87,9%	81,4%	6,5%	2,3%	9,8%	20.121
<i>Phytophthora</i>	<i>capsici</i>	31,6%	31,6%	0,0%	0,5%	67,9%	19.805

Genus	Species	Assembly Version
<i>Phytophthora</i>	<i>sojae</i>	V3.0
<i>Phytophthora</i>	<i>ramorum</i>	pr102_1
<i>Hyaloperonospora</i>	<i>arabidopsidis</i>	HyaAraEmoy2
<i>Phytophthora</i>	<i>infestans</i>	ASM14294v1
<i>Peronospora</i>	<i>halstedii</i>	V2
<i>Pseudoperonospora</i>	<i>cubensis</i>	MSU-1
<i>Peronospora</i>	<i>tabacina</i>	968-s26
<i>Peronospora</i>	<i>farinosa</i>	H7
<i>Saprolegnia</i>	<i>parasitica</i>	cbs_223.65_2
<i>Phytophthora</i>	<i>capsici</i>	11

S4 Table. Genome sizes and repeat content of different assembled oomycete genomes.

Genus	Species	Assembly size (Mb)	Number of genes	GC content	Repeat content	Reference
<i>Phytophthora</i>	<i>infestans</i>	240,00	17.797	50,96%	74%	https://www.nature.com/articles/nature08358#s1
<i>Phytophthora</i>	<i>sojae</i>	95,00	19.027	54,60%	39%	http://science.sciencemag.org/content/313/5791/1261.long
<i>Phytophthora</i>	<i>ramorum</i>	65,00	15.743	53,85%	28%	http://science.sciencemag.org/content/313/5791/1261.long
<i>Peronospora</i>	<i>tabacina</i>	63,10	11.310	41,17%	24%	https://apsjournals.apsnet.org/doi/10.1094/MPMI-05-15-0112-R#b4
<i>Hyaloperonospora</i>	<i>arabidopsidis</i>	100,00	14.321	47,21%	43%	http://science.sciencemag.org/content/330/6010/1549.long
<i>Phytophthora</i>	<i>capsici</i>	64,00	19.805	50,42%	19%	https://apsjournals.apsnet.org/doi/10.1094/MPMI-02-12-0028-R
<i>Albugo</i>	<i>laibachii</i>	32,70	13.032	44,34%	22%	https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.1001094
<i>Pythium</i>	<i>ultimum</i>	42,80	15.290	48,27%	7%	https://genomebiology.biomedcentral.com/articles/10.1186/gb-2010-11-7-r73
<i>Peronospora</i>	<i>effusa (Pfs1)</i>	30,48	8.927	48,25%	21%	-
<i>Peronospora</i>	<i>effusa (Pfs13)</i>	32,10	8.607	47,63%	33%	https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-018-5214-8
<i>Plasmopara</i>	<i>viticola</i>	101,30	17.014	45,00%	26%	https://www.nature.com/articles/srep46553
<i>Plasmopara</i>	<i>halstedii</i>	75,30	15.469	45,29%	39%	https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-015-1904-7
<i>Albugo</i>	<i>candida</i>	34,50	15.824	43,00%	17%	https://bmcgenomics.biomedcentral.com/articles/10.1186/1471-2164-12-503#Sec2

S5 Table. Putative annotations of the *Pfs* proteins as obtained with ANNIE. In addition, the presence of a N-terminal signal peptide for secretion, WY motif, TM motif and overlap with a repeat region are listed for each protein coding gene.

Table S5 is available online:

<https://doi.org/10.17026/dans-zey-8aec>

2

S6 Table. Overview of the host translocated effectors (RxLR and CRN) identified in the genome of *Pfs1*. Also, their respective functional domains and locations are listed per effector. Selected effectors that were used in the gene intergenic distance analysis are listed in the second tab.

Table S6 is available online:

<https://doi.org/10.17026/dans-zey-8aec>

S7 Table. Secreted CAZyme domains per species.

Table S7 is available online:

<https://doi.org/10.17026/dans-zey-8aec>

S8 Table. Secreted Pfam domains per species.

Table S8 is available online:

<https://doi.org/10.17026/dans-zey-8aec>

Supplemental Figures

2

S1 Fig. GC plot of various oomycete assemblies on contigs larger than 1kb.

Figure S2. PCA on absolute numbers of secreted CAZyme domains.

Figure S3. PCA on absolute numbers of secreted Pfam domains.

Figure S4. Secreted cell wall degrading proteins (CAZymes).
Numbers (a) of literature curated plant cell wall degrading enzymes per species. (b) The same data represented as fraction of the total number cell wall degrading protein domains per species.

Figure S5. Secreted pathogenicity associated Pfam domains.

Occurrence of Pfam domains known to be involved in pathogenicity within the secretome of each species. Figure (a) shows the absolute number of Pfam domains, while (b) shows the number relative to the total number of Pfam domains per species.

3

Identification of plant interactors of host- translocated effectors of *Peronospora effusa*

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Abstract

The obligate biotrophic oomycete *Peronospora effusa* (*Pfs*), which causes downy mildew on spinach, has an arsenal of host-translocated effectors that are presumed to manipulate plant cell processes during infection. Comparative analysis of its predicted proteome showed that 39 of them are unique to *Pfs* as they have no detectable orthologs in other species tested. Of the other candidate host-translocated effectors, 10 are downy mildews-specific, 23 occur in downy mildews and other Peronosporales species, and 40 are also found in more distantly-related oomycetes such as *Pythium* and *Albugo* species. Twenty-six candidate effectors were used as baits in a yeast two-hybrid screen of an Arabidopsis prey library. The screen revealed 40 interactions between 18 *Pfs* proteins and putative plant interactor proteins. An interaction network supplemented with reported protein-protein interactions from the plant-pathogen literature showed that 9 interactors of *Pfs* effectors were also targeted by effectors of other species. These highly connected plant proteins like CSN5A, TCP14, and FBN1A likely play important regulatory roles in the plant cell during infection. Other *Pfs* effectors interact with less connected proteins. Some of these, like HBI1, two NAC transcription factors and two prenylated Rab acceptor (PRA1) proteins, have been linked to disease susceptibility in other species before but for most of them their biological function is unknown. This study identified the first plant proteins that interact with effectors of the spinach downy mildew and provides a first glimpse into the possible targets of host-translocated effectors of *Pfs* and their potential role in the infection process.

Introduction

Phytopathogens secrete proteins and other molecules, named effectors, to establish and maintain successful infections. Effectors have evolved to manipulate plant processes to the benefit of the pathogen. An important activity of host-translocated effectors is to suppress plant immunity [198]. The plant immune system recognizes and responds to pathogen-associated molecular patterns (PAMPs) using receptors that trigger immune responses [80]. Pattern-triggered immunity (PTI) encompasses callose deposition, altered hormone signaling and gene transcription and the release of reactive oxygen species (ROS) and antimicrobial compounds, all to limit infection of plant tissues by pathogens [41, 42, 43].

Effector proteins of phytopathogens act in plant tissues either in the extracellular space (apoplastic effectors) or inside host cells (host-translocated effectors). Known activities of apoplastic effectors include inhibition of hydrolytic enzymes and proteases, induction of necrosis and protection of the pathogen cell wall [40], [199]. In oomycete phytopathogens two main types of host-translocated effector proteins are RxLR and Crinkler (CRN) effectors [55]. RXLR and CRN effectors are characterized by the presence of a signal peptide, a conserved RxLR or LFLAK (CRN) domain at the N-terminus and a variable C-terminal effector domain which is responsible for the function of the effector in the host cell [57, 66]. The RxLR and CRN motifs are often accompanied by EER and HVL motifs respectively [56, 200] and together they are likely required for the translocation into host cells [56, 65]. Recently, it was shown that in an oomycete the RxLR motif likely guides effectors to a non-conventional secretion and translocation route that bypasses the oomycete Golgi system [69].

The C-terminal effector domain of CRN and RxLR effectors is responsible for the virulence function and usually lacks known protein domains or considerable conservation with other proteins [40]. However, a conserved tertiary structure, an α -helical fold, termed the WY-fold is found in *Phytophthora* and downy mildew species [60, 61, 77]. The WY-

fold is hypothesized to provide molecular stability to the proteins, while maintaining flexibility to evolve and avoid recognition by the plant immune system [77]. For a number of host-translocated effectors it was shown that they interact with plant proteins to suppress immunity, cell death and RNA silencing [90, 103, 201, 202]. However, the virulence function of most host-translocated oomycete effectors is currently unknown.

Effector protein repertoires are highly variable between species. Effector genes are continuously lost (deleted), gained, and modified resulting in fast changing and dynamic gene repertoires and little conservation between effector repertoires of different species [75, 199]. RxLR effector proteins, for instance, have evolved and expanded within the Peronosporales resulting in hundreds of RxLR protein-encoding genes in the genomes of species like *Phytophthora infestans* (563), *Plasmopara viticola* (>190) and *Peronospora tabacina* (120) [22, 58, 60, 203]. Rapid modification of effector proteins helps to avoid recognition by the plant immune system because pathogen success depends primarily on its ability to avoid recognition.

Although effector repertoires of different species have limited similarity, a number of host-translocated RxLR effectors are reported to be conserved among pathogenic oomycete species. Possibly, these effectors are especially important for pathogen virulence and are therefore under negative selection to maintain their function. Proof of concept of this idea was shown for the *Hyaloperonospora arabidopsidis* (*Hpa*) effector HaRxL96 and its homolog PsAvh163 from *Phytophthora sojae*. Both effectors suppress immunity in soybean and enhance susceptibility to virulent and avirulent *Hpa* when stably expressed in Arabidopsis. These findings demonstrate that evolutionarily conserved effectors from different oomycete species can have similar functions, even in non-host plant species [204]. The similar function of conserved effectors likely results from interaction with conserved plant proteins. Studying the plant proteins that are targeted by effectors can provide insights into the plant processes that effectors interfere with.

An extensive interaction network of *Arabidopsis* proteins and pathogen effectors of different species was published in 2011 [205, 206]. The systematic yeast two-hybrid (Y2H) screens underlying this network resulted in the finding that 18 *Arabidopsis* proteins are targeted by effectors from multiple evolutionary distinct phytopathogens, including the oomycete *Hpa* [206]. The network provided a start for unravelling plant protein function and possible manipulation of plant processes by pathogen host-translocated effectors.

Here we set out to characterize the host-translocated RxLR and CRN repertoire of the spinach downy mildew *Peronospora effusa* (*Pfs*) and identify interacting plant proteins. *Pfs* is an obligate biotrophic oomycete that was first identified in 1824. Resistance-breaking isolates of the species have rapidly evolved over the last 3 decades following the introduction of new resistant spinach cultivars [30, 31, 32, 188]. The genome of the reference race 1 of *Pfs* encodes 99 candidate RxLR and 13 candidate CRN effectors. In a two-step approach we first classified the host-translocated effector repertoire based on their conservation with proteins of other oomycete species. This information was used to make a selection of interesting host-translocated effector proteins. In the second step we performed Y2H interaction screens on 26 *Pfs* effectors to identify interacting plant proteins that could represent effector-targets.

Results and Discussion

The *Pfs1* RxLR and CRN effector repertoire

The predicted proteome of *Pfs1* was previously mined for candidate RxLR and CRN host-translocated effectors using a regex script and HMM searches for WY folds and CRN motifs (**Chapter 2**). A total of 99 RxLR and 14 CRN-candidate effectors were identified by these criteria (**Fig. 1**). Note that one candidate effector, Pfs1|3487, has both a canonical RxLR and LFLAK motif and hence qualifies as RxLR as well as CRN candidate effector. The total number of proteins that are considered candidate effectors therefore totals 112, not 113.

We found 64 secreted proteins with a canonical RxLR motif. Of these, 21 have WY domains and EER-like motifs, 7 have WY domains, and 13 have an EER motif. A total of 35 secreted proteins with degenerate RxLR-like motifs (28 xLR and 7 RxL) were found of which 28 lacked the first arginine (xLR) and 7 lacked the second (RxL). Please note that these 35 proteins were selected because they contained additional domains commonly found in RXLR effectors; of them 17 have an EER-like motif, 17 have one or more WY domains and one protein has both (**Fig. 1a**). The genome of *Pfs1* has 5 canonical CRN effectors of which only two were recognized by the CRN HMM search. In addition, 6 secreted proteins with a degenerate LFLAK domain (3 L.LAK or 3 LF.AK) were found. Finally, 3 proteins were identified by the CRN HMM search as potential effectors and were hence included, despite lacking a recognizable translocation domain. Six of the candidate CRN effector proteins had an HVL motif (**Fig. 1b**). The inclusion of proteins that have degenerate translocation domains is justified because the exact requirements of translocation signals are still under debate.

A

B

3

Figure 1. RxLR and CRN (-like) motifs observed in candidate host-translocated effectors identified in the genome of *Pfs1*. The candidate host-translocated effectors are grouped based on the variation in the putative translocation domain (RxLR or LFLAK). The number of proteins that have additional effector features are shown. For (degenerate) RxLR (A) the presence of a WY domain (green), EER-like motif (grey) or both (blue-green) is shown. For candidate CRN effectors the presence of an HVL (grey) motif and the recognition of the protein by the CRN HMM (green) or both (blue-green) are depicted.

Secretome comparison identifies conserved and species-specific host-translocated effectors.

The analysis of orthogroups (described in Chapter 2) identified orthology between *Pfs* proteins and proteins of other species. Here we use this data to study the conservation of host-translocated effectors of *Pfs*. Of the 112 effectors, 19 neither had orthologs (in other species) nor

paralogs (within *Pfs*). These unique *Pfs* host-translocated effectors were therefore not placed in any orthogroup. The other 93 candidate effectors were assigned to 50 orthogroups of which 46 groups had RxLR effector candidates, 3 groups had CRN effector candidates and 1 group had both RxLR and CRN candidates. The latter has one predicted *Pfs* protein that qualifies as a RxLR as well as a CRN effector. One should note that also other proteins reside in these groups that do not all have the features of host-translocated-effectors, but nevertheless are orthologous.

We differentiated the orthogroups that have candidate *Pfs* host-translocated effectors into four classes; the *Pfs*-specific (not conserved), downy mildew-specific, Peronosporales-specific and broadly conserved orthogroups. The *Pfs*-specific effectors also include those that are not in any orthogroup at all. Of 112 *Pfs* host-translocated effectors 39 are *Pfs*-specific, 10 downy mildew-specific, 23 Peronosporales-specific, and 40 are more broadly conserved as they are in orthogroups that include proteins from *Albugo* and/or *Pythium* species (**Fig. 2**). In the next paragraphs these four different effector conservation classes are further discussed while highlighting some interesting orthogroups.

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Figure 2. Classification of the 112 candidate *Pfs* host-translocated effectors into four conservation classes.

Based on a comparative analysis including 18 oomycete species, the candidate host-translocated effectors of *Pfs* were classified as species-, downy mildew-, Peronosporales-specific or broadly conserved.

All of the 39 *Pfs*-specific candidate host-translocated effectors are RxLR proteins (**Table S1**). Nineteen are not related to any other protein within the proteome of *Pfs1*. The other 20 effectors were found in orthogroups that have between 3 and 17 paralogous *Pfs* proteins, both predicted effectors and other proteins. One interesting *Pfs*-specific effector group has eight proteins, of which seven are candidate RxLR effectors. Manual curation of the eighth protein identified an alternative gene model that has a signal peptide and is therefore also a candidate effector [207]. Polymorphisms, observed between *Pfs* races, in two of the candidate effectors in this group are associated with the breaking of resistance locus *RFP2* [207].

The 10 downy mildew-specific host-translocated effectors are in 6 small orthogroups that have up to 12 proteins. Each group is only represented by two or three of the six downy mildew species represented in the study (**Table 1**). No *Plasmopara* proteins were found in these groups, which is in line with recent findings indicating that these species are not monophyletic with the other *Hyaloperonospora/Peronospora* downy mildew species [27]. In orthogroup OG0009188, the number of *Pfs* proteins is largely expanded. The orthogroup of twelve has eleven *Pfs* and one *P. tabacina* protein (**Fig. 3**). Three of the *Pfs* proteins are predicted to be secreted, of which only two are candidate effectors because the third one (Pfs1|10501) lacks a detectable RXLR sequence. Although the majority of proteins are not predicted to be secreted, 11 of them have WY-folds typical for RxLR effectors. Six of them have an additional degenerative RxLR motif. Manual curation led to an N-terminal extension for only one protein, Pfs1|4091 (**Suppl information 1**). The extended protein has a signal peptide and combined with the xLR and 3 WY domains, it is a candidate host-translocated effector.

Table 1. The total number of proteins in downy mildew-specific orthogroups containing candidate RxLR effectors.

Orthogroup	<i>Pfs</i>	<i>P. belbahrii</i>	<i>P. tabacina</i>	<i>Hpa</i>	<i>Pl. halstedii</i>	<i>Pl. viticola</i>	<i>Phytophthora</i>	Other
OG0009188	11 (2)	0	1	0	0	0	0	0
OG0010260	2 (2)	6 (4)	0	0	0	0	0	0
OG0010880	5 (2)	0	0	1 (1)	0	0	0	0
OG0010559	2 (1)	5	0	0	0	0	0	0
OG0010838	2 (2)	3 (3)	0	1 (1)	0	0	0	0
OG0013057	1 (1)	1 (1)	0	0	0	0	0	0

The number of candidate host-translocated RxLR effectors in each species is indicated between brackets.

Figure 3. Phylogenetic tree of downy mildew proteins in orthogroup OG0009188.

Details on the next page

Figure 3. The tree includes a schematic representation of the protein showing the signal peptide (N-terminal square), RxLR like sequences and the WY domains (rounded squares) for each protein. Three proteins have a signal peptide (N-terminal filled square). Manual curation of *Pfs1*|4091 identified an additional start codon that yields a protein with a signal peptide (open square). Proteins are drawn to scale, except for the WY domain signs that are not drawn to scale but start at the accurate position. The number of amino acids is indicated at the C-terminus of the protein. Neighbor-Joining tree, Jukes-Cantor distance model. Number of substitutions per site are displayed at the branches).

The 23 candidate Peronosporales-specific RxLR effectors of *Pfs* grouped into 9 orthogroups. These groups contain proteins of both downy mildew and *Phytophthora* species, but not from more distantly-related species. Orthogroup OG0000009 is a major group that totals over 350 proteins, of which the numbers of downy mildew proteins (1 - 35) are lower compared to that of *Phytophthora* species (69 – 100). The group contains the PSR2 proteins of *P. sojae* and *P. infestans* that have WY domains, enhance virulence, and are known to suppress RNA silencing activity in plants [95, 110]. PSR-effectors are a rare example of conserved host-translocated effector proteins in oomycetes. *Pfs* has 35 proteins in this group of which 14 are candidate effectors. All but two *Pfs* proteins have at least one WY-fold, a feature they share with the proteins of other species in this orthogroup. The protein alignment shows that only some residues in the WY-folds and their flanking amino acids are conserved between the proteins (**Supporting information 1**). Only 3 (0.2%) of the amino acids in the alignment are identical in all proteins. The 21 *Pfs1* proteins that are not considered host-translocated effectors either lack a signal peptide (7) or RxLR motif (14).

Peronosporales-specific orthogroup OG0007491 is a group of 16 proteins that harbors HaRxL44, a known *Hpa* RxLR effector that was described to interact with Mediator Subunit 19a (MED19a) of Arabidopsis [91]. The interaction was found to result in the degradation of MED19a, leading to enhanced ethylene/jasmonic acid signaling and ultimately suppression of salicylic acid triggered immunity. The orthogroup contains two *Pfs1* proteins that have duplicated within the *Pfs* genome. Recent duplication also occurred within the genomes of the other downy mildew species *Peronospora belbahrii* and *Hpa* (**Fig. 4a**).

The reported RxLR-like motif 'RFL' of HaRxL44 is conserved in all proteins in this group except Pfs1|12209 ('RSL') [91]. Remarkably, the *Hpa* effectors have a downstream canonical RxLR motif that is accompanied by an EER(-like) motif in HaRxL44, HaRxL45 and HaRxLL108. The canonical RxLR motif and EER-like motif are not conserved between the *Hpa* and *Pfs* proteins (nor other proteins in the group, not shown) (**Fig. 4b**). To our knowledge, it has not been tested whether either or both of the motifs are required for translocation of HaRxL44.

Twelve of the orthologs in this group, including the *Pfs* proteins, have a predicted nuclear localization signal (NLS) (**Fig. 4b**). NLS are stretches of basic amino acids that are recognized and bound by importin- α , a host protein that ultimately mediates the import into the nucleus [208]. Therefore, it is likely that the effectors target the host nucleus, which was indeed confirmed for HaRxL44, 45 and HaRxLL108 [209, 210].

Figure 4. Phylogenetic tree of the Peronosporales-specific orthogroup OG0007491 of *Hpa* protein HaRxL44.

The majority of the proteins have a signal peptide (square) and a nuclear localization signal (checkmark). All proteins have a conserved RxLR-like (RxL) motif (open triangle). The *Hpa* proteins have an additional canonical RxLR motif (filled triangle). Most proteins have an EER or EER-like motif (closed vs. open circle). The alignment (b) of the *Hpa* and *Pfs* proteins shows that the RxL but not the RxLR motif is conserved between *Hpa* and *Pfs* proteins. The domains are highlighted in yellow (signal peptide), green (RxL motif), light green (RxLR motif), red (EER(-like)) motif and blue (basic amino acid residue-rich region contain a predicted NLS area). The *Pfs* proteins and HaRxL44 are marked bold. (Neighbor-Joining tree, Jukes-Cantor distance model. Number of substitutions per site are displayed at the branches).

The final class, the broadly conserved effectors, has a total of 40 candidate effectors, that is all 13 CRN and 27 of the RxLR proteins. These broadly conserved effectors are in large groups that also contain proteins from more distantly-related oomycetes, *Pythium* and *Albugo*, besides before mentioned downy mildew and Peronosporales species. The genomes of *Pythium* and *Albugo* species have small numbers of RxLR-like proteins and their contribution to virulence is not yet understood. A role in virulence is likely, because *Albugo* RxLR effectors were shown to suppress immunity and recently *Pythium* RxLR-like proteins were found to induce defense response in *N. benthamiana* [63, 64]. CRN effectors have evolved earlier in evolution and are known to exist within the order of Peronosporales as well as Albuginales and the Pythiales/*Pythiaceae* species [56]. Seven of the thirteen candidate *Pfs* CRN effectors reside in one orthogroup (OG0000033). The group has 234 proteins from *Pfs*, *Hpa*, *Plasmopara*, *Phytophthora*, *Pythium* and *Albugo* species. The other *Peronospora* species, *Pta* and *Pbe* are not represented in this group. The *Pfs* proteins are divided into four groups in the tree (**Fig. 5**). All 7 *Pfs* candidate CRN proteins are in one group (IV), together with 8 other *Pfs* proteins. No proteins of other species are present in this group, indicating that the expansion of these paralogs occurred within *Pfs*. *Hpa*, the closest relative of *Pfs* in this orthogroup, has one clade of 7 proteins that has specifically expanded within *Hpa*. The *Hpa* proteins are CRN homologs that have a RxLR motif (RELRLFLAK or a degenerative variant of that) [75]. The expansion of CRN proteins in downy mildew species is surprising because a number of CRN proteins are known to cause cell death, a condition that would be disadvantages for these obligate biotrophs [56].

Tree scale: 0.1

Figure 5. Rooted tree of the broadly conserved proteins of orthogroup OG0000033 that contains 7 candidate *Pfs* host-translocated CRN effectors. The total of 24 *Pfs* proteins (orange) are in four groups (I-IV). All 7 candidate host-translocated CRN effectors (*) are in clade IV that has expanded within *Pfs*. Eight clades without *Pfs* proteins (114 proteins) are collapsed. *Phytophthora* = blue, *Plasmodium* = red, *Hpa* = purple *Pythium* = grey, *Albugo* = green. The tree is created using DendroBLAST within Orthofinder and is rooted at the *Albugo* clade [211]. The tree was created using ITOL [212].

Selection of candidate host-translocated effectors for interaction studies

The challenge in effector biology is to understand how individual host-translocated effectors contribute to infection of the host. We used the orthology data to focus our research on a selection of host-translocated effectors that are interesting because of their conservation with other species and the presence of canonical translocation motifs. In total, 40 candidate host-translocated effector proteins of *Pfs1* were selected for functional analysis (**Table S2**). Of them, 6 are *Pfs*-specific, 4 only occur in downy mildew species, 14 are conserved within the Peronosporales and 17 are broadly conserved. The selected proteins include 28 RxLR, 2 CRN effectors and *Pfs1*|3487 that has both a RxLR and CRN translocation domain. In addition, we selected 2 CRN-like proteins without a signal peptide, 4 candidate host-translocated effectors that were predicted from previous *Pfs1* genome assemblies and one protein with degenerate RxLR and CRN motifs but no other effector features. Finally, 4 proteins were selected because they are in orthogroups with selected candidate *Pfs* host-translocated effectors. These proteins have multiple WY domains but lacked a RxLR(-like motif). From now on, all above mentioned proteins will be referred to as 'effector baits'.

3

Yeast two-hybrid screening identifies *Pfs* effector-interacting plant proteins

The effector baits were amplified from mRNA derived from *Pfs1*-infected spinach plants. Effector baits were cloned, without the signal peptide coding sequence, into the gateway pDEST22 vector for expression in yeast. In total, 33 effector bait sequences were successfully cloned and transformed into yeast. None of the candidate effector-fusions auto-activated the *HIS* reporter gene in the absence of a prey protein. The effector-constructs were used as baits in a yeast two-hybrid screen with a prey library of oligo-dT primed cDNA of Arabidopsis.

Out of 33 cloned effector baits, 26 were successfully mated in the yeast two-hybrid screens. For the six baits that were not mated

successfully the required number of colonies (>1 million) to be screened was not sufficient. The effector baits usually had highly-specific interactions with plant proteins. For eight effector baits only one plant interactor was found, while 10 interacted with multiple unique plant proteins. Four plant proteins were found to interact with multiple effector baits. For 9 effector baits, no interactors were obtained from the screen.

Selected bait and prey vectors were retransformed into yeast to verify their interaction. A total of 40 unique plant proteins (51 unique colonies) were found to interact with 18 effector candidates (**Table 2** and **Table 3**). The identification of plant proteins that interact with *Pfs* effectors provide a first insight into the possible virulence targets of *Pfs*. Importantly, these results need to be verified in planta, to assess the biological relevance. Within the Y2H system, both bait and prey proteins are directed to the nucleus, so it is possible to identify interactions between proteins that are spatially separated under natural conditions [213]. The further verification of the subcellular localization of *Pfs* effectors and their interacting plant proteins is further discussed in **chapter 4**.

Table 2. Number of interacting plant proteins per candidate *Pfs* effector detected in Y2H screening (ranked by number of plant interactors).

Effector	Motifs and folds	Conservation	Orthogroup	# of plant interactors
Pfs1 10492	RxLR EER WY	Peronosporales	OG0000009	8
Pfs1 6786	RxLR EER WY	<i>Pfs</i> -specific	OG0010381	7
Pfs1 1800	RxLR EER	Peronosporales	OG0007294	5
Pfs1 3869	RxLR	Downy mildew	OG0010260	5
Pfs1 9071	LFLAK	Broad	OG0000033	5
Pfs1 3172	RxLR EER TM	Downy mildew	OG0010838	3
Pfs1 3210	xLR EER	Peronosporales	OG0006720	3
Pfs1 7215	RxLR	Broad	OG0000021	3
Pfs1 3487	RxLR LFLAK HVL	Broad	OG0000437	2
Pfs1 4005*	LFLAK	Broad	OG0000657	2
Pfs1 10453	RxLR EER	Broad	OG0000023	1
Pfs1 10763*	RxLR EER WY	<i>Pfs</i> -specific	OG0007292	1
Pfs1 2936	RxLR	Broad	OG0000062	1
Pfs1 3069	xLR WY	Peronosporales	OG0000009	1
Pfs1 805	RxLR WY	Peronosporales	OG0000009	1
Pfs1 8598	xLR EER	Broad	OG0003089	1
Pfs1 9976	LF.AK HVL	Broad	OG0000222	1
TR14313**	RxLR EER WY	Downy mildew	OG0001021	1
Pfs1 10418	xLR	Broad	OG0000255	0
Pfs1 129	WY no RxLR	Peronosporales	OG0000009	0
Pfs1 4263	RxLR EER WY	<i>Pfs</i> -specific	OG0010381	0
Pfs1 9414	RxLR EER WY	<i>Pfs</i> -specific	OG0033384	0
Pfs1 964*	LFLAK	Broad	OG0000723	0
Pfs1 9237	RxLR EER WY	<i>Pfs</i> -specific	OG0007292	0
Pfs1 8028	RxLR EER WY	Peronosporales	OG0000009	0
Pfs1 8288a	RxLR WY	Peronosporales	OG0000009	0
Pfs1 8804	RxLR EER	Broad	OG0004556	0
Pfs1 1192	RxLR EER WY	Peronosporales	OG0000009	ND
Pfs1 12059	RxLR WY	Peronosporales	OG0000009	ND
Pfs1 13089	RxLR EER WY	Peronosporales	OG0000009	ND
Pfs1 8208a	RxLR EER WY	Peronosporales	OG0010393	ND
Pfs1 8804	RxLR EER	broad	OG0004556	ND
Pfs1 8905*	RxLR EER WY	<i>Pfs</i> specific	OG0007292	ND

Asterisks (*) mark proteins without signal peptides. 'EER' indicates EER-like motifs, TM is a transmembrane domain and '.' is any amino acid except leucine (L). **The full name of TR14313 is TR14313|c0_g2_i1|m.24305-00001. For proteins marked 'ND' (not done) no successful mating was performed.

Table 3. *Pfs* effector-interacting Arabidopsis proteins as identified by yeast two hybrid screening.

Gene ID	Protein name	Effector	Length (aa)	colonies (#)	Retransformation	Pfam
AT1G07730	Disease res.-resp.	Pfs1 10492	389	2	weak	PF03018
AT1G18500	methylthio. syn.	^{1.} Pfs1 3487	631	1,2	strong	PF08502 , PF00682
AT1G22920	CSN5A	Pfs1 3487	357	1	intermediate	PF01398
AT1G33060	NAC014	Pfs1 3172	652	1	strong	PF02365
AT1G55190	PRA1.F2	Pfs1 9071	189	1	strong	PF03208
AT3G13720	PRA1.F3	Pfs1 9071	188	1	strong	PF03208
AT1G60870	MEE9	Pfs1 10492	147	1	-	-
AT2G18300	HBI1	Pfs1 6786	364	2	strong	PF00010
AT2G19390	serine/arginine rich	Pfs1 1800	1211	3	strong	-
AT2G25510	TM protein	Pfs1 10492	103	1	-	-
AT2G27720	60S ribosomal	Pfs1 1800	130	3	weak	PF00428
AT3G01340	TFPD	Pfs1 10492	302	2	weak	PF00400
AT3G01500	Carb. anhyd. 1	Pfs1 1800	347	1	intermediate	PF00484
AT3G02470	upstr.orf9/SAMDC	Pfs1 6786	366	1	strong	PF01536
AT3G11100	VFP3	Pfs1 1800	249	1	intermediate	PF13837
AT3G11930	USPA	^{2.} Pfs1 1800	226	5	strong	PF00582
AT3G13800	AT3G13800	^{3.} Pfs1 1800	361,366	1	intermediate	PF12706
AT3G47620	TCP14	^{4.} Pfs1 6786	489	1-3	strong/weak	PF03634
AT3G48560	AHAS	Pfs1 6786	670	2	strong	3 domains⁶
AT3G49580	LSU1	^{5.} Pfs1 3869	94	1,1	strong/weak ⁵	-
AT3G57560	NAGK	Pfs1 3869	347	3	strong/weak	PF00696
AT4G01150	Curv. thylakoid 1A	Pfs1 9071	164	1	intermediate	PF14159
AT4G04020	FBN1A	Pfs1 9976	318	1	intermediate	PF04755
AT4G13200	Uncharacterized	Pfs1 6786	185	1	weak	-
AT4G16250	phytochrome D	Pfs1 7215	1164	1	strong	5 domains⁷

Table 3. continued

Gene ID	Gene name	Effector	Length (aa)	colonies (#)	Retransformation	Pfam
AT4G27740	<i>Yippee family</i>	Pfs1 3210	105	1	weak	PF03226
AT4G29680	<i>alkaline phosph.</i>	Pfs1 4005	496	1	weak	PF01663
AT4G35070	AT4G35070	Pfs1 10492	265	1	-	-
AT4G35110	<i>PEARL4</i>	Pfs1 10492	401	2	strong	PF05278
AT4G36120	<i>DUF869</i>	Pfs1 10492	996	2	-	PF05911
AT4G36870.2	<i>Bhl2</i>	Pfs1 1800	739	1	-	PF05920, PF07526
AT5G03300	<i>ade. kinase 2</i>	Pfs1 3869	345	1	strong	PF00294
AT5G04410	<i>NAC078</i>	Pfs1 3172	567	4	intermediate	PF02365
AT5G08330	<i>TCP21</i>	Pfs1 3869	239	1	-	PF03634
AT5G12150	<i>Rho GTPase</i>	Pfs1 6786	827	1	-	3 domains⁸
AT5G40190	<i>RNA ligase</i>	Pfs1 3869	183	1	strong	PF13563
AT5G47090	<i>coiled-coil</i>	Pfs1 6786	310	1	-	PF09747
AT5G51110	<i>RAF2</i>	Pfs1 6786	220	3	strong	PF01329
AT5G53180	<i>PTB</i>	Pfs1 3869	534	1	-	PF00076
AT5G56100	<i>Glycine-rich</i>	Pfs1 9071	150	2	strong	-

Pfam domains that are bold are within the smallest fragment that interacted with the candidate host-translocated effector.

1. AT1G18500 was found to interact with two candidate effectors Pfs1|3210, Pfs1|805.
2. USPA was found to interact with three candidate effectors Pfs1|10763, Pfs1|3172, Pfs1|4005.
3. AT3G13800 gene product was found to interact with two candidate effectors Pfs1|3172, TR14313|c0_g2_1|lm.24305-00001.
4. TCP14 was found to interact with eight candidate effectors; Pfs1|10453, Pfs1|2936, Pfs1|3069, Pfs1|3210, Pfs1|3487, Pfs1|3869, Pfs1|17215 and Pfs1|8595.
5. LSU1 interacted with two candidate effectors; Pfs1|2936 (weak) and Pfs1|17215 (strong).
6. The AHAS protein has 3 domains; **PF02775**, PF02776, PF00205.
7. AT4G16250 has five domains; **PF00989**, PF01590, PF00512, PF02518, PF00360.
8. Rho GTPase has three domains; **PF14389**, PF00169, PF00620

Interaction network of *Arabidopsis* proteins targeted by host-translocated *Pfs* effectors

A public database, EffectorK [214] was mined for experimentally-verified interactions between the *Arabidopsis* proteins identified in the *Pfs* effector Y2H screens and other proteins. The EffectorK interaction dataset includes experimentally-confirmed protein-protein interactions from 2035 peer-reviewed publications and contains *Arabidopsis*-*Arabidopsis* as well as *Arabidopsis*-effector protein interactions. Effectors from bacterial (*Pseudomonas*, *Agrobacterium*, *Xanthomonas*, *Ralstonia*), fungal (*Golovinomyces*) and oomycete (*H. arabidopsidis*) phytopathogens are represented [205, 206, 214, 215, 216, 217, 218]. The *Pfs* effector interactome supplemented with the published interactions was visualized in a *Pfs* interaction network (**Fig. 6**).

We obtained 1220 literature curated interactions between the *Pfs* effector-interactors and other proteins from the database, of which 1089 with plant proteins and 131 with effectors of other pathogen species. The interactions between proteins can give an idea about the function of *Pfs* effector-interactors in the plant. Possibly the host-translocated effectors interfere with this function.

Previous studies suggested that effectors, of a wide range of pathogens, target similar plant proteins [206]. These proteins are characterized by their high connectivity within the protein interaction network [219]. The *Pfs* Y2H interaction network is centered around six 'hubs' of highly connected proteins; low sulfur upregulated 1 (LSU1), TEOSINTE BRANCHED, CYCLOIDEA AND PROLIFERATING CELL FACTORS 14 (TCP14), virF interacting protein 3, (VFP3), constitutive photomorphogenesis 5A (CSN5A), fibrillin, and Prenylated Rab acceptor 1 (PRA1). Proteins in each of these hubs are targeted by effectors of other pathogens as well.

Figure 6. Interaction network of Arabidopsis interactors identified by Y2H interaction with *Pfs* effectors. Details on the next page.

Figure 6 continued

The network includes published interactions between Arabidopsis (blue) *Pfs* (yellow), *Hpa* (orange), fungal (*Golovinomyces*, green) and bacterial (*Pseudomonas*, *Agrobacterium*, *Xanthomonas*, *Ralstonia*, red) effectors. The interactions identified in this study are indicated with dashed blue lines and the highly connected proteins are highlighted (light red box). *LSU1* = low sulfur upregulated 1, *TCP14* = TEOSINTE BRANCHED, CYCLOIDEA AND PROLIFERATING CELL FACTORS 14, *VFP3* virF interacting protein 3, *Pra1* = prenylated Rab acceptor 1, *FBN1A* = Fibrillin precursor protein 1A, *CSN5A* = constitutive photomorphogenesis 5A.

Nine of the *Pfs1* effector protein-interactors identified in the Y2H screens were previously described to interact with effectors from other species (**Table 4**). *CSN5A*, *PRA1.F3* (Prenylated Rab acceptor 1.F3), *TCP14* and fibrillin 1a (*FBN1a*) even interact with effectors from species of three kingdoms of life represented in the database. *VFP3* and *LSU1* are targeted by bacterial and *Pfs* effectors. These nine candidate effector proteins are highly connected within the overall Arabidopsis interactome and are likely important regulators of cellular processes and possibly linked to plant immunity or other aspects of the infection process.

Table 4. Nine *Pfs* effector-interactors of Arabidopsis are targeted by host-translocated effectors of other microbes.

<i>Pfs</i>-interactor protein		Effectors			
Gene code	Protein name	<i>Pfs</i>	Bacteria	<i>Golovinomyces</i>	Other
AT1G22920	CSN5A	Pfs1 3487	18	9	13
AT3G13720	PRA1.F3	Pfs1 9071	4	1	1
AT3G47620	TCP14	8	4	23	27
AT4G04020	FBN1a	Pfs1 9976	1	1	1
AT5G08330	TCP21	Pfs1 3869	-	-	1
AT5G51110	Pterin dehydratase	Pfs1 6786	-	-	1
AT3G01500	Carbonic anhydrase 1	Pfs1 1800	-	-	1
AT3G11100	VFP3	Pfs1 1800	2	-	-
AT3G49580	LSU1	2	9	-	-

*The number of Arabidopsis proteins that were reported to interact with *Pfs* effector-interactors.

CSN5A, that is found to be targeted by Pfs1|3487 in our study, is part of the eight sub-unit COP9 signalosome that regulates developmental processes and hormonal signaling [220]. CSN5A was previously found to interact with as many as 29 effectors of *Hpa* and *Pseudomonas*. *Csn5a-2 cul3a* (silencing of *CUL3* reduces the pleiotropic effects of CSN5A silencing) plants were shown to overaccumulate PR1, a common marker for PTI and enhanced resistance against *Hpa* [206]. Silencing of the CSN5A gene in Arabidopsis was shown to reduce auxin and jasmonic acid signaling resulting in reduced defense against *Manduca sexta* and *Botrytis cinerea* [221]. However, one should remain cautious as both CSN5a and TCP14 were reported to interact with the GAL4 DNA binding domain and the interactions in our study were weak so they could be an artefact of Y2H screening [222, 223].

The majority of the *Pfs* effector-interacting proteins were less connected in our network. As many as 27 Arabidopsis interactors have between one and five connections with other proteins. Six of these lesser connected host-translocated effectors are connected to the bigger 'hubs' by one of their interactors but also have interactors that are not linked to the hubs. An interesting example is host-translocated effector Pfs1|6768 that is linked to the fibrillin centered hub of the network through its interaction with Rubisco accumulation factor 2 (RAF2, AT5G51110). RAF2 is required for Rubisco biogenesis, its link to fibrillin is unknown [224]. Of the other six interactors of Pfs1|6786, five do not interact with any protein other than Pfs1|6786. Remarkably, gene expression of five of the Pfs1|6786 interacting protein encoding genes, including RAF2, were reported to be down-regulated upon treatment of Arabidopsis plants with salicylic acid (SA), suggesting a response to immune signaling [225]. The interacting proteins include transcriptional regulators like the negative regulator of defense *BhLh64/HBI1* (AT2G18300) [226], a coiled-coil protein of unknown function (AT5G47090) and an upstream open reading frame that encodes a peptide that regulates SAMDC1 transcription [227, 228]. Overexpression of *SAMDC1* was shown to elevate spermine levels and induce defense-related gene expression and higher resistance to *Pseudomonas syringae* and *Hpa* [229]. The other PFS1|6786 interactor-proteins are involved in metabolic processes like amino acid synthesis (*acetolactate synthase (AHAS)*, AT3G48560) or have no known functions (AT4G13200, AT5G12150) [225, 230, 231].

Although the function of the Pfs1|6786 interactors are reasonably well described, the function of the majority of lesser connected *Pfs* interactor-proteins is unknown. For instance, Pfs1|10492 was found to interact with seven Arabidopsis proteins. The proteins are not reported to interact with each other and for five of them, their molecular function is unknown. One of the two proteins that have been more extensively studied is *Pfs* effector-interactor protein AT3G01340. It interacts with its paralog AT2G30050 that links this effector to the CSN5A signaling hub of the network. AT3G01340 and AT2G30050 are the paralogs of the budding

yeast Sec13 protein that mediates the formation of COPII coated vesicles that transport proteins from the ER to the Golgi system [232]. The other studied interactor AT2G25510 does not have a known molecular function but was associated with defense against *H. arabidopsidis* because of its high expression levels in Arabidopsis *downy mildew resistant 5 and 6* (*dmr5* and *dmr6*) mutants identified in a ethyl methane sulfonate (EMS) screen [233].

Other *Pfs* host-translocated effectors with a link to a large hub in the network and additional independent interactors are Pfs1|9071, Pfs1|3869, Pfs1|1800. The low complexity interaction network of these proteins makes them interesting targets for further functional analysis as their function may be easier to comprehend than that of highly connected proteins.

3

Four candidate host-translocated effectors are not connected to the main network at all. They form a separate cluster because Pfs1|3172 shares interactor proteins with the other three host-translocated effectors. Pfs1|4005, Pfs1|10763 and Pfs1|3172 all target AT3G11930, a protein that has a universal stress protein domain A (USPA) [234]. This domain is omnipresent in both prokaryotes and eukaryotes and is thought to be involved in the response to environmental stimuli. The network shows that it interacts with AT2G47710, that has a USPA domain as well. The protein is targeted by three *Pfs* host-translocated effectors but not any of the other effectors in the network, so it may be a *Pfs*-specific interactor target. Pfs1|3172 and TR14313|c0_g2_i1|m.24305-00001 both interacted with AT3G13800 in the Y2H screen. AT3G13800 has a Metallo-hydrolase/oxidoreductase superfamily protein domain but its molecular function is otherwise unknown. Besides the shared Arabidopsis interactor proteins, Pfs1|3271 interacts with two NAC transcription factors, NAC014 and NAC078. Both NACs have a C-terminal transmembrane domain, just like Pfs1|3172. NACs in this family have been shown to be effector targets of *P. infestans*, *Hpa* and *Bremia lactucae* effectors [86, 169, 206]. Their possible function in immunity will be discussed in **chapter 4**.

Prioritizing plant interactors for further validation

The Y2H-based effector interaction network provides a first insight into the cellular processes that may be targeted by host-translocated *Pfs* effectors. Further research is needed to understand the role of the effectors and their targets in the infection process. We used the Y2H and interaction network to select fourteen interactors, targeted by eight candidate effectors, that are of particular interest for further characterization (**Table 5**). In the selection we prioritized Y2H interactors that were represented by multiple unique gene fragments and/or showed strong interaction with the effector upon retransformation, over weaker interactors represented by single fragments. Interactions between multiple unique fragments and an effector protein served as independent replicas of the interaction. We selected nine effector-interactors that had less than ten connections in the network. Five other interactors had between fourteen and 110 connections but could be interesting because they have been published to interact with effectors of other species or are known to affect disease susceptibility. The further characterization of these fourteen high confidence interactors and their eight effector proteins is the topic of **chapter 4** of this thesis.

Table 5. Selected interactors of candidate host-translocated effectors of *Pfs* for further functional studies (Chapter 4).

Gene ID	Protein name	Length (aa)	Unique Colonies Y2H (#)	Pfam	Interacting effectors
AT1G33060	NAC014	652	1	PF02365	Pfs1 3172
AT1G55190	PRA1.F2	189	1	PF03208	Pfs1 9071
AT3G13720	PRA1.F3	188	1	PF03208	Pfs1 9071
AT2G18300	HBI1	364	2	PF00010	Pfs1 6786
AT2G19390	SR*-rich protein	1211	3	-	Pfs1 1800
AT3G11930	USPA	226	5	PF00582	Pfs1 10763, Pfs1 3172
AT3G13800	AT3G13800	361	1	PF12706	Pfs1 3172
AT3G48560	AHAS	670	2	PF02775 /PF02776/PF00205	Pfs1 6786
AT3G49580	LSU1	94	1,1	-	Pfs1 7215
AT3G57560	NAGK*	347	3	PF00696	Pfs1 3869
AT4G04020	FBN1a	318	1	PF04755	Pfs1 9976
AT5G04410	NAC078	567	4	PF02365	Pfs1 3172
AT5G51110	RAF2	220	3	PF01329	Pfs1 6786
AT5G56100	Glycine-rich protein	150	2	-	Pfs1 9071

The Pfam domains indicated in bold are found in the protein fragments present in the prey clones obtained in the screen. SR* = serine arginine, NAGK = N-acetyl-L-glutamate kinase.

Conclusion

Comparative analysis of the candidate RxLR and CRN effectors of *Pfs1* revealed that 39 effectors are *Pfs* specific, 10 are downy mildew specific, 23 are Peronosporales specific and 40 more broadly conserved. Candidate effector Pfs1|10382 is in an orthogroups with *H. arabidopsis* effector HaRxL44 and possibly has a similar function. Using Y2H assays, 40 Arabidopsis proteins that interact with 26 *Pfs* candidate effectors were identified, to our knowledge representing the first known plant interactors of *Pfs* effectors. The interaction data were supplemented with published interactions with other proteins, providing an interaction network that gave insight into connectivity and possible processes in which the plant interactors may be involved. Based on the interaction strength in the Y2H assays and the information in the interaction network, fourteen high confidence plant interactors and eight corresponding *Pfs1* were selected to study their interaction in planta.

3

Material and Methods

Computational analysis of secreted proteins for comparative genomics

The clustering of orthologous proteins using Orthofinder, the secretome prediction and the effector identification were described in chapter 2. The proteome of *Pfs* was compared to that of four downy mildew, four *Phytophthora*, two *Albugo* and 5 *Pythium* species (**Chapter 2, Table S1**).

Selection of candidate host-translocated effectors for functional validation

Candidate host-translocated effectors were prioritized based on their conservational status and the presence of known motifs and domains in their amino acid sequence. Candidate host-translocated effectors that are conserved between downy mildew or Peronosporales species and canonical effectors were prioritized over candidate host-translocated effectors that were unique to *Pfs* or had degenerative motifs. Three

additional candidate host-translocated effectors were identified by manual annotation of genes in the final assembly of *Pfs1* (**Supplemental data 1**).

Yeast-two hybrid library construction

An oligo-dT primed *Arabidopsis thaliana* Col-0 cDNA library was produced using Invitrogen Custom Services (Invitrogen, Carlsbad, California). Ten day old seedlings were exposed to five different treatments; (1) infection with *H. arabidopsidis* Cala2 (incompatible, harvested 3 days post infection (dpi)) or (2) *H. arabidopsidis* Noco2 (compatible, (3 dpi)), (3) treatment with BTH (24 hours, sprayed with 0,1 mg/μl), (4) infiltration with the NLP3-derived peptide pattern nlp24 (24 hours), or (5) untreated. Plant material was harvested for RNA isolation using phenol/chloroform extraction. RNA samples were mixed at a 1:1:1:1:1 ratio, using 2 mg of RNA for each sample. A three frame uncut cDNA library was made using oligo-dT primers and cloned into pENTR22 entry vectors using Gateway technology [235]. Cloning of the cDNA library resulted in 168×10^6 colony forming units with an average insert size of 1.5 kb. The inserts were then recombined into Y2H prey vector pDEST22 to create GAL4 activation domain fusions. Transformation into *E. coli* DH10B resulted in 7.9×10^6 colony forming units with an average insert size of 1.55 kb.

Yeast-two hybrid bait construction

Peronospora effusa reference race 1 (*Pfs1*) was provided by the Dutch breeding company Rijk Zwaan Breeding BV in 2014. The growth conditions and infection protocol are described in chapter 2. At 4, 5, 6 and 7 days post infection leaf samples were harvested for mRNA isolation using the Qiagen mRNA isolation kit. cDNA was generated using oligo-dT primers at standard settings. Effector coding sequences were amplified from the predicted signal peptide cleavage site (SignalP v4.0, setting 0.34). A start codon was added to the forward primer (**Table S3**). The effectors were recombined into the Y2H bait vector pDEST32 to create DNA-binding domain (DBD) effector protein fusions. All entry clones were verified by Sanger sequencing. The insert size of the destination vectors was confirmed using colony PCR.

Yeast strains and transformation

The bait and prey vectors in this study were transformed into yeast strain Y8800 and Y9300, respectively. To make competent yeast cells, yeast was grown overnight in 250 ml of YEPD at 20°C while shaking. The medium was transferred to a 28°C shaking incubator and grown until the OD₆₀₀ was between 0.2 and 0.8. The yeast cells were collected by centrifuging the medium at 1800 rpm for five minutes. The YEPD was discarded and the cells were washed in 50 ml ddH₂O and spun down twice before they were dissolved in 50ml TE/LiAc (10 mM Tris, 1 mM EDTA, 100 mM LiAc). After a final centrifugation, the now competent cells were dissolved into TE/LiAc to a final OD₆₀₀ of 50. For a single transformation reaction, 20 µl of competent yeast cells was added to a premixed reaction mix of 82 µl 60% PEG, 11 µl of 10x TE, 13 µl 1 M LiAc, 20 µl of salmon sperm DNA and 200 ng of plasmid DNA. The salmon sperm DNA was heated at 95°C for five minutes and kept on ice prior to use. Transformation reactions were incubated at 30°C for 30 minutes and transformed to a 42°C water bath for 15 minutes. One ml of ddH₂O was added to each tube and the cells were spun down at 5000 rpm for 30 seconds to collect the yeast. The yeast pellet was resuspended in ddH₂O and plated on synthetic complete (Sc) plates with the appropriate marker (Sc-Trp for bait and Sc-Leu for prey). Plates were incubated at 30°C and colonies would be visible after three days.

The library transformation was done in a similar fashion, just increasing the volume of the competent cells to 9.6 ml and the other components of the reaction to ratio. A total of 121,8 µg of library DNA was used. The medium was plated on Sc -Leu-Trp plates to select for cells that obtained the bait as well as a prey vector. The transformation yielded >1x10⁶ colony forming units (cfu's) The yeast strains were harvested in YEPD + 20% glycerol. 1ml aliquots of OD₆₀₀ 40 were stored at -80°C.

Library screen

The library screen was performed using the mating method [236]. A yeast library aliquot of 1 ml was thawed on ice, transferred to 100 ml YEPD and incubated on a shaker at 28°C for one hour. Cells were centrifuged at 1800 rpm for 5 minutes and washed in ddH₂O twice. Cells were resuspended in YEPD to OD₆₀₀ = 3.

The pDEST32 bait vectors with effector proteins were grown overnight in 20 ml of YEPD with ampicillin in 50 ml Falcon tubes while shaking at 20°C until they reached the exponential growth stage (0.2 – 0.8 OD) and collected by centrifugation as described above.

For each mating reaction 3 ml of library and 6 OD (so, in case of OD₆₀₀ = 0.5, 12 ml is used.) of bait transformed yeast were mixed, centrifuged, resuspended in 300 µl of ddH₂O and plated on a YEPD (+ 100 µg/ml ampicillin) plate. After 4 hours of incubation at 30°C yeast was collected from the plates, suspended in ddH₂O and collected by centrifugation. The cells were resuspended in 600 µl ddH₂O and plated on synthetic complete (SC) plates without leucine (Leu) tryptophan (Trp) and histidine (His) supplemented with ampicillin (Amp). A 1:10,000 dilution was plated onto SC – Leu – Trp to monitor the number of diploid colonies screened. A minimum of 1 million colonies was screened for each bait. After four days of incubation the colonies were picked from the Sc -Leu -Trp -His plates and suspended in 15 µl ddH₂O. The dissolved colonies were then spotted on two fresh SC -Leu -Trp -His. One plate was used for replica plating onto SC -Leu -Trp – His plates supplemented with 2 or 5 mM of the histidine biosynthesis inhibitor 3-aminio-1,2,4 -triazole (3-AT) (ForMedium) to test the strength of the interaction. Plates were incubated for two days and pictures were taken to document the growth of the yeast.

The other plate was used for colony PCR. Yeast colonies were lightly picked and transferred to 30 µl of 0.02 M NaOH. The samples were heated (99°C, 10 minutes), cells were collected using centrifugation and 1 µl of the supernatant was used for colony PCR. The colony PCR reactions were 10 µl each and DNA was amplified using

Dreamtaq DNA polymerase (ThermoScientific) and primers for pDEST22 (Forward; TATAACGCGTTTGGGAATCACT, Reverse; ACCTTGATTGGAGACTTGAC). Prey PCR products were purified (Agencourt AMPure XP beads) and Sanger sequenced.

Sequences were analyzed using Geneious v 9. Arabidopsis coding sequences that were out of frame with the GAL4-AD domain or where less than 50 amino acids in length were discarded. The corresponding full-length Arabidopsis genes were identified by finding the best hit in the NCBI non-redundant database using BlastN.

For every identified gene a representative colony was grown in 3 ml of YEPD. To isolate the plasmids the culture was centrifuged (2000 rpm, 2 minutes), resuspended in 1 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). After another round of centrifugation, the cells were suspended in 200 µl Resuspension buffer with RNase A (Macherey Nagel NucleoSpin Plasmid EasyPure, Mini kit for easy plasmid DNA purification), 15 U of Zymolase-20T (Amsbio #120491-1) and 2 µl of 2-Mercaptoethanol. Samples were incubated at 37°C for an hour before plasmid isolation with the GenElute plasmid miniprep kit (Sigma). Plasmids were amplified via transformation into DH5α cells. The obtained plasmid DNA (from a single colony) were used in the retransformation assay.

The interaction between bait and prey was verified by transforming both plasmids into yeast strain Y8930. Colonies were selected on SC -Leu -Trp -His plates and replica plated on SC -Leu -Trp -His + 2 and 5 mM 3-AT.

Network analysis

All datasets from the newly published EffectorK database were downloaded and mined for interactions that involved the Arabidopsis proteins that interacted with the *Pfs* effector baits in this study [214]. A network was created using cytoscape v3.7.9. and improved using Photoshop v1.9.1 to highlight the more connected interactors.

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Supplemental Tables.

Table S1. RxLR and CRN effectors of *Pfs1*

Table S1 is available from:

<https://doi.org/10.17026/dans-zey-8aec>

Table S2. Pfs1 proteins selected for use as bait in yeast two hybrid screening with a library of oligo-dT primed arabidopsis proteins.

Protein	Category	Orthogroup	Conservation in orthofinder	RxLR or CRN	Amplified (cDNA)	Screened (Y2H)
Pfs1 10492	conserved	OG0000009	Peronosporales	RxLR EER WY	yes	yes
Pfs1 3069	conserved	OG0000009	Peronosporales	xxLR WY	yes	yes
Pfs1 8028	conserved	OG0000009	Peronosporales	RxLR EER WY	yes	yes
Pfs1 805	conserved	OG0000009	Peronosporales	RxLR WY	yes	yes
Pfs1 8288a	conserved	OG0000009	Peronosporales	RxLR WY	yes	yes
Pfs1 7215	conserved	OG0000021	broad	RxLR	yes	yes
Pfs1 10453	conserved	OG0000023	broad	RxLx EER	yes	yes
Pfs1 9071	conserved	OG0000033	broad	LFLAK	yes	yes
Pfs1 2936	conserved	OG0000062	broad	RxLR	yes	yes
Pfs1 9976	conserved	OG0000222	broad	LFxAK HVL HMM	yes	yes
Pfs1 3487	conserved	OG0000437	broad	RxLR LFLAK HVL	yes	yes
Pfs1 8598	conserved	OG0003089	broad	xxLR EER	yes	yes
Pfs1 3210	conserved	OG0006720	Peronosporales	xxLR EER	yes	yes
Pfs1 3869	conserved	OG0010260	downy mildew	RxLR	yes	yes
Pfs1 4263	canonical	OG0010381	Pfs specific	RxLR EER WY	yes	yes
Pfs1 6786	canonical	OG0010381	Pfs specific	RxLR EER WY	yes	yes
Pfs1 3172	conserved	OG0010838	downy mildew	RxLR EER	yes	yes

Table S2. continued

Protein	Category	Orthogroup	Conservation in orthofinder	RxLR or CRN	Amplified (cDNA)	Screened (Y2H)
Pfs1 9414	canonical	OG0033384	Pfs specific	RxLR EER WY	yes	yes
Pfs1 9237	canonical	OG0007292	Pfs specific	RxLR EER WY	yes	yes
Pfs1 1192	conserved	OG0000009	Peronosporales	RxLR EER WY	yes	no
Pfs1 12059	conserved	OG0000009	Peronosporales	RxLR WY	yes	no
Pfs1 8804	conserved	OG0004556	broad	RxLR EER	yes	no
Pfs1 13089	conserved	OG0000009	Peronosporales	RxLR EER WY	yes	no
Pfs1 3228*	conserved	OG0000350	broad	RxLx LFxAK	yes	no

Protein	Category	Orthogroup	Conservation in orthofinder	RxLR or CRN	Amplified (cDNA)	Screened (Y2H)
Pfs1 8208a	conserved	OG0010393	Peronosporales	RxLR EER WY	yes	no
Pfs1 12056	conserved	OG0000009	Peronosporales	RxLR WY	no	no
Pfs1 3286	conserved	OG0000009	Peronosporales	RxLR WY	no	no
Pfs1 10814	conserved	OG0000132	broad	RxLR	no	no
Pfs1 1257	conserved	OG0001590	broad	RxLR	no	no
Pfs1 1135	conserved	OG0003900	broad	RxLR	no	no
Pfs1 668	conserved	OG0005391	broad	RxLx EER	no	no
Pfs1 3683	conserved	OG0010260	downy mildew	RxLR	no	no

Table S2. continued

CRN peptide	without	signal			
Pfs1 4005**	conserved	OG0000657	broad	LFLAK	yes
Pfs1 964**	conserved	OG0000723	broad	LFLAK	yes
RxLR without signal peptide in final assembly					
TR14313 c0_					
g_2					
i1 m.24305**	conserved	OG0001021	downy mildew	RxLR EER WY	yes
Pfs1 10763**	canonical	OG0007292	Pfs specific	RxLR EER WY	yes
Pfs1 8905**	canonical	OG0007292	Pfs specific	RxLR EER WY	no
Pfs1 1800**	conserved	OG0007294	Peronosporales	RxLR EER	yes
Proteins that are related to effectors					
Pfs1 12416***	conserved	OG0000009	Peronosporales	WY no RxLR	no
Pfs1 129***	conserved	OG0000009	Peronosporales	WY no RxLR	yes
Pfs1 2926**	conserved	OG0000132	broad	xxLR	no
Pfs1 10418*	conserved	OG0000255	broad	xxLR DEK	yes

The category (conserved, canonical, else) of each protein is indicated. Proteins that are not true putative effectors are shaded grey. The domains that define the putative effectors are outlined (RxLR (-like), WY, EER, LFLAK (-like), HVL). The final two columns show whether the amplification of the protein from cDNA and the yeast two hybrid assay were successful (>1 million colonies).

* Protein is not a putative effector because it has a degenerative RxLR (and LFLAK) but no additional domains (WY,EER,(HVL))

** Protein is not a putative effector because it has no signal peptide (in the final assembly of Pfs1).

Table S3. Primers

Gene name	Forward primer
pfs1 10492	GGGGACAAGTTTGTACAAAAAGCAGGCTCAATGAGTCCAAGTACTATGAAG
pfs1 1192	GGGGACAAGTTTGTACAAAAAGCAGGCTCAATGACCCCCAAGTC
pfs1 3286	GGGGACAAGTTTGTACAAAAAGCAGGCTCAATGGTGAAACTCATAAGTC
pfs1 3487	GGGGACAAGTTTGTACAAAAAGCAGGCTCAATGCTCTCGGTGCG
pfs1 8028	GGGGACAAGTTTGTACAAAAAGCAGGCTCAATGAGTCCAAGCACTGT
pfs1 8288a	GGGGACAAGTTTGTACAAAAAGCAGGCTCAATGAATTCGCGCC
pfs1 8804	GGGGACAAGTTTGTACAAAAAGCAGGCTCAATGTTGTTGTGCTATG
pfs1 10453	GGGGACAAGTTTGTACAAAAAGCAGGCTTCATGACCAATTTGGAACGACAAACCC
pfs1 805	GGGGACAAGTTTGTACAAAAAGCAGGCTTCATGAGTCCAAGCACTGTGAGC
pfs1 2936	GGGGACAAGTTTGTACAAAAAGCAGGCTTCATGCAACACCTAGTGGCTCCAC
pfs1 10418	GGGGACAAGTTTGTACAAAAAGCAGGCTTCATGCACCAAGTTGTGCGGAATCC
pfs1 8598	GGGGACAAGTTTGTACAAAAAGCAGGCTTCATGCGAAATCAATTACGGGTGCG
pfs1 3683	GGGGACAAGTTTGTACAAAAAGCAGGCTTCATGGATGTTCCGCTTCCCTACAG
pfs1 12056	GGGGACAAGTTTGTACAAAAAGCAGGCTTCATGGCACAATCGAGCACTGTCAT
pfs1 3210	GGGGACAAGTTTGTACAAAAAGCAGGCTTCATGGCCAATCTTCGTTCCGAAAAGA
pfs1 668	GGGGACAAGTTTGTACAAAAAGCAGGCTTCATGGGTAAAGACTATTACGAGATCTGGG
pfs1 3228	CACCATGGTAACGTTAATATGTGCGA
pfs1 9071	CACCATGATGCTAAAGCTTTTTTGCCTCG
pfs1 964	CACCATGATGGCAAAGCTGTTGTGCGT
pfs1 4005	CACCATGATGTACATGATGTCACTAGCTC
pfs1 9976	GGGGACAAGTTTGTACAAAAAGCAGGCTTAATGGCCTGCACGATTAAGAAAG
pfs1 13089	CACCATGGGCGACACTCCCATCCCT
TR14313*	CACCATGACAAGATCACCTGAGGATGC
pfs1 1800	CACCATGGCTGCCCCGGCAGCTCA
pfs1 8905	CACCATGGTAATGATATGGAGTCCACAG
pfs1 4263	CACCATGGGTGCACATACAGAGCCGT

Gene name	Forward primer
pfs1 9237	CACCATGGTTGTGCTAATATGGAGTCCA
pfs1 10763	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGTTGTGCTAATATGGAGTCCA
pfs1 6786	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCATGTACAGATGACCAATATG
pfs1 3172	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTGCGGCTGCTTCATTCGTG
pfs1 9414	CACCATGGATCTGTACCAGAGGATGC
pfs1 1135	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCGACCAACAGCACACCTCT
pfs1 1257	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTTTGTGAATTACCTCTTGCTTTTCTG
pfs1 3069	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGATAACAACCTCCGTCGTG
pfs1 129	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAGCCAACACCTTTCTG
pfs1 12416	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGACCAAAGTCGAGCCAACAACCTT
pfs1 3869	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATGTCCCGCACTTCGCGCATC
pfs1 7215	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCCGAAGGGACCGAGCTGACAC
pfs1 10814	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCGGGTGTTGGGTCTCCG
pfs1 2926	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGACTCGCTACAGAGCGAAAGAGG
pfs1 12059	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTTGCTCGCATATTCAGGTTT
Gene name	Reverse primer
pfs1 10492	GGGGACCACCTTTGTACAAGAAAGCTGGGTCCTATCAATTTTTCATCGGCGGCT
pfs1 1192	GGGGACCACCTTTGTACAAGAAAGCTGGGTCCTACTAACCATGAACATCGAAATTGCT
pfs1 3286	GGGGACCACCTTTGTACAAGAAAGCTGGGTCCTATTAAAACATCTTTATCAGTGCATCAGC
pfs1 3487	GGGGACCACCTTTGTACAAGAAAGCTGGGTCCTATCACTGGCCGATTTTCGTCTTG
pfs1 8028	GGGGACCACCTTTGTACAAGAAAGCTGGGTCCTACTAATTTTCCGTGGTGACTCG
pfs1 8288a	GGGGACCACCTTTGTACAAGAAAGCTGGGTCCTATAAGCCACTTTCGTATCAGCT
pfs1 8804	GGGGACCACCTTTGTACAAGAAAGCTGGGTCCTATTACCCACTTGTGTCGTCAA
pfs1 10453	GGGGACCACCTTTGTACAAGAAAGCTGGGTCCTATTACAAAAGACACTCAAACCACTGC
pfs1 805	GGGGACCACCTTTGTACAAGAAAGCTGGGTCCTATCAACATCTTCTTTATCCACGAGT
pfs1 2936	GGGGACCACCTTTGTACAAGAAAGCTGGGTCCTATTAAGCGAAGTCTGAGCGATCG

Table S3. continued	
Gene name	Reverse primer
pfs1 10418	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATCAGTTACTCACAATATGGTGGTG
pfs1 8598	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATCATTCTCGCTAAACTCTGTCC
pfs1 3683	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATTACAATATACTTAGGTTGATGAGGCC
pfs1 12056	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATTACTTTTCCGAGTAAGGTACTACA
pfs1 3210	GGGGACCACTTTGTACAAGAAAGCTGGGTCTACTACATACTCCCATTACTCGGCG
pfs1 668	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATTACTTCATCGAAATAAATTGCGA
pfs1 3228	TCAATCGACCGCCGGAGTC
pfs1 9071	TTATGACGTAAAAATGTCTCAAG
pfs1 964	TCATCTTGAAGCTTGCCCTTCC
pfs1 4005	TTATAACAGTGTGAGACCACTG
pfs1 9976	TCATTTCACAATTGAGTCATCG
pfs1 13089	TCACGCGTATTGAGCCTTAAT
TR14313*	CTACTTGTGCCCCAGAAACG
pfs1 1800	CTATGGCGATTCGTCATGGT
pfs1 8905	CTACTGACCATCGTCTTTTCG
pfs1 4263	TCTAAGGTATTAAATCTTTCCCA
pfs1 9237	CTACTGACCATCGTCTTCTC
pfs1 10763	GGGGACCACTTTGTACAAGAAAGCTGGGTCTACTGACCATCGTCGATAC
pfs1 6786	GGGGACCACTTTGTACAAGAAAGCTGGGTCTACTCCGGAAGCGACTTTA
pfs1 3172	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAAACGGTAGTCGTCGATC
pfs1 9414	TCACGAGCTTAGGTCGAGTT
pfs1 1135	GGGGACCACTTTGTACAAGAAAGCTGGGTCTACTATTTCTCATGTCTTTCGTTGTCTGA
pfs1 1257	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATTAGGCAAGAGCTGTACGTTGG
pfs1 3069	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACTCTGCTTCCCGCATTC
pfs1 129	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAACTCGATTCTTGTTGCC
pfs1 12416	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAATCTTCTCCCTCTTTGTCTT
Gene name	Reverse primer
pfs1 3869	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACAGATTCACAATAGCATCCAA
pfs1 7215	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACGAGCACTTCTCCGATT
pfs1 10814	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAATTTATAGCGGTCCG
pfs1 2926	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATTACGGCCCTCATCAT
pfs1 12059	GGGGACCACTTTGTACAAGAAAGCTGGGTCTACCTGTCTTGTGGTACAAATCCA

Primers used for amplification of effectors for Gateway BP and entry cloning.

Supplemental information 1

Upon manual curation an additional start codon was identified (bold) for protein Pfs1|1800-00002 and Pfs1|4091. The newly predicted sequences have a signal peptides (*italics*). The putative translocation domain is underlined.

>Pfs1|1800

MRLLCVLVM~~AVAAL~~TASNSAAPAAQATDDIKLWQANPQDRIDTMDALHIK-
TRPK**RML**RGVSNEDYDVDNEVDNDSSTSDTFLEDRIQQKLAKKSVRLQLY-
EVWYDCRFTVEKVS~~KHLT~~ISSSNKLFPTYHALKKGYERYCARPHDKPIRPVS-
DETCESYLAQILDNHDESP

>Pfs1|4091

MRLDSILFVAVLFANLSISSTRSMSTVVKSVTFNQEDVPAHGL-
GVKTTNEERTLGSPVVEKAAEEITSNVPESEGLVNLQNEMEKRLRGEDK-
SIMDNPHFNNWVRDLAKTIDTSSAAKEVITKVSELYGDEGLLKMLNILKDEV-
GEVEGLQGELKSALMASWEKQEMSARGVFEQLKLDQETDHTLYIQL
LTMWVRYTKENNLETKTVITSYDGNISLMLFEGLSEIEGTEEIVKSLQDSLMT-
FWAGRELSAEDLFSTLKLDNGGSKSNYDMWVKYVAQEFDLQPTKEVILKS-
SEVFGEEGLLKMLNALDLKEVGQDIQSELTALMASWKEKEKKPVEVLM-
LLESELMPEFNNHKVNIGRIKMWNQYVNENFEYPGLVELLAMGQIKNIAHK-
TIILEDLKQINLAAKDARKNSENAE

Although the Pfs1|10763 has a 33 amino acid hydrophobic N-terminus (*italics*), the signal prediction for this protein is just below the threshold. The effector bait for this protein lacks the hydrophobic part. The putative translocation domain is underlined.

>Pfs1|10763

MACTIKKVKHLFWYMLVTVVVLWSPQTMVKMVASQHAPRDARENRRL-
RPSSYMKEGVNGGDEERRLNIDLNLPALPDNLFKVLKIDPEVGEKIEATPE-
VGEKLEAKVPEVDENFEGWIPEVDEKIEATPEVDENVKSLLKNTRLHEWANF
PKDTTVEEYINAAIVKLTTELVTKNGKETVASMLKAAKNDPATKQIAANLERIL-
VLSHELNEDAFKMLSVGQADLRAFLASPLLKNYLELHLQLTGDFSLGPLVT-
MLLEHYSDAALAKKLEVHSKDAAAEMAEVSTYHTDHWENTYSGEILFER-
LGLKKAGIHMVDSPVWDTWTMYMAMRKGINKFAEPDLAEVAIPVLRKNFH-
DKVLLDWFNAAKTDAGVTRAGFLKTALDEARTAEGKAPLTISKRKGIDNELL-
VPTKRQRIDDGQ

4

Imaging *Peronospora effusa* effector-host protein interactions in planta

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Abstract

Phytopathogenic oomycetes secrete effector proteins into plant tissue to establish and maintain their infection. These effectors can either act in the apoplast or translocate into host cells and function there. Host-translocated effectors, like RxLR and Crinkler (CRN) effectors, are known to interact with plant components like RNA and proteins to manipulate cell processes. The genome of *Peronospora effusa* (*Pfs*), the obligate biotrophic oomycete that causes downy mildew on spinach, encodes over 100 candidate RxLR and CRN effectors. Previously, interacting *Arabidopsis* proteins of 18 effectors were identified by yeast two-hybrid screening. Eight candidate effectors that interacted with fourteen *Arabidopsis* protein fragments were selected for further functional validation. The subcellular localization of the effectors and their interactors was visualized by expression of fluorescent fusion proteins in *Nicotiana benthamiana* leaves. All but two of the 14 tested *Arabidopsis* interactors and their spinach orthologs localized with the candidate effector in the same subcellular compartment. The subcellular localization of six plant interactors and two effector proteins was altered upon co-expression of the effector and interactor proteins. Furthermore, seven plant proteins showed a more stable signal when co-expressed with the effector.

Introduction

Peronospora effusa (*Pfs*) is an obligate biotrophic oomycete that causes downy mildew on spinach and is a major threat to spinach production worldwide [30, 32]. Its hyphae can penetrate the leaf tissue and grow between plant cells, which they invaginate to form haustoria, the sites of interaction between the oomycete and plant cells [37]. The pathogen secretes proteins and other molecules to establish and maintain a successful infection [237]. These secreted proteins are called effectors and have evolved to establish compatibility between the pathogen and the spinach host. Effectors can either function between (apoplast) or within plant cells (host-translocated effectors) [40]. Host-translocated effectors are known to interact with plant proteins to suppress immunity and alter cellular processes for the benefit of the pathogen [238]. Two types of host-translocated effectors are known in oomycetes, the RxLR and Crinkler (CRN) effectors [56, 65].

It was shown that RxLR and CRN effectors of downy mildew species localize to many different subcellular compartments in plant cells. *Hyaloperonospora arabidopsidis* RxLR effectors were found to mainly target two cellular compartments; the nucleus (66% of tested effectors) and membranes [209]. Similar targeting of host-translocated effectors was found in the grapevine downy mildew *Plasmopara viticola* and sunflower downy mildew *Plasmopara halstedii* [239]. Remarkably, *P. viticola* also has three effectors that localize to chloroplasts and mitochondria [240], while a number of *P. halstedii* effectors were found to target chloroplasts, the tonoplast, endoplasmic reticulum (ER) and the Golgi system. Several CRN effectors were found to localize to the plant nucleus upon their expression in planta [56]. The next challenge is to elucidate why individual host-translocated effectors show such a specific localization in plant cells and how that contributes to pathogen virulence.

Previously we identified 99 RxLR and 14 CRN candidate genes in the genome of the *P. effusa* reference race 1 (*Pfs*1) (**Chapter 1 and 2**). In a first step towards understanding the function of effectors in plant cells, Arabidopsis protein fragments that interact with 18 candidate effectors

were identified in a yeast two-hybrid (Y2H) screen (**Chapter 3**). Here we describe the further analysis of eight candidate effectors and their 14 interacting *Arabidopsis* proteins. The spinach orthologs of the *Arabidopsis* proteins were identified and the subcellular distribution of all plant and effector proteins was assessed using transient in planta expression of fluorescent protein fusions. Co-expression of host interactors and effectors showed that subcellular localization of 12 *Arabidopsis* and all 14 spinach proteins overlapped with that of the effector, supporting their presumed interaction in planta. Furthermore, upon co-expression we observed the re-localization of two effector proteins and six interacting plant proteins. Another 7 plant proteins were stabilized upon co-expression with the effector. These results strongly suggest that the identified protein-protein interactions are indeed occurring in planta.

Results

Identification of spinach orthologs of *Arabidopsis* interactors

4

Orthologs of the *Arabidopsis* interactors were identified in the published proteome of spinach variety Sp75 [241] using a reciprocal BlastP search (TAIR10 *Arabidopsis* proteome). For ten *Arabidopsis* proteins we identified spinach proteins that gave a reciprocal best hit, suggesting they are orthologs (**Table 1**). For four other *Arabidopsis* interactors, low sulphur upregulated 1 (LSU1, At3g49580), serine-arginine rich protein (SR-rich, At2g19390), glycine-rich protein (glycine-rich, At5g56100), HOMOLOG OF BEE2 INTERACTING WITH IBH 1 (HBI1, At2g18300), the highest-scoring spinach proteins were more closely related to other *Arabidopsis* proteins in the reciprocal blast, suggesting a multiple-to-one orthology between the *Arabidopsis* and spinach proteins. The relations between the *Arabidopsis* and spinach proteins that were not reciprocally matched were clarified using protein trees generated with all significantly related predicted proteins of both species as identified by a BlastP search (**Fig. S1**).

In the case of HBI1 we found a 2-to-1 orthology between HBI1, At4g34530 and spinach protein (Spo) Spo06524. Even though Spo06524 is the spinach protein with the highest similarity to HBI1, it is more similar to the Arabidopsis HBI1 paralog At4g34530. In addition, a 2-to-1 orthology was found between At2g19390, At4g29790 and Spo24468 and a 6-to-1 orthology was observed for At5g56100 and five Arabidopsis paralogs with a single spinach protein, Spo12707. However, the similarity significance score of At5g56100 and Spo12707 (BlastP E-value; $1.48e^{-03}$) is above $1e^{-05}$ and their potential orthology should thus be considered with caution (**Fig. S1**). For LSU1 the protein tree did not improve our understanding of orthology to spinach because of the unresolved branches. The best BlastP hit, Spo22402, was therefore included as the ortholog. Both Arabidopsis Prenylated Rab acceptor 1 interactors, PRA1.F2 and PRA1.F3, had the same spinach protein, Spo20420, as a reciprocal best hit. In total, 13 spinach proteins, referred to as “protein orthologs”, were added to the 14 Arabidopsis interactors for further analysis. The protein alignments between the Arabidopsis and spinach orthologs are shown in **Fig. S2**.

Table 1. Spinach protein orthologs or best BlastP hits of Arabidopsis effector-interactors.

Arabidopsis		Best BlastP hit		Reciprocal Best BlastP hit		Reciprocal best hit
effector-interactor	At-code	Spinach	E-value	Arabidopsis	E-value	
NAC014	At1g33060	Spo22390	3.85e ⁻⁸⁸	At1g33060	7.51e ⁻⁸⁸	Yes
PRA1.F2	At1g55190	Spo20420	1.50e ⁻⁶⁰	At1g55190	1.06e ⁻⁶¹	Yes
USPA	At3g11930	Spo22595	8.65e ⁻⁶⁵	At3g11930	5.69e ⁻⁶⁶	Yes
PRA1.F3	At3g13720	Spo20420	3.10e ⁻⁶²	At3g13720	1.10e ⁻⁵⁵	Yes
AT3G13800	At3g13800	Spo20405	0	At3g13800	0	Yes
AHAS	At3t48560	Spo20077	0	At3g48560	0	Yes
NAGK	At3g57560	Spo15075	5.17e ⁻¹⁵²	At3g57560	1.03e ⁻¹⁵¹	Yes
FBN1a	At4g04020	Spo20112	2.96e ⁻¹²⁷	At4g04020	4.39e ⁻¹²⁷	Yes
NAC078	At5g04410	Spo16524	3.86e ⁻¹²⁸	At5g04410	1.85e ⁻¹²³	Yes
RAF2	At5g51110	Spo14154	4.10e ⁻⁶⁵	At5g51110	7.89e ⁻⁶⁹	Yes
SR-rich	At2g19390	Spo24468	7.11e ⁻¹²¹	At5g22450	4.59e ⁻¹⁶⁷	No
LSU1	At3g49580	Spo22402	3.22e ⁻¹⁰	At5g24655	1.33e ⁻¹⁸	No
Glycin rich	At5g56100	Spo12707	1.48e ⁻⁰³	At4g25140	4.09e ⁻⁵²	No
HBI1	At2g18300	Spo06524	1.75e ⁻⁵²	At4g34530	8.32e ⁻⁶⁴	No

4 Subcellular localization of candidate *Pfs* host-translocated effectors and their interactors in planta

The coding sequence of the candidate host-translocated effectors (without signal peptide), Arabidopsis interactors and spinach orthologs were cloned into plant expression vectors for making fusions to fluorescent proteins (FPs). Effectors were fused to yellow fluorescent protein (YFP) and the interacting plant proteins to cyan fluorescent protein (CFP) at their N-terminal unless otherwise indicated. The fusion proteins were produced in *Nicotiana benthamiana* by transient *Agrobacterium*-mediated expression to examine the subcellular localization of the proteins. Originally, we attempted to produce the fusion proteins in spinach leaves, but found that the level of expression was much lower compared to *N. benthamiana*, and in most cases insufficient for microscopic analysis of

subcellular localization (**Fig. S3**). Hence, the gene fusion proteins were visualized in *N. benthamiana* leaves.

Six candidate host-translocated effectors were found to localize in punctate structures (PS) (**Fig. 1**). Two of the six PS-localized effector fusions, Pfs1|1800 and Pfs1|3869, were also detected in the nucleus and cytoplasm, and one (Pfs1|3172) also in the endoplasmic reticulum (ER). PS in plant cells could represent structures like Golgi associated vesicles, lipid bodies [242], autophagosomes [243] or vacuoles [244]. The PS to which the candidate host-translocated effectors localize are very similar in size and shape to Golgi vesicles, as demonstrated by their resemblance to the Golgi marker, soybean α -1,2-mannosidase I [245, 246] (**Table 2**). However, we cannot be entirely sure as PS are also observed in mitochondria and chloroplasts as demonstrated by the localization of the yeast cytochrome oxidase subunit IV (ScCOX41–29-GFP) that serves as a mitochondrial marker in *N. benthamiana* [247].

Of the eight tested effector fusion proteins, two localized elsewhere. Pfs1|10763 was located solely and strongly in large nuclear speckles, while Pfs1|9071 was visible in the nucleus and cytoplasm (**Fig. 1**). The experimentally-determined localization of the fusion proteins was compared to the predicted effector localization as assigned by the machine learning algorithm LOCALIZER, that has a specific configuration for effector proteins and by the deep learning algorithm Deeploc. Surprisingly, the predicted and experimental localization matched only for Pfs1|3172 that localizes to Golgi-like PS (**Table 2**).

Figure 1. Subcellular localization of eight candidate *Pfs1* effectors in *N. benthamiana*.

Pfs1 effectors fused to YFP (N-terminal) were produced in *N. benthamiana* leaves by *Agrobacterium*-mediated transient expression. Protein localization is indicated in the figure. Images were taken ~48 hours after infiltration. Images are representative for $n \geq 2$ observations, except for Pfs1|6786 (5 cells) and Pfs1|3869 (3 cells) that were only imaged once. Scale bars = 20 μm .

Table 2. Experimental and predicted subcellular localization of N-terminal YFP fluorescent protein fusions of individually expressed candidate effector proteins of *Pfs1* in *N. benthamiana*

Effector	Subcellular localization	LOCALIZER	Deeploc
Pfs1 9071	nucleo-cytoplasmic	-	mitochondrion (soluble)
Pfs1 9976	punctate structures (PS)	nucleus	cytoplasm (soluble)
Pfs1 1800	PS, nucleo-cytoplasmic	-	plastid (membrane)
Pfs1 10763	large nuclear speckles	nucleus	extracellular (soluble)
Pfs1 6786	PS	-	mitochondrion (membrane)
Pfs1 3172	ER	-	Golgi (membrane)
Pfs1 3869	PS, nucleo-cytoplasmic	-	extracellular (soluble)
Pfs1 7215	PS	-	extracellular (soluble)

LOCALIZER predicts the localization of effector proteins to chloroplast, mitochondria (transit peptide) and nucleus (nuclear localization signal (NLS)). For effectors, it does not consider the first 20 amino acids. Note that effector protein sequences with the signal peptide sequences removed were used for Deeploc predictions.

Similarly, the Arabidopsis interactors and their spinach orthologs were each individually expressed in *N. benthamiana* in the absence of the corresponding effector protein. The observed localization was compared to subcellular localization data retrieved from SUBA4 (Table 3) [248]. The SUBA consensus (SUBAcon) prediction is the localization retrieved from the combined predicted and experimentally observed (if available) subcellular localizations. The data from the SUBA4 experimental database are separately shown. For six of the fifteen Arabidopsis proteins the observed and reported localization overlapped, but for eight interactors a different localization was observed. For NAC078, (NO APICAL MERISTEM (NAM), *ARABIDOPSIS THALIANA* TRANSCRIPTION ACTIVATION FACTOR (ATAF1/2) and CUP-SHAPED COTYLEDON (CUC2) 078, At5g04410) we were not able to determine the localization, as the fusion protein could not be detected.

Nine out of fourteen Arabidopsis interactors were found to localize in PS in the imaged *N. benthamiana* cells (Fig. S4, Table 3). All PS-localized interactors were also found in other subcellular compartments, like chloroplasts (1x), the ER (4x), the ER, nucleus and cytoplasm (1x), the

ER and nucleus (1x) the ER and cytoplasm (1x), and nucleus (1x). Two interactors, NAC014 (At1g33060) and HBI1 (At2g18300) were observed in nuclear speckles. Nuclear speckles are visible as dot-like structures within the nucleoplasm [249]. Both interactors were hard to observe as their protein levels were low if present at all, suggesting that the fusion proteins may be unstable, or their cellular levels strongly regulated. Universal stress protein A (USPA) (At3g11930) was found to be soluble and was observed in the cytoplasm and nucleus. Acetohydroxyacid synthase (AHAS, At3g48560) was observed in plastids.

Ten of the fourteen tested spinach fusion proteins localized at least partially to the same cell compartments as their Arabidopsis orthologs. Three could not be compared because the spinach (3x) or Arabidopsis (1x) proteins could not be visualized due to low intensity of the fluorescent signal upon expression of the fusion proteins. For a single Arabidopsis-spinach pair, the protein localizations were remarkably different: the Arabidopsis AHAS fusion protein was observed in chloroplasts and PS while its ortholog Spo20077 localized to the PS only.

Table 3. Subcellular localization of individually expressed Arabidopsis and spinach interactors fused to CFP and expressed in *N. benthamiana*

Arabidopsis		Experimental localization	SUBAcon prediction	SUBA4 experimental localization
Gene code	Name			
At1g33060	NAC014	(low expression), nuclear speckles	nucleus	
At1g55190	PRA1.F2	ER, PS	vacuole	Golgi, vacuole
At2g18300	HBI1	(low expression), nuclear speckles	nucleus	
At2g19390	S-R rich	cytoplasm, nuclear speckles, ER (not shown)	nucleus	
At3g11930	USPA	cytoplasm	nucleus	cytoplasm, extracellular, nucleus, pl. membr.
At3g13720	PRA1.F3	PS, ER	ER	cytoplasm
At3g13800	AT3G13800	ER, PS	plastid	mitochondrion
At3g48560	AHAS	chloroplast, PS	plastid	Golgi, plastid
At3g49580	LSU1	PS, ER, cytoplasm	nucleus	
At3g57560	NAGK	PS, ER, nucleus	plastid	extracellular, plastid
At4g04020	FBN1a	PS, nucleio-cytoplasm	plastid	Golgi, mitochondrion, nucleus, plastid
At5g04410	NAC078	ND	nucleus	
At5g1110	RAF2	nucleus	plastid	plastid
At5g56100	Glycin rich	ER, PS	vacuole	

Table 3. continued

Spinach	At ortholog	Overlap between orthologs
Spo22390	AT1G33060	ND
Spo20420*	AT1G55190	ER, PS, nucleus, cytoplasm
Spo06524	AT2G18300	nuclear speckles, nucleolus
Spo24468	AT2G19390	PS, nucleus
Spo22595	AT3G11930	cytoplasm
Spo20405	AT3G13800	PS
Spo20077	AT3G48560	PS, ER, cytoplasm
Spo22402	AT3G49580	PS, cytoplasm
Spo15075	AT3G57560	ND
Spo20112	AT4G04020	PS, ER, nucleoplasm
Spo16524	AT5G04410	ND
Spo14154	AT5G51110	cytoplasm, nucleus
Spo12707	AT5G56100	PS, ER, cytoplasm

The fluorescent protein was fused to the C-terminal of At3g48560 and Spo2077. Spo20420* is the best protein ortholog of PRA1.F2 and PRA1.F3.

Co-localization of candidate effectors and their interactors

The results above describe the subcellular localization of the individual candidate effectors and their interactors. It is important to determine the subcellular localization of effector and corresponding interactor proteins when they are co-expressed in the same cells. When such co-localization experiments demonstrate the presence of both interaction partners in the same subcellular compartment, it supports their proposed interaction. Also, co-expression may alter the localization or protein stability of one or either interaction partners. We observed that all but two of the Arabidopsis and all spinach interactor fusions tested localized, at least partially, to the same subcellular compartment as the corresponding candidate host-translocated effector (**Table 4**). The two exceptions, PRA1.F2 (At1g55190) and PRA1.F3 (At3g13720) localize to the ER and PS, whereas the candidate effector Pfs1|9071 is observed in the nucleus. The observation that these proteins do not co-localize, and their localization is not altered by co-expression suggests that PRA1.F2/F3 and Pfs1|9071 do not interact *in planta* and could thus be considered false positive Y2H interactors. Interestingly, the localization of the ortholog of PRA1.F2 and PRA1.F3 in spinach, Spo20420, was in the ER like PRA1.F2/F3, but also in the cytoplasm. Therefore, the spinach ortholog co-localized with effector Pfs1|9071 in the cytoplasm. The presence of intact Spo20420 fused to CFP was confirmed by Western blot detection, indicating that the cytoplasmic signal is not derived from free CFP resulting from fusion protein break-down (**Table S1**).

Table 4. Subcellular localization of Pfs1 candidate effectors and Arabidopsis + spinach interactors upon co-expression in *N. benthamiana*.

Arabidopsis target		Effector	Localization Interactor	Localization Effector	co-localization
At1g33060	NAC014	Pfs1 3172	Nucleus, nucleolus, cytoplasm, PS	nucleus, nucleolus, cytoplasm, PS, ER	yes
At1g55190	PRA1.F2	Pfs1 9071	ER	nucleoplasm	no
At2g18300	HBI1	Pfs1 6786	nucleoplasm, nuclear speckles	nucleoplasm, nuclear speckles	yes
At2g19390	S-R rich	Pfs1 1800	Nucleo-cytoplasm, nucleolus	nucleoplasm, cytoplasm, ER	yes
At3g11930	USPA	Pfs1 10763	nucleus, cytoplasm	nucleus	yes
At3g13720	PRA1.F3	Pfs1 9071	PS, ER	nucleus	no
At3g13800	AT3G13800	Pfs1 3172	ER, PS	ER, PS	yes
At3g48560	AHAS	Pfs1 6786	nuclear speckles, PS, chloroplast	nuclear speckles, PS	yes
At3g49580	LSU1	Pfs1 7215	nucleo-cytoplasm, PS	nucleo-cytoplasm, PS	yes
At3g57560	NAGK	Pfs1 3869	PS, nuclear membrane	PS, nucleus-cytoplasm	yes
At4g04020	FBN1a	Pfs1 9976	PS, nucleoplasm	PS, nucleoplasm	yes
At5g04410	NAC078	Pfs1 3172	large PS, cytoplasm nucleus	PS, ER	yes
At5g51110	RAF2	Pfs1 6786	Nucleo-cytoplasm, nucl. speckles, PS	nucleus, nuclear speckles, PS	yes
At5g56100	Glycin rich	Pfs1 9071	ER, PS, cytoplasm	ER, PS, nucleo-cytoplasm	yes

Table 4. continued

Spinach target						
Spo22390	At1G33060	Pfs1 3172	nucleolus, PS, nuclear speckles	nuclear membrane, PS, ER	yes	yes
Spo20420*	At1g55190	Pfs1 9071	cytoplasm	cytoplasm, nucleoplasm	yes	yes
Spo06524	At2g18300	Pfs1 6786	nuclear speckles and PS	nuclear speckles and PS	yes	yes
Spo24468	At2g19390	Pfs1 1800	PS, nucleus	PS, nucleus, ER	yes	yes
Spo22595	At3g11930	Pfs1 10763	Nucleoplasm, nuclear speckles	nucleoplasm	yes	yes
Spo20405	At3g13800	Pfs1 3172	PS	PS, ER	yes	yes
Spo20077	At3g48560	Pfs1 6786	PS	PS	yes	yes
Spo22402	At3g49580	Pfs1 7215	PS	PS	yes	yes
Spo15075	At3g57560	Pfs1 3869	PS, nuclear speckles	PS, nucleus, cytoplasm or ER	yes	yes
Spo20112	At4g04020	Pfs1 9976	nucleoplasm, PS, nuclear membrane	nucleus	yes	yes
Spo16524	At5g04410	Pfs1 3172	large PS, cytoplasm	Large PS, cytoplasm	yes	yes
Spo14154	At5g51110	Pfs1 6786	PS, cytoplasm	PS, cytoplasm	yes	yes
Spo12707	At5g56100	Pfs1 9071	ER, PS	ER, PS, nucleoplasm	yes	yes

* Spo20420 is the protein ortholog of both PRA1.F2 and PRA1.F3.

Stabilization of interacting proteins upon effector co-expression

Three Arabidopsis (NAC014, NAC078 and HBI1) and three spinach fusion proteins (NAC04, NAC078 and NAGK-ortholog Spo15075) were barely visible by confocal microscopy when individually expressed in *N. benthamiana*. The fluorescent signal was generally low, and if fluorescence was observed, it was often concentrated in large (as opposed to the small PS) cytoplasmic dots. The origin of the large dots is unclear but they resemble the subcellular localization of proteasome core particle subunit PAG1 [250] which could suggest that the proteins are targeted for degradation by the proteasome.

Surprisingly, all of these unstable interactors could be successfully imaged when co-expressed with their cognate candidate host-translocated effector. The fluorescent signals of the effector co-infiltrated plant fusion proteins were more abundant within the transformed cells compared to the signals of the individual interactors.

Protein stabilization after co-expression with the candidate host-translocated effector Pfs1|3869 was observed for spinach protein Spo15075. When co-expressed the Spo15075 fusion protein was clearly visible in PS, nucleoplasm and nuclear speckles, while individually it was only observed in large fluorescent dots (**Fig. 2**). The presence of the candidate effector appears to stabilize the interactor signal. Co-localization of the interactor and candidate effector occurred in the PS and the nucleoplasm but not in the nuclear speckles. For the Arabidopsis ortholog ACETYLGLUTAMATE KINASE (NAGK, AT3G57560) there was no difference if it was expressed individually or together with the candidate effector; in both cases it localized to the PS, the nucleoplasm and possibly the ER.

Figure 2. Co-localization of Arabidopsis NAGK (At3g57560) and its protein ortholog Spo15075 with candidate effector Pfs1|3869 in *N. benthamiana*.

Plant interactors fused to CFP and candidate effectors fused to YFP (N-terminus) were transiently expressed in *N. benthamiana*. The Spo15075 fusion protein was observed in large cytoplasmic speckles when expressed independent of effector Pfs1|3869 but was clearly visible in PS, nucleoplasm and nuclear speckles upon co-expression. Subcellular localization and intensity of the Arabidopsis NAGK fusion protein was not altered upon co-expression with the candidate effector. Images were taken ~72 hours after infiltration and are representative of >8 cells. Scale bars = 20 μ m.

4

Another example of stabilization of interactor proteins is for the NAC transcription factors NAC014 and NAC078. Despite numerous attempts, no clear localization was found for NAC014 and NAC078 nor for the corresponding orthologs Spo22390 and Spo14154, respectively, due to low intensity of the signal. In our studies, NAC014 and Spo14154 (NAC078 ortholog) could be weakly detected in nuclear speckles in one sample (**Fig. 3**) but this was not reproduced. When co-expressed with Pfs1|3172, the interactor fusions could be visualized (**Fig. 3**). NAC014 was observed in the nucleus (nuclear membrane and nucleolus),

cytoplasm, ER and PS. Co-localization with Pfs1|3172 was observed in all compartments. The spinach NAC014 ortholog, Spo22390, was primarily observed in PS that clearly overlay the ER, and in the cytoplasm. Co-localization with the effector was observed in the cytoplasm. NAC078 localized in the nucleus and cytoplasm, PS and in large cytoplasmic speckles. Pfs1|3172 co-localized with NAC078 in the large cytoplasmic speckles, nucleus and cytoplasm. The effector was clearly localized in the ER together with a weak NAC078 signal. The localization of the NAC078 ortholog, Spo16524, resembled that of NAC078 but with a more pronounced ER localization.

In a study in lettuce (*Lactuca sativa*), it was previously shown that NAC transcription factors interact with host-translocated effectors of the downy mildew pathogen *Bremia lactucae*. It was found that a truncated fusion protein of LsNAC069, lacking the NAC domain, was more stable compared to the full length protein [169]. Therefore, a truncated version of Arabidopsis NAC014 expressed as a CFP-tagged fusion protein was expressed in *N. benthamiana*. All NAC protein fragments that interacted with Pfs1|3172 in the Y2H assays lacked the NAC domain and showed a strong interaction with the effector so the NAC domain is not required for that (**Chapter 3**). The truncated protein fusion was, indeed, more stable and showed a strong signal in the ER, cytoplasm and PS when individually expressed (**Fig. 4**). Also, the presence of the truncated but not the full-length protein could be confirmed by western blot analysis using an antibody against the FP (**Table S1**). When co-expressed with effector Pfs|3172 co-localization was observed in the ER, PS and potentially the nucleus.

Figure 3. Stabilization of NAC transcription factor fusions by co-expression with candidate effector Pfs1|3172 in *N. benthamiana*.

Plant interactors fused to CFP and candidate effectors fused to YFP (N-terminus) were transiently expressed in *N. benthamiana*. The subcellular localization of both Arabidopsis and spinach NAC transcription factors was observed at higher intensity when co-expressed with candidate effector Pfs1|3172. Images were taken ~48 hours after infiltration. Images are representative of >8. Scale bars = 20 μ m.

Figure 4. Subcellular localization of individually and co-expressed full length and truncated NAC014 (At1g033060) proteins in *N. benthamiana*.

Plant interactors fused to CFP and candidate effectors fused to YFP (N-terminus) were transiently expressed in *N. benthamiana*. (A) The signal of the truncated protein fusion is stronger compared to the full length one. (B) Co-localization of the truncated NAC014 and *Pfs1* candidate effector Pfs1|3172 is observed in the ER, PS, and potentially in the nucleus. Images were taken ~48 hours after infiltration and are representative of 3 cells (NAC014, 2 technical replicates) 10 cells (truncated NAC014), 4 cells (co-expression). Scale bars = 20 μ m.

Co-expression of effector and interactor candidates results in re-localization of proteins

RxLR effector candidate Pfs1|6786 was found to interact with multiple *Arabidopsis* proteins in the Y2H screen (**Chapter 3**). Interestingly, the co-expression studies showed that the presence of the effector alters subcellular localization of the *Arabidopsis* AHAS and spinach Spo6524 (HBI1 ortholog) proteins. Moreover, the subcellular localization of the candidate effector itself changed upon co-expression with the interactors. The effector fusion protein predominantly localized in PS when expressed individually and shifted to a predominantly nuclear localization when co-expressed with AHAS (At3g48560), HBI1, Spo06524, and Rubisco Assembly Factor 2 (RAF2, At5g51110) (**Fig. 5**). Within the nucleus, the candidate effector was observed in the nucleoplasm, and in nuclear speckles, but remained visible in PS as well (**Fig. 5**).

Pfs1|6786 interactors AHAS and Spo6524 (HBI1 ortholog), changed localization upon co-expression as well. The signal of AHAS shifted from plastids and PS when expressed individually to nuclear speckles, PS and plastids when co-expressed with Pfs1|6786. The spinach ortholog (Spo20077) did not clearly localize to the plastids nor changed localization when co-expressed with Pfs1|6786. Nevertheless, Spo20077 co-localized with Pfs1|6786 to the PS. Presently, it is not clear whether the observed PS were located in the cytoplasm, plastid, mitochondria or the ER-Golgi system.

In a first experiment, Spo06524 (HBI1 ortholog) localized to plant nuclear speckles and nucleolus when expressed individually and shifted to PS and nuclear speckles when co-expressed with Pfs1|6786. Imaging the *Arabidopsis* HBI1 fusion protein was particularly difficult due to a low fluorescent signal, resulting in the observation of only four transformed cells in five independent replicates. Co-expression of Pfs1|6786 and HBI1 seemed to stabilize HBI1 and alter the subcellular localization of HBI1 ortholog Spo06524. The HBI1 fusion protein was observed in nuclear speckles, both in the individual and co-expression assays. Co-

expression resulted in relocalization of the predominantly PS-localized Pfs1|6786 effector to the nucleus. Co-expression of effector candidate and interactors seemed to stabilize HBI1 and alter the subcellular localization of the HBI ortholog Spo06524.

The final interactor that altered the subcellular localization of Pfs1|6786, RAF2, showed a strong nuclear signal in individual- and in co-expression experiments. However, cells that showed PS localization of RAF2 and Pfs1|6786 and no nuclear signal from Pfs1|6786 upon co-expression were observed as well. The ortholog Spo14154 predominantly localized to the nucleus when expressed individually, but co-expression with Pfs1|6786 resulted in a PS signal and large fluorescent speckles in the cytoplasm. Both for RAF2 as for Spo14154, the subcellular localization of the fluorescent signal was unstable, and the obtained results did not allow to draw a conclusion about their localization and potential re-localization. Remarkably, both effector Pfs1|6786 and RAF2 were strongly concentrated in the nucleolus and nuclear speckles when co-expressed. The concentration of the fluorescent signals in nuclei in the Spo14154 co-localization and the RAF2/Spo14154 individual localization experiments was too strong to observe the subnuclear localization of the proteins (**Fig. 5**).

Figure 5. The subcellular localization of candidate effector Pfs1|6786 and four out of six of its candidate interactor proteins changed upon co-expression in *N. benthamiana* cells.

The subcellular localization of candidate effector Pfs1|6786 and its three tested Arabidopsis interactors (A) and their spinach orthologs (B) are shown. The subcellular localization of Pfs1|6786 changed from PS to the nucleus (nucleoplasm, nucleolus and nuclear speckles) upon co-expression with AHAS, HBI1 and RAF2 (a, dashed line). The subcellular localization of AHAS (A), Spo06524 (b) and possibly RAF2 (A) and Spo14154 (B) were altered upon co-expression. The expression of HBI was stabilized upon co-expression. The co-expression experiments are representative of 2 cells (HBI1), 7 cells (Spo06524), 8 cells (Spo14154). Images were taken ~48 hours post infiltration. Scale bars = 20 μ m.

Co-expression of candidate RxLR effector Pfs1|10763 with its interactors USPA and ortholog Spo22595 induced re-localization and stabilization of the plant proteins (**Fig. 6**). During individual expression, both plant proteins were visible in the cytoplasm. The signal of Spo22595 could only be observed in a few cells. USPA was occasionally visible in the nucleoplasm. USP and Spo22595 are small ~ 25 and 23 kDa proteins. Even when fused to the 27 kDa CFP protein, they might passively move through the nuclear pores into the nucleus [251, 252]. Upon co-expression with Pfs1|10763, both interactors showed a very strong nuclear signal. The Arabidopsis interactor still occurred in the cytoplasm, whereas the spinach ortholog was only visible in the nucleus.

Figure 6. Nuclear co-localization of Arabidopsis USPA (At3g11930) and its protein ortholog Spo22595 with candidate effector Pfs1|10763 in *N. benthamiana*.

Plant interactors fused to CFP and candidate effectors fused to YFP (N-terminus) were transiently expressed in *N. benthamiana*. The expression of USP and Spo22595, shifted from cytoplasm to nuclear upon co-expression with Pfs1|10763. Images were taken ~48 hours after infiltration and are representative of >4 cells for the individual samples. Scale bars = 20 μ m.

Re-localization of both the interactor and candidate host-translocated effector protein was observed upon co-expression of candidate CRN effector Pfs1|9976 and Fibrillin 1a (FBN1a) (AT4g04020). The FBN1a and Pfs1|9976 fusion proteins re-localized from cytoplasm to the nucleoplasm, whereas localization to PS was maintained (**Fig. 7**). Co-expression of Pfs1|9976 and FBN1a directed the proteins towards the nucleus. Co-expression of FBN1a ortholog Spo20112 and Pfs1|9976 resulted in relocation of the candidate effector but not the interactor to the nucleus. Spo20112 was observed in PS, ER and nucleus so it co-localized with Pfs1|9976 in the nucleus.

Figure 7. Co-localization of Arabidopsis FBN1a (At4g04020) and its protein ortholog Spo15075 with candidate effector Pfs1|9971 in *N. benthamiana*.

Plant interactors fused to CFP and candidate effectors fused to YFP (N-terminus) were transiently expressed in *N. benthamiana*. The expression of FBN1a, not Spo15075, shifted from PS and cytoplasm to PS and nucleus upon co-expression with Pfs1|9971. Pfs1|9971 re-localized from PS to the nucleoplasm upon co-expression with both FBN1a and Spo15075. Images were taken ~72 hours after infiltration and are representative of >5 cells. Scale bars = 20 μ m.

Finally, the SR-rich protein (At2g19390) showed a subnuclear re-localization from the nuclear speckles to the nucleoplasm where it co-localized with the RxLR effector Pfs1|1800 (**Fig. 8**). The ortholog Spo24468 was also observed in the nucleus when expressed individually.

Figure 8. Co-localization of Arabidopsis SR-rich protein (At2g19390) and its protein ortholog Spo24468 with candidate effector Pfs1|1800 in *N. benthamiana*.

Plant interactors fused to CFP and candidate effectors fused to YFP (N-terminus) were transiently expressed in *N. benthamiana*. The expression of At2g19390, not Spo24468, shifted from nuclear speckles and cytoplasm to nucleo-cytoplasm upon co-expression with Pfs1|1800. Images were taken ~48 hours after infiltration and are representative of >5 cells. Scale bars = 20 μ m.

The interactor proteins that did not stabilize or re-localize upon co-expression with the candidate host-translocated effectors are summarized in **Fig. S5**. LSU1 and its ortholog Spo22402 co-localized with Pfs1|7215 in the nucleus and cytoplasm and PS. At5g56100 and Spo12707 co-localized in the ER and PS and also in the nucleus and cytoplasm with Pfs1|7215. The co-localization of candidate effector and interactor support their putative interaction *in planta*.

Discussion

Oomycete phytopathogens translocate effectors into plant cells to establish infection [56, 65]. Inside the plant cells, RxLR and CRN proteins interact with plant proteins and other components to manipulate plant processes, like immune signaling, metabolism and protein transport to the benefit of the oomycete [41, 42, 43]. Recently, it was demonstrated that delivery of RxLR effector Pi04314, an RxLR effector of *P. infestans* was independent of the conventional ER-to Golgi secretion system and that the RxLR motif was required for translocation [69]. CRN effectors translocation is dependent on the N-terminal LFLAK domain but the exact mechanism of translocation is unknown [56]. Within plant cells, host-translocated effectors target different subcellular compartments where they interfere with plant processes. Some effectors contain sorting signals like nuclear localization signals or transit peptide-like sequences that target the protein to different organelles within the plant cell [56, 253, 254, 255]. Other effectors lack these sorting signal and possibly piggy-back on interacting host proteins that are targeted to specific compartments.

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We visualized the subcellular localization of the first eight candidate host-translocated effectors of *Pfs1* by detecting fluorescent effector-fusion proteins in *N. benthamiana* epidermal cells using confocal microscopy. RxLR effectors Pfs1|3869, Pfs1|1800, Pfs1|10763 and CRN effector Pfs1|9071 localized to the nucleus or showed a nucleocytoplasmic localization. Studies in other species showed that the nucleus and cytoplasm are common sites of effector function. All CRNs studied so far and the majority of RxLR effectors of other oomycete species localized in the nucleus or cytoplasm [56, 240, 256]. A multitude of subnuclear localizations are reported for both RxLR and CRN effectors, something we also observe in our small subset of effectors. In the individual subcellular localization experiments we found Pfs1|3869, Pfs1|1800 and Pfs1|10763 in the nucleoplasm and Pfs1|10763 in large nuclear speckles. Additionally, in co-expression experiments, candidate effectors were observed in small nuclear speckles (Pfs1|6786), the nuclear membrane and nucleolus (Pfs1|3172). The other effectors localized to the ER (Pfs1|3172) and

PS (Pfs1|6786 and Pfs1|7215, Pfs1|9976). Co-expression of the fusion proteins with molecular markers for ER, Golgi, plastoglobuli, lipid bodies, autophagosomes or vacuoles should be performed to confirm the origin of the PS. Remarkably, candidate host-translocated CRN effector Pfs1|9976 did only localize to the nucleus when co-expressed with FBN1a, but not in experiments expressing only the effector - where it was observed in PS.

The subcellular localization observed for seven out of eight of the effectors did not match the predicted DeepLoc localizations. The DeepLoc predictions are based on a neural network-trained on experimentally verified eukaryotic proteins derived from Uniprot. The prediction does not explicitly take into account known sorting signals like signal or transit peptides. In studies of other oomycete species, poor prediction of subcellular localization was also encountered for host-translocated effectors [148]. The latter is addressed by LOCALIZER, a machine learning method that uses a sliding window approach to predict NLS, and chloroplast/mitochondrial transit peptides [257]. As opposed to other prediction methods like TargetP [258], LOCALIZER does not assume that a transit peptides starts at the first residue after the signal peptide. This likely improves the prediction because it was shown that signal and transit peptides are in some cases separated by so-called ‘pro-domains’ of varying length [257, 259]. LOCALIZER predicted nuclear localization for Pfs1|9976 and nuclear and chloroplast localization for Pfs1|10763, which is not in line with the observed localization in the individual expression experiments. However, Pfs1|9976 relocalized to the nucleus upon co-expression with FBN1a. The prediction of localization of effector proteins is likely complicated by translocation from one organism to another and the ability of the usually small effector proteins to co-migrate through the cells with interacting proteins. Additionally, the signal peptide is likely removed during translocation and it is unknown whether the N-terminus is further processed during transport [260].

Within plant cells, host-translocated effectors interact with plant proteins to manipulate host processes to the benefit of the oomycete. A prerequisite for interaction is that the effector and interactor reside in

the same subcellular compartment. We found that twelve of the fourteen *Arabidopsis* and all of the thirteen spinach orthologs localized at least partially to the same subcellular compartments as the effector that was used as bait to identify them in the Y2H screen, supporting their proposed interaction (**Chapter 3**). Interestingly, the fluorescent signal of interactor-fusion proteins that were barely visible upon individual transient expression, stabilized upon co-expression with the candidate effector. This suggests that the presence of, or interaction with the effector stabilizes the plant proteins. Additionally, some interactor proteins and two candidate host-translocated effectors (Pfs1|6786, Pfs1|9976) showed a different subcellular localization in the co-expression compared to individual expression experiments.

It is tempting to speculate and hypothesize on the putative processes that are affected by the *Pfs* effectors. The plant proteins targeted by the candidate host-translocated *Pfs1* effectors can be roughly divided into three functional classes, based on their alleged molecular function. The functional classes are; cellular programming, metabolic homeostasis and “other” or “unknown” for proteins that have no clear possible link to immunity. Manipulation of cellular programming can be accomplished at different levels; directly through manipulation of transcription or posttranscriptional processes or indirectly through manipulation of signaling pathways, e.g. in hormonal or immune signaling (**Chapter 1**). Below, a number of effector interactions and their possible impact on cellular processes are freely discussed (sometimes providing speculative ideas).

Cellular programming – manipulation of hormone and immune signaling

Candidate CRN effector Pfs1|9976 was found to interact with FBN1a, a fibrillin protein that is found in plastids, primarily in lipid particles called plastoglobuli [261]. Plastoglobuli have a membrane lipid monolayer and contain lipids and proteins [262]. Their exact biological function is unclear, but they were observed to grow during developmental processes

and stress responses [262]. Plastids, particularly chloroplasts are sites of synthesis of phytohormones (jasmonic and salicylic acid), defense compounds, reactive oxygen species and nitric oxide and thus play an important role in plant immunity [263].

We observed the FBN1a fusion protein in PS that possibly represent plastoglobuli. The spinach ortholog of FBN1a was also observed in the ER and nucleus. Localization of fibrillin in the nucleus was reported before (**Table 1**). *FBN1a* orthologs in species other than *Arabidopsis* were found to be differentially expressed upon pathogen invasion. *FBN1a* orthologs were upregulated upon infection with obligate biotrophic as well as necrotic fungal and bacterial pathogens in cucumber, tomato and grape [264, 265]. RNA silencing of *FBN1a* ortholog *LeCHRC* (*Lycopersicon esculentum* carotenoid-associated protein) in tomato plants increased susceptibility to the necrotrophic fungus *Botrytis cinerea*, while RNAi lines overexpression *LeCHRC* (sense) were more resistant to infection [264]. FBN1a can be considered a common effector target as it was found to interact with effectors from bacterial, fungal and oomycete species (**Chapter 3**, [219]). Interestingly, a role for FBN1a was implicated in jasmonic acid biosynthesis. The application of exogenous methyl jasmonate was shown to abolish the effect of knock-down of *FBN1-2*. Also, *FBN1a* was found to co-localize with the biosynthetic enzyme allene oxide synthase in plastoglobules [266]. Jasmonic acid antagonizes salicylic acid-mediated immune responses against biotrophic pathogens [267]. We observed re-localization of FBN1a and Pfs1|9971 from PS to the nucleoplasm upon co-expression with candidate host-translocated CRN effector Pfs1|9971. Interestingly, the effector nor de interactor were observed in the nucleus when expressed individually. Given the published information, a re-localization of FBN1a away from plastoglobuli does not provide a clear benefit to *Pfs*. If FBN1a positively regulates jasmonic acid synthesis than re-location results in lower levels of the hormone. Additional factors like timing, co-expression with other effectors and cell type targeting may affect the outcome of binding of FBN1a by Pfs1|9971. Alternatively, translocation of proteins from chloroplast to the nucleus

is observed during retrograde signaling. Chloroplasts may function as environmental sensors that mediate stress, like pathogen infection, by signaling that leads to alternative regulation of genes [263]. However, other than translocation of FBN1a to the nucleus upon co-expression with CRN effector Pfs1|9976 there is no indication of such a role for FBN1a.

Candidate host-translocated RxLR effector Pfs1|7215 interacts with LSU1. As its name implies, LSU1 proteins are upregulated in response to S-deficiency [268]. Mutant *lsu1* plants produce reduced amounts of reactive oxygen species (ROS) resulting in impaired stomatal closure in response to low sulphur conditions [269]. LSU1 is an effector target of evolutionary diverse pathogens, suggesting that the protein has a yet unknown role in plant immunity [206, 219]. Possibly effectors manipulate LSU1 to alter ROS production, and hence the redox state of the cell, which serves as a stress signal and plays an activating role in plant immunity [270]. Plastid localized bacterial effectors were shown to sabotage the activating interaction between LSU1 and the superoxide dismutase FSD2, hampering the conversion of O_2 to H_2O_2 . *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 infection resulted in reduced chloroplast localization of LSU1 during low sulfur (S) stress. Plants that lacked *LSU1* expression showed a moderately enhanced susceptibility to *Pst* under low S-stress but not under standard conditions [269]. We observed the LSU1 fusion protein primarily in the cytoplasm and in PS. Spinach ortholog Sp022403 was only observed in PS. Both proteins co-localized with Pfs1|7215 in PS and LSU1 also in the nucleo-cytoplasm. The co-localization between Pfs1|7215 and LSU1 suggests that *Pfs*, through effector Pfs1|7215, could interfere with ROS production and stomatal closure as well.

Cellular programming – transcriptional manipulation

Pfs1|3172 is a candidate RxLR effector that has a C-terminal transmembrane domain and interacted with two membrane-bound NAC transcription factors in the Y2H screens. The transcription factors have a highly conserved N-terminal DNA-binding NAC domain and a more variable C-terminal transcriptional regulation domain [271]. More than 100 NAC transcription factor genes are present in the genome of Arabidopsis. At least nine of them have been linked to different aspects of immunity, like hormone signaling and stomatal closure [272]. The identification of AtNAC014 and AtNAC078 as interactors of Pfs1|3172 may add these two candidates to this list. The transmembrane domain anchors the transcription factors to the plasma- or ER-membrane rendering it inactive as a transcription factor [273]. The membrane-bound NACs are proteolytically cleaved upon developmental and environmental cues, enabling the NAC transcription factor to move into the nucleus to regulate gene transcription [86, 169].

We observed co-localization of the host-translocated effector and the (truncated) NAC014 transcription factor in the ER, PS and potentially in the nucleus. The full-length spinach and Arabidopsis NACs stabilized upon co-expression with the effector and were observed in the ER (NAC014), PS and nucleus/cytoplasm (Spo22390, NAC078). The observed subcellular localization patterns resemble that of known oomycete effectors that interact with tail anchored NAC transcription factors of lettuce (LsNAC069) (*Lactuca sativa*) and potato (StNTP1, StNTP2) (*Solanum tuberosum*). These effectors were found to prevent *Phytophthora* culture filtrate-induced re-localization of the NAC transcription factors to the nucleus. *N. benthamiana* plants in which *NbNTP1*, *NbNTP2* were silenced, showed increased susceptibility to *P. infestans* infection and silencing of the *Pi03192* effector gene reduced virulence of *P. infestans* [86]. Higher susceptibility against the corresponding oomycete *B. lactucae* was not observed in *LsNAC069*-silenced *L. sativa* transformants [169]. The *B. lactucae*, *P. infestans* and in the current study also the *Pfs* host-translocated effectors, all interact

with tail-anchored NAC transcription factors from species other than their host, (i.e., *P. infestans* Pi03192 interacts with lettuce NACs, *Bremia* effectors interact with Arabidopsis and potato NACs and *Pfs* effectors interact with Arabidopsis NACS) suggesting that tail-anchored NAC transcription factors are conserved oomycete effector targets.

HBI1 is a bHLH transcription factor that plays an important role in the growth-defense trade-off in plants as it positively regulates leaf cell expansion and negatively regulates ROS production upon exposure to PAMPs [226, 274]. HBI1 interacted with candidate RxLR effector Pfs1|6786. Interestingly, the subcellular localization of Pfs1|6786 changed from predominantly PS to predominantly nuclear upon co-expression with HBI1, Spo6524 (HBI1 ortholog), AHAS, and RAF2. We found that the HBI1 protein is stabilized in the nucleus by the presence of effector Pfs1|6786. When expressed individually, the HBI1 fusion protein was hard to observe due to low signal and if visible was observed in the cytosol and nucleus. Stabilization of HBI1 could be beneficial for *Pfs1*, as HBI1 is a negative regulator of PAMP-triggered immunity. Despite its clear role in basal immunity, HBI1 is not targeted by many pathogen effectors, in fact, to our knowledge Pfs1|6786 is the first effector reported to target and stabilize HBI1. The spinach ortholog of HBI1, Spo06524 shows a similar, nuclear distribution when individually expressed but is in small PS, just like the effector when co-expressed. One should note that Spo06524 is not a reciprocal best hit of HBI1 and may have a different function.

4

Cellular programming – posttranscriptional manipulation

RxLR effector Pfs1|1800 was found to interact with a protein (At2g19390) of unknown function that is rich in serine and arginine dipeptides. Serine-arginine (SR)-rich proteins are known to be involved in alternative splicing of mRNAs [275] However, the plant interactor of Pfs1|1800 lacks RNA binding domains that characterize known SR proteins. In At2g19390, the SR dipeptides are distributed throughout the protein, rather than at the C-terminus in SR-rich proteins with known splicing activity [276].

We found that At2g19390 was localized to small nuclear speckles while ortholog Spo24486, localizes to large nuclear speckles. Nuclear speckles are sites of storage and assembly of splicing factors that are observed near active transcription sites. Active cells possess many small speckles, whereas large speckles are associated with inhibition of transcription and splicing [249]. Upon co-expression with effector Pfs1|1800, the subnuclear localization of the At2g19390 protein changes from speckles to the nucleoplasm. Co-expression of the spinach ortholog and effector leads to their co-localization in large nuclear speckles. It will be interesting to test if the effector interferes with RNA processing in the plant cell nucleus.

RxLR effector Pfs1|10763 interacts with the At3g11930 protein that has a universal stress protein A domain (USPA). USPA is an ancient domain that was likely present in the last universal common ancestor of all extant life [277]. Bacterial USPA proteins mediate cell survival under a variety of abiotic stresses [278]. The Arabidopsis genome encodes 44 proteins with USP domains [234], of which At3g53990 was shown to exhibit a redox-dependent chaperone function that enhances plant tolerance to heat shock and oxidative stress [279]. Comparative analysis showed that the interactor USPA has conserved ATP binding residues [234], but functional data on this protein is lacking. Co-expression with Pfs1|10763 resulted in a change of subcellular localization of At3g11930 from nucleo-cytoplasmic to nuclear, where it is present in large nuclear speckles. The spinach ortholog Spo22595 was unstable but showed a clear signal from the nucleoplasm in the presence of the effector, which is also detected there. The altered localization of the plant interactors when the effector is present prompts further research into the function At3g11930 during downy mildew infection and its role as a potential effector target.

Manipulation of metabolic homeostasis

AHAS or Acetohydroxyacid synthase is a protein that catalyzes the first step in the synthesis of the branched-chain amino acids valine, leucine and isoleucine [280]. A study in tomato showed that a near-isogenic line carrying the Ol-1 resistance locus expresses an AHAS homolog that is involved in resistance to the powdery mildew fungus *Oidium neolycopersici*. The resistance seems to be the result of altered AHAS-mediated amino acid homeostasis, i.e. altered levels of branched-chain amino acids in cells, rather than a basal or resistance gene-based defense mechanism [230]. Tomato plants treated with the AHAS-inhibiting herbicide chlorsulfuron were more susceptible to *Oidium neolycopersici* infection. Chlorsulfuron treatment was shown to reduce levels of valine and leucine in soybean suspension cultures. The observed plastid localization when individually expressed is in line with published research [280]. We observed a shift of the AHAS-fusion protein to the nucleoplasm when co-expressed with Pfs1|6786, while it was still visible in plastids/PS. The outcome of interference of Pfs1|6786 with the synthesis of branched-chain amino acids and an effect of that on *Pfs* virulence is hard to predict, because amino acid synthesis requires multiple proteins and is negatively regulated by the amino acids produced [230]. Also, the plant needs the amino acids for its own metabolism and functioning. The spinach ortholog Spo20077 was observed in the cytoplasm and PS but not in the plastid lumen. Co-expression did not alter the localization of Pfs1|6786 nor Spo20077.

RAF2 is a Rubisco assembly chaperone that was first identified in maize. RAF2 associates with the Rubisco small subunit and possibly facilitates assembly with the large subunit by releasing the small subunit from the chaperone complex [224]. Surprisingly we observed RAF2 primarily in the nucleus rather than in chloroplasts, both in the individual and co-expression experiments where the presence of RAF2 appears to direct Pfs1|6786 to the nucleus as well. Likely, the N-terminal fluorescent tag prohibited the transit into the chloroplasts. The signal is not only in the nucleoplasm, which could occur by simple diffusion but also strongly

in nuclear speckles - it is possible that RAF2 resides in the nucleus as well. Localization was observed in the majority of the cells (>10) and a band of the correct molecular weight (of the protein including the transit peptide) was observed on the western blot. The localization of the N-terminal fusion protein to the nucleus, therefore, seems genuine. It will be important to look into mesophyll cells that have more chloroplasts, using C-terminally fused fluorescent proteins, to see whether the protein and effector localize to chloroplasts as well.

The final metabolism-related interactor protein is NAGK, a protein that is involved in the synthesis of arginine in chloroplasts. Under high nitrogen conditions, NAGK is bound by the plastid signal transduction protein PII to induce arginine synthesis. Binding between these proteins is reduced when little nitrogen is available or when arginine levels increase [281]. Arginine is the substrate for production of nitric oxide (NO) by nitrogen oxide synthase NOS1 [282]. PAMP-treatment in Arabidopsis plants was shown to trigger a NO-burst which is emergingly recognized as a plant PAMP-triggered immunity reaction, analogous to that in animals [282]. Plants with a mutated *AtNOS1* are not able to convert arginine to NO and were more susceptible to *Pseudomonas syringae* pv. tomato DC3000 [282]. We observed NAGK in the ER and PS. The protein was expressed with an N-terminal tag that likely prohibits the entry of the fusion protein into the chloroplast, although the PS structures may represent chloroplast plastoglobuli. When co-localized with the effector, NAGK localizes to PS of different sizes and no signal is observed from the ER. The spinach ortholog stabilized upon co-expression with Pfs1|3869 and was observed in PS, nucleoplasm and nuclear speckles.

Other effector-interacting proteins

Little is known about the interactor At5g56100 that is annotated as 'glycine-rich/oleosin'. Oleosins are proteins that locate on the surface of lipid bodies that store lipids in seeds to facilitate their germination and seedling growth [283]. However, this protein is oleosin-like and it is not known whether it localizes to these bodies. We observed the protein in the ER and in PS in epidermal cells. It co-localizes with the candidate host-translocated CRN effector in the ER, PS and cytoplasm. Despite its limited similarity, the subcellular localization pattern of spinach ortholog Spo12707 is similar to that of At5g56100. Little is known about the mode of action of CRN effectors. A sparse example of CRN-interacting proteins is described for *P. sojae* effectors CRN115 and CRN63 that were shown to interact with plant catalases to regulate H₂O₂ production elicited by CRN63 and overcome host immunity [103] .

The final protein that was found to co-localize with an effector is At3g13800, a protein with a Metallo-beta-lactamase (IPR001279) domain of unknown function. At3g13800 and its spinach ortholog co-localize with the membrane bound Pfs1|3172 effector in the ER and in PS. Further research should focus on the molecular role of At3g13800 and its effect on plant susceptibility and pathogen infection.

Conclusion

In this chapter we visualized the subcellular localization of the first eight candidate host-translocated effectors of *Pfs*. We found that in most cases the effectors co-localize with their putative interacting plant proteins and their identified spinach orthologs. Co-expression in some cases led to stabilization and re-localization of either or both of the interacting proteins within plant cells. Future research should validate the interaction between the proteins in planta and set out to understand the molecular roles of the plant proteins in immunity and the ways the host-translocated effectors manipulate these functions.

Methods

Reciprocal blast analysis of candidate spinach orthologs

A reciprocal blastP search was performed to identify the spinach protein orthologs of the Arabidopsis interactor proteins. Arabidopsis and spinach protein Blast libraries were created using TAIR10_pep_20110103_representative_gene_model" (arabidopsis.org) and 'spinach protein sequences V1' (<http://www.spinachbase.org/>) in Geneious 9.1.8 (<https://www.geneious.com>) [284]. The Arabidopsis interactor proteins were used as the query in the first search, the best spinach protein hits (E-value < 1.0e-05) were considered candidate protein orthologs. The reciprocal blast was performed using the significant spinach hits on the library of Arabidopsis proteins. In case no reciprocal best hit was identified, a protein tree was created to clarify possible multiple-to-one or multiple-to-multiple orthology. For each Arabidopsis interactor all significant reciprocal BlastP hits were aligned (MUSCLE [285] and Neighbor-Joining trees (Tamura-Nei distance model) were created using standard settings (Geneious 9.1.8). Trees were visualized using iTOL [212].

Prediction of subcellular localization

The subcellular localization of candidate effectors without their signal peptides was predicted using LOCALIZER 1.0.4 [257] and Deeploc 1.0 [286] using standard settings. The subcellular localization of interacting Arabidopsis proteins was retrieved from Suba4 [248].

Cloning

mRNA was isolated from infected Arabidopsis Col-0 and spinach Viroflay plants using the Qiagen mRNA isolation kit according to protocol. cDNA was generated using Maxima H Minus Reverse Transcriptase and oligo(dT) primers at standard settings. Primers were designed to include attachment sites to aid Gateway BP or TOPO cloning (**Table S2**). Effectors and interactors were amplified from cDNA of infected plants and cloned into a pGEM-T-Easy using BP clonase according to the Gateway protocol or in the pENTR/D-TOPO vector according to the TOPO cloning kit. Vectors were transformed into *E. coli* DH5 α for selection using ampicillin as a selection marker. Correct insertion of the coding sequences into the vector was verified by Sanger sequencing (Macrogen). The entry vectors were used to transfer the genes of interest to YFP- and CFP-containing expression vectors from the pB7WG2 vector series for expression from the 35S promoter of fluorescent fusion proteins in planta [235].

4

Transformation of *Agrobacterium* for expression of fusion proteins in planta

The pB7WG2 series expression vectors were transformed into *Agrobacterium tumefaciens* strain AGL1 using heat shock to allow for large scale transformation of plasmids. Cells were grown in 200 ml of LB with 25 μ g/ml rifampicin and 15 μ g/ml tetracycline at 28°C overnight until the cells were in the exponential growth phase (OD₆₀₀ = 0.5-0.8). Cells were chilled on ice for 10 minutes and collected at 4700 rpm at 4°C. Supernatant was discarded and the cells were suspended in 2 ml of ice cold 20 mM CaCl₂ and aliquoted in 100 μ l samples that were snap frozen in liquid nitrogen. To transform the *Agrobacterium* cells, between 100

and 1000 ng of plasmid was added to each thawed aliquot which was then again snap frozen in liquid nitrogen for 5 minutes. Cells were heat shocked for 5 minutes at 37°C degrees, transferred to a 15 ml tube with 1 ml of LB and incubated for 1 hour at 28°C while shaking. The sample was plated on LB supplemented with the appropriate antibiotics at 28°C to form colonies within two days. The insert size of the transformed *Agrobacterium* colonies was verified using colony PCR with plasmid specific primers.

Transient expression in *N. benthamiana*

N. benthamiana plants were grown in long day conditions (16 hours of light at 22 °C and 8 hours of darkness at 20 °C). *Agrobacterium* with the desired plasmid was grown overnight at 28°C in LB + 50 nM spectinomycin (spec), 50 nM carbenicillin (carb) & 20 nM rifampicin (rif). Cells were pelleted and resuspended into 7.5 ml of induction medium (1x M9 salts, 1% glucose) + appropriate antibiotics (spec, carb, rif) and incubated for 3 hours at 28°C. Cells were pelleted and resuspended in infiltration medium (0.5x Murashige & Skoog salts, 10 mM 2-(N-Morpholino) ethanesulfonic acid sodium salt (MES), 0.5% sucrose, 0.5% fructose and 150 µM of acetosyringone). Three leaves of 3 week-old short day grown spinach or five week-old *N. benthamiana* plants were infiltrated with *Agrobacterium* at an OD600 of 0.6. Effectors and targets were expressed separately and in combination, with the OD600 kept at 0.6 for the mixtures. The infiltrated plants were grown for 48 hours before examination under the confocal microscope. The number of technical replicates and the number of transformed cells observed per construct are indicated in **Table S2**.

Confocal microscopy

Expression of fluorescent proteins was assessed under a Zeiss® LSM700 confocal microscope using the 63x objective with oil emersion (Objective Plan-Apochromat 63x/1.40 Oil DIC). Excitation for CFP was set at 405 and YFP at 488 nm. The emission wavelength for both CFP and YFP was set at 523 nm with a detection limit of 493-550 nm. Images were analyzed with ZEN lite v2.3 software (Zeiss microscopy) and the overview figures were created using Photoshop v. 1.9. **Table S3** lists the number of technical replicates and numbers of cells visualized per protein and per host-translocated effector–plant interactor combination.

Western blot detection of effector protein and plant protein fusions

Three leaf punches (6mm) of the infiltrated *N. benthamiana* plants used in the confocal assays were snap frozen in liquid nitrogen and grinded using a mortar and pestle. 100 µl of lysis buffer (10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.5 mM EDTA, 0.25% (v/v) Triton-X 100, 0.25% (v/v) Tween-20, 5 mM DTT, 4% (w/v) polyvinylpyrrolidone) was added to the grinded sample and incubated at 4°C for 30 minutes while shaking. After incubation the sample was centrifuged (4°C for 30 minutes, 7000 rpm) and the supernatant for another 10 minutes under the same conditions. Laemlli buffer (33,3 µl) was added and the sample was cooked for 10 minutes at 95°C and centrifuged for 0 minutes at 6600 rpm. Samples were loaded to a pre-cast Invitrogen Bolt Bis-Tris Plus gradient gel 4-12%. Gel loading and western blotting was performed according to standard protocol. Poncou staining was performed to validate the total level of the proteins. A horse radish peroxidase conjugated GFP antibody (Chromotek) was used to visualize the fusion proteins in the sample.

Acknowledgements

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Supplemental tables

Table S1. Western blot using an antibody against the fluorescent protein for detection of intact fusion proteins in *Nicotiana benthamiana* leaves.

	Protein	Size (KDa)	observed in western blot
Arabidopsis	AT1G33060 (1)	54	no
	AT1G33060_trunc	53	yes
	AT1G55190	21	no
	AT2G18300 (1)	41	no
	AT2G19390 (1)	133	no
	AT3G11930 (2)	25	yes
	AT3G13720	21	no
	AT3G13800 (1)	40	no
	AT3G48560	73	yes
	AT3G49580 (1)	11	yes
	AT3G57560	37	no
	AT4G04020	35	yes
	AT4G35110 (3)	45	yes (low amount)
	AT5G04410	19	no
	AT5G51110 (1)	24	yes
	AT5G56100	16	yes
Spinach	Spo22390	22	no
	Spo22390 (variant)	22	yes
	Spo20420*	22	yes
	Spo06524	52	no
	Spo24468	137	no
	Spo22595	23	no
	Spo20405	40	yes
	Spo20077	73	yes
	Spo22402	14	yes
	Spo15075	44	yes
	Spo20112	35	yes
	Spo18795	40	not available
	Spo16524	19	no
	Spo14154	24	yes
	Spo12707	16	no

Table S1 continued

Pfs1 effectors	pfs1 9071	11	yes
	pfs1 9976	69	no
	pfs1 1800	20	yes
	pfs1 10763	48	yes
	pfs1 6786	41	yes
	pfs1 3172	18	yes
	pfs1 10492	80	not available
	pfs1 3869	20	yes
	pfs1 7215	19	yes

Table S2. Overview of the number of technical replicates and the number of transformed cells observed in the(co-)expression experiments of fluorescent fusion proteins in *N. benthamiana* leaves.

Gene code	Protein name	Individual expression		Co-expression	
		Techn. repl.	Cumulative # cells	Techn. repl.	Cumulative # cells
At1g33060	NAC014	2	3	1	8
At1g55190	PRA1.F2	1	9	1	4
At2g18300	HBI1	5	4	1	2
At2g19390	S-R rich	1	9	1	5
At3g11930	USPA	2	4	1	7
At3g13720	PRA1.F3	1	8	1	5
At3g13800	AT3G13800	1	5	1	8
At3g48560	AHAS	1	7	2	9
At3g49580	LSU1	3	13	3	17
At3g57560	NAGK	1	6	1	9
At4g04020	FBN1a	1	7	1	7
At5g04410	NAC078	2	3	2	9
At5g51110	RAF2	1	13	1	10
At5g56100	Glycin rich	3	16	2	20
AT1G33060_ trunc.	NAC014 trunc.	2	10	1	4

Table S2. continued

Gene code	Protein name	Individual expression		Co-expression	
		Techn. repl.	Cumulative # cells	Techn. repl.	Cumulative # cells
Spo22390	AT1G33060	2	2	1	3
Spo20420*	AT1G55190	2	7	1	6
Spo06524	AT2G18300	2	5	1	7
Spo24468	AT2G19390	2	7	3	10
Spo22595	AT3G11930	2	5	1	7
Spo20405	AT3G13800	1	4	1	6
Spo20077	AT3G48560	1	4	2	8
Spo22402	AT3G49580	2	16	3	16
Spo15075	AT3G57560	2	2	1	8
Spo20112	AT4G04020	1	7	1	5
Spo16524	AT5G04410	2	3	1	9
Spo14154	AT5G51110	1	4	1	8
Spo12707	AT5G56100	3	9	3	10
Pfs1 3172		2	10		
Pfs1 9071		3	13		
Pfs1 6786		1	5		
Pfs1 1800		2	13		
pfs1 10763		2	15		
Pfs1 7215		4	18		
Pfs1 3869		1	3		
Pfs1 9976		2	7		

Supplemental figures

Figure S1. Protein tree of Arabidopsis effector-interactors, their protein paralogs and spinach orthologs.
Green dots represent the last shared branch between the spinach and Arabidopsis protein.

Figure S2. Protein alignments of *Arabidopsis* interactor proteins and the selected spinach Sp75 orthologs

Available at:

<https://doi.org/10.17026/dans-zey-8aec>

Figure S3. Expression of fusion proteins in *N. benthamiana* (left) and spinach variety *Viroflay* (right).

Each line represents the same protein in each of the plant species. Pictures are taken at 3 days post infection. Scale bars are 20 μ m.

Figure S4. Subcellular localization of Arabidopsis interactors and their spinach orthologs in *N. benthamiana*.

Plant interactors fused to CFP (N-terminus) were produced in *N. benthamiana*. AHAS and ortholog Sp020077 were fused at its C- terminus to CFP. Images were taken ~48 to 72 hours after infiltration. Scale bars = 20 μ m.

Figure S4 (continued). Subcellular localization of Arabidopsis interactors and their spinach orthologs in *N. benthamiana*.

Plant interactors fused to CFP (N-terminus) were produced in *N. benthamiana*. AHAS and ortholog Sp020077 were fused at its C- terminus to CFP. Images were taken ~48 to 72 hours after infiltration. Scale bars = 20 μ m.

Figure S4 (continued). Subcellular localization of Arabidopsis interactors and their spinach orthologs in *N. benthamiana*.

Plant interactors fused to CFP (N-terminus) were produced in *N. benthamiana*. AHAS and ortholog Spo20077 were fused at its C- terminus to CFP. Images were taken ~48 to 72 hours after infiltration. Scale bars = 20 μ m.

Figure S4 (continued). Subcellular localization of Arabidopsis interactors and their spinach orthologs in *N. benthamiana*.

Plant interactors fused to CFP (N-terminus) were produced in *N. benthamiana*. AHAS and ortholog Sp020077 were fused at its C- terminus to CFP. Images were taken ~48 to 72 hours after infiltration. Scale bars = 20 μ m.

Figure S5. Co-localization of 5 Arabidopsis interactors and their spinach orthologs with candidate effectors of *Pfs*.

Plant interactors fused to CFP and candidate effectors fused to YFP (N-terminus) were transiently expressed in *N. benthamiana*. Co-localization was observed for all interactors except PRA1.F2 and F3. Subcellular localization of neither the interactors nor the effector candidate changed upon co-expression. Images were taken ~48 hours after infiltration and are representative of >5 cells. Scale bars = 20 μ m.

Figure S5 continued. Co-localization of 5 Arabidopsis interactors and their spinach orthologs with candidate effectors of *Pfs*.

Plant interactors fused to CFP and candidate effectors fused to YFP (N-terminus) were transiently expressed in *N. benthamiana*. Co-localization was observed for all interactors except PRA1.F2 and F3. Subcellular localization of neither the interactors nor the effector candidate changed upon co-expression. Images were taken ~48 hours after infiltration and are representative of >5 cells. Scale bars = 20 μ m.

5

General discussion

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General discussion

The molecular interaction between phytopathogenic oomycetes and plants is a well-orchestrated and highly dynamic process [287, 288, 289]. As a basis for studying the molecular interactions between *Peronospora effusa* (*Pfs*) and spinach we sequenced reference race 1 of the spinach downy mildew (**Chapter 2**). During infection oomycetes secrete large numbers of effector proteins that help suppress the plant immune response and establish and maintain the infection [40]. Two types of host-translocated effectors are known in downy mildew species; the RxLR and CRN effectors [56, 120, 121]. In my thesis I describe a comparative analysis that revealed the conservation of these host-translocated effectors between oomycete species. In addition, plant proteins interacting with *Pfs* effectors were identified and characterized to get insights into possible molecular mechanisms enabling *Pfs* infection. Here I discuss the underlying genomic evolution of pathogenicity in oomycetes and put our findings on RxLR and CRN effectors in a broader context of susceptibility of spinach to *Pfs*.

Evolutionary genomics of oomycete phytopathogens

Evolution of pathogenicity

Intriguingly, the ability of eukaryotic microbes to cause disease (on plants) evolved independently in fungal and oomycete species [290] Additionally, species with intermediate lifestyles, like the hemi-biotrophic *Phytophthora* species, are observed. *Phytophthora* infection has an initial biotrophic phase that is succeeded by a necrotrophic one [291].

It is generally thought that early saprotrophic species (that feed on already death plant tissue) have evolved to live off living plant tissue [290] Both saprotrophic and pathogenic fungi and oomycetes have filamentous hyphae and soilborne and waterborne spores, traits that may form the basis for the evolution of pathogenicity in both kingdoms [292]. Pathogenic species of both kingdoms have developed specialized feeding hyphae (haustoria) to accommodate their interaction with plants.

Now, the growing number of available genomes of phytopathogens allows studying genomic signatures of pathogen lifestyles. Both the necrotrophic and biotrophic lifestyles require specialized genes and well-orchestrated gene expression to enable the successful infection of plants [293].

Comparing genomes of phytopathogenic species to that of saprotrophic ones provides insight into the molecular differences that underly the pathogenic lifestyle. Proteomes of phytopathogenic oomycetes and fungi were found to contain more hydrolytic enzymes, proteins with domains involved in carbohydrate metabolism, nutrient uptake, signaling and suppression of the host immunity response compared to non-pathogenic eukaryotic species [53, 294]. Many of the enriched domains were found in secreted proteins that are anticipated to be directly involved in virulence [53]. Among them are the host-translocated RxLR and CRN effectors in oomycetes that are discussed in the ‘effector biology’ part of this discussion.

Functional differences between proteomes of necrotrophic and (obligate/hemi) biotrophic species are also observed. They include enrichment of transporter functions in necrotrophs, enzymes involved in amino acid metabolism in hemi-biotrophs and nucleic acid metabolism and signal transduction (GTPase, lysophospholipase and tyrosine kinase activity) in obligate biotrophic species [294]. Also, obligate biotrophs were shown to have lost genes involved in metabolic pathways for the production of nutrients that can be obtained from the host. For examples, genes encoding sulfite, nitrite and nitrate reductases, a nitrate transporter and genes required for synthesis of arachidonic acid and polyamine oxidase were not found in the *Hyaloperonospora arabidopsidis* genome [59]. The nitrate reductases are also missing from the genomes of *Pfs* and other downy mildew species [29, 137, 187, 290]. The loss of genes encoding enzymes involved in processing and uptake of inorganic nitrates is also observed in *Albugo laibachii* and several fungal species (*Melampsora larici-populina*, *Blumeria graminis*, *Puccinia* f. sp. *graminis*) [290, 295]. It is the only example of a pathway that is lost in a broad range of pathogens, as the majority of the metabolic pathways are maintained in

pathogen genomes [290]. These losses are considered a consequence of the obligate biotrophic lifestyle rather than a causal factor [197].

In **chapter 2** we compared the secretomes of oomycete phytopathogens with different lifestyles. We found that evolutionary distinct biotrophic species, i.e. *Albugo*, *Peronospora* and *Plasmopara* showed similar sets of secreted proteins in terms of presence of Pfam domains. Secretomes of obligate biotrophs had fewer Nep-1 like proteins, proteins with RxLR domains (PF16810) and proteins with Sel1 repeat domains (PF08238) compared to hemi-biotrophic *Phytophthora* species. The reduction or loss of especially cytotoxic NLPs may aid the obligate biotrophic life, as they cause cytolysis and cell death [177]. Reduced numbers of proteins with PAN/Apple (PF14295), subtilase (PF00082) and Thrombospondin type 1 (PF00090) domains were observed in the secretomes of the obligate biotrophs compared to *Pythium* species. The PAN/Apple and subtilase domains are implicated in evasion of recognition and degradation of plant antimicrobial compounds respectively [179, 180]. PF00090-containing proteins have no known function and are abundant in the secretome of *Pythium* and *Phytophthora* species but absent the biotrophic species except for *Plasmopara viticola*.

Genome size

An interesting observation is that many filamentous pathogens have larger genomes than their non-pathogenic relatives [190, 296]. This observation contrasts the general idea of parasites and symbionts that evolve compact minimal genomes that lack unnecessary genes and entire metabolic pathways because metabolites can directly be obtained from the host [297, 298]. Among the largest phytopathogen genomes sequenced are those of the oomycete *P. infestans* (240 Mb) and powdery mildew fungus *Blumeria graminis* (174 Mb) [58, 299]. Genome expansion is largely attributed to repetitive DNA, as a result of the amplification of transposable elements [58, 295, 296]. Transposable elements are mobile stretches of repetitive DNA that can ‘travel’ and amplify within genomes thereby altering DNA content and gene activity. Transposable

elements increase genome size and induce genomic variation that may be advantageous for the adaptation of species [190]. The repetitive elements are often unevenly distributed across genomes resulting in distinct gene-dense, repeat-poor and gene-sparse repeat-rich genome areas [172, 300].

The gene-sparse, repeat-rich regions were found to evolve more rapidly than the rest of the genome resulting in an evolutionary pattern termed the 'two-speed genome' [190, 300]. These dynamic regions serve as a cradle for adaptive evolution while the rest of the genome contains more conserved genes that function in basal growth and developmental processes. Host-translocated RxLR and CRN effector genes often reside in the repeat rich areas of the genome resulting in their large expansion and rapid evolution [58]. The *Pfs* host-translocated effectors are modestly enriched in the gene sparse regions of the genome of *Pfs1* but the gene density difference between the effectors and core genes is not as strong as in the *P. infestans* genome (**Chapter 2**).

Pathogens with flexible genomes can more easily cope with resistant hosts by losing or altering genes and can even adapt to infect a new host plant species. Assuming that it is only a matter of time before a host plant species evolves resistance to a pathogen, pathogens with flexible genomes are able to more frequently jump to a susceptible or new host and are thus more likely to persist over evolutionary time. This flexibility is proposed to outweigh the disadvantages of a large and costly genome [190].

Genome flexibility is argued to be beneficial especially for biotrophic pathogenic species as they depend on their host and have to avoid recognition by the ever adapting host immune system [290]. However, genome sequencing revealed an opposing trend in the genome sizes of downy mildews of the *Peronospora* genus and other obligate biotrophic oomycete and fungal species (**Chapter 2**, [60, 137, 187, 301, 302]).

The *Peronospora* species sequenced so far, including *Pfs*, have small genomes, ranging between from ~30 to 60 Mb [60, 137]. We found that the assembled *Pfs1* reference genome size is 32.4 Mb (the estimated size based on *k*-mer analysis 36.2Mb), which is comparable to the *Pfs* genome size reported by Fletcher et al (~39 Mb), based on measured nuclear DNA using flow cytometry [187]. We estimated the repeat content of *Pfs1* to be ~27% (8.8Mb) (**Chapter 2**) which is low compared to that of for instance *P. infestans* (74%), similarly to the repeat content of other *Peronospora* species [58]. The relatively low content of repetitive DNA may be underestimated as the genome is quite fragmented and repetitive DNA is particularly hard to assemble. Given the proposed advantages of large flexible genomes, this trend in downy mildews is remarkable. Yet, *Peronospora* is a successful genus with many species that are virulent on a wide range of plant species [17].

Alternatively to genome variation by repetitive DNA and transposable elements, sexual reproduction could increase variation in the genomes of downy mildews [290]. Genetic recombination in smut fungi is facilitated strictly through sexual reproduction, which may explain why their genomes are small yet flexible enough to adapt. Although oomycetes can reproduce sexually they are thought to primarily propagate through asexual reproduction, suggesting that the contribution of sexual reproduction to genetic variation is smaller compared to that in smut fungi [8]. It is not clear how sufficient genome flexibility is maintained in these relatively small downy mildew genomes. Possibly, the small genomes reflect reduction of unnecessary genes while maintaining enough flexibility required for survival. We observed that a smaller proportion (~4-7%) of the proteome of *Hyaloperonospora/Peronospora* is secreted compared to *Phytophthora* species (~7-10%) (**chapter 2**). The secretomes of these oomycete species are functionally comparable in terms of carbohydrate active enzymes but the numbers and thus diversity of secreted proteins is reduced in downy mildew species. This reduction may help to avoid recognition by the plant immune system and contribute to the reduced genome size.

Genomics and taxonomy

Systematics is perhaps best described as the science that satisfies the human desire to order its natural surroundings into conceivable, orderly units. The idea that similar species descent from a common ancestor, first proposed by Charles Darwin, gave way to phylogenetic inference of the 'tree of life'. Systematics classifies species into monophyletic groups, i.e. groups that are derived from a common ancestor (first proposed by Henning) and names them accordingly (taxonomy) [303]. In taxonomy species are organized into ranks; genera, then families, orders, classes and so on in order of inclusiveness. Traditionally, taxonomy is based on morphological and biological traits [304]. The emergence of molecular genetics provides an additional, powerful trait to help solve evolutionary relationships. In many cases, molecular genetic data showed that traditionally recognized clades are not monophyletic. In those cases, morphological and biological similarity may result from convergent evolution or genetic drift rather than ancestry, thereby complicating the definition of families, genera and even species.

Within the oomycetes these discrepancies in taxonomy are especially evident in the large and diverse taxa of *Pythiaceae* and Peronosporales. While some authors divide the crown oomycete species into three orders; Pythiales, Peronosporales and Albuginales [4, 292], others prefer to place the phytopathogenic *Pythium* species in a family (*Pythiaceae*) within the Peronosporales order [6, 20]. This lack of consensus is nicely illustrated by the number of hits obtained from Google Scholar searches for the different orders and families and combinations of those (**Table 1**). The combination '*Pythiaceae* -Peronosporales' (4.540) is used slightly more often in scientific literature than 'Pythiales, Peronosporales' (3.790). Remarkably, 4.200 hits are obtained for the combination 'Pythiales, *Pythiaceae*'; a *Pythiaceae* family within a Pythiales order.

Table 1. Usage of the taxonomic classifications ‘*Pythiaceae*’ or ‘*Pythiales*’ in scientific publications (based on Google Scholar).

Query	Number of hits
Pythiales	5.870
<i>Pythiaceae</i>	8.170
Peronosporales	13.200
Peronosporales, Pythiales	3.790
Peronosporales, <i>Pythiaceae</i>	4.540
Pythiales, <i>Pythiaceae</i>	4.200

Number of Google Scholar hits for different queries, obtained in May 2020.

It is widely accepted that the Pythiales or *Pythiaceae* are not monophyletic but there is a lack of consensus on how to deal with the taxonomy of the many species [20, 305]. Following Hennings idea of taxonomy informed on monophyletic groups, there is no basis for defining an order of Pythiales that includes all currently identified *Pythium* species. Multigene phylogenies show that *Pythium* species like *Pythium ultimum* and *Pythium splendens* are not monophyletic with the other *Pythium* species but are more closely related to *Phytophthora* species. For the same reason, a monophyletic *Pythiaceae* family (that encompasses all *Pythium* species) within the Peronosporales order is not supported [20]. Dividing the *Pythium* species into two families within the Peronosporales order or renaming *Pythium* clade I,F,G species to solve the polyphyly would provide an intuitive organization of *Pythium* species that will aid further communication about the crown oomycete taxa.

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Similarly, the large group of *Phytophthora* species is now widely accepted to be paraphyletic, with the downy mildew species embedded among the different *Phytophthora* clades, asking for re-evaluation of their taxonomy [306] In multiple gene/protein and full genome comparisons in this (**Chapter 2**) and other studies [27, 173], downy mildew species were suggested not be monophyletic. The lineage of downy mildew species with pyriform haustoria (in the genera *Plasmopara*, *Bremia*) was found not to be monophyletic to the lineage that encompasses the graminicolous grass-infecting downy mildews, the downy mildews with colored conidia

(like *Peronospora*) and the brassicolous downy mildews. Future studies including more species and using curated sets of conserved genes are needed to get an unambiguous answer to the Peronosporales phylogeny.

Effector biology

Genomes of phytopathogenic oomycetes encode large numbers of effector proteins. These proteins are secreted from the oomycete and can function either outside or inside the plant cell. Many effectors have evolved to manipulate plant processes to avoid or suppress plant immunity [80, 198], e.g the responses triggered by recognition of pathogen-associated molecular patterns (PTI) or effectors (ETI). Two types of host-translocated effectors are known from Peronosporales species, the RxLR and CRN effectors. For some of those effectors of Peronosporalean species other than *Pfs* the molecular role in virulence was studied in more detail (**Chapter 1**) but the function of the majority of RxLR and CRN proteins is still unknown.

The sequencing and assembly of the *Pfs1* reference genome cleared the way for identifying its host-translocated effectors and studying their individual contribution to the interaction with the spinach host. We identified 99 candidate host-translocated RxLR and 14 candidate CRN effectors in the secretome of *Pfs1* (**Chapter 2**). The work of Joël Klein, that is complementary to the work described in this thesis, described the search polymorphisms in effectors that are associated with the breaking of resistance in spinach [207]. Resistance to specific races of pathogens is generally mediated by intracellular R-proteins that recognize host-translocated effector proteins [287]. Intracellular nucleotide-binding leucine-rich repeat receptors (NB-LLR receptors) directly or indirectly recognize effectors [307]. The recognition triggers an immune response termed effector-triggered immunity (ETI) [80]. However, pathogens such as *Pfs* did not evolve effectors to be recognized, but rather to function in manipulation of host processes to allow successful infection. In this thesis I describe the study of the molecular interaction between individual RxLR and CRN effectors and plant proteins in a first step towards understanding

how these effectors contribute to disease.

The combined works provide an integrated view of *Pfs* effectors related, on the one hand to breaking of resistance, and on the other hand to the molecular interactions with plant proteins.

The search for resistance breaking effectors in the *Pfs*

The recognition of an effector mediated by a plant R-gene confers a very strong immune response that induces resistance to the pathogen [308]. When recognition occurs, this overrules the virulence function of the effector and the activated plant immune system reduces the fitness of the pathogen. The detection of effectors by the plant leads to diversifying selection pressure so that recognized effectors may be lost or altered or new effectors suppressing the immune response evolve. Individual effectors are usually not crucial for survival and there is redundancy in effector repertoires [200, 309]. In *Pfs*, these dynamics lead to the continuous emergence of new races that break R-gene mediated resistance of newly bred spinach varieties [30, 32]. Within the work of Joël Klein the genomes of 16 races and 8 field isolates of *Pfs* have been sequenced [207].

Effector polymorphisms between the races were used to identify effectors that may be recognized by resistance loci using a differential set of spinach lines. Most strikingly, deletion of candidate effector *Pfs*1|1792 was found to be associated with the breaking of resistance conferred by *RPF3* and polymorphisms in three other effectors were associated to breaking *RPF5* (effector *Pfs*1|3839) and to *RPF2* (effectors *Pfs*1|6786 and *Pfs*1|9985) resistance. Systematic screening of effector proteins can be employed to identify R-genes, in cultivated and wild plant species, by assessing their response to effectors and linking it to genomic loci in the plants [310]. This is especially instrumental in resistance breeding of crops.

The downside of R-gene mediated resistance is that though it is very effective, it is usually not durable [288]. Many resistances rely on

the recognition of single effectors that are often not crucial for pathogen success [311]. Pathogens have large effector sets that have redundant functions, so upon recognition they can easily lose or alter effectors to avoid recognition.

Towards the virulence function of *Pfs* effectors

To better understand the role of individual host-translocated effectors I studied their possible virulence function by identifying their putative plant targets. In other words, how do effectors interfere with plant processes to make plants susceptible to *Pfs*? To this end, I first studied if effectors are present in related species to identify those that are conserved between species. Little conservation of effector proteins between related species is observed, potentially as a result of positive selection to avoid the recognition by the plant immune system [75]. Still, a number of RxLR effectors are conserved among phytopathogenic oomycetes, possibly because they contribute significantly to virulence [312]. Although conserved effectors may be the ‘odd ones out’ due to positive selection to avoid recognition, the fact that some are conserved makes them interesting targets. These effectors may target conserved interacting plant proteins that are involved in the infection process and contribute to plant disease susceptibility. The identification of Arabidopsis proteins that interact with *Pfs* effectors supports the idea of conserved plant targets across species (**Chapter 3**). These so called “Achilles heel” effectors can be used to identify and understand molecular manipulation of plants [308].

We identified the potential virulence targets of 18 candidate *Pfs* host-translocated effectors from a yeast two-hybrid library of Arabidopsis protein fragments (**Chapter 3**). The spinach ortholog of each Arabidopsis prey protein was identified within the proteome of spinach line Sp75 [241]. The interaction between the spinach orthologs and the *Pfs* host-translocated effectors will have to be confirmed in future Y2H screens. For six plant preys and their spinach orthologs fused to fluorescent proteins we showed that they reside in the same subcellular compartment as the

effector when co-expressed in *N. benthamiana*. For five of them we found that the presence of the candidate effector induced re-localization of the target to a different subcellular compartment (**Chapter 4**). The subcellular localization of two effectors, Pfs1|6786 and CRN Pfs1|9976, also changed upon co-expression with the plant interactors. Altered localization upon co-expression is suggestive of physical interaction between the proteins. A next step would be to confirm the interaction in planta by using co-immunoprecipitation (co-IP) or Fröster resonance energy transfer by fluorescence lifetime imaging (FRET-FLIM) [313].

Once the interaction is established the question that remains is what the effect is of binding of the plant protein by the candidate effector on processes in the plant and susceptibility to *Pfs*. Studies in **chapter 4** provide a first idea on the processes affected. The effector-interactor proteins roughly fall into 5 categories; immune signaling (hormonal, transcriptional, ROS) defense, post-transcriptional regulation, metabolism, and other. To our knowledge Pfs1|6786 and Pfs1|3869 are the first oomycete effectors that could be involved in the manipulation of amino acid metabolism.

The outcome of the interaction between effector and protein depends on the function of the plant protein and the effect of the interaction on that functionality. Effectors are commonly observed to target proteins to suppress immunity, thus enhancing plant susceptibility [198]. Additionally oomycete pathogens exploit plant proteins termed susceptibility genes (S-genes) to promote disease [90, 99, 314]. Host genes that contribute to susceptibility of plants to pathogens are termed susceptibility (S) genes [315]. Examples include Rho-like GTPases (*OsRAC4* and *OsRAC5*) that are required for full susceptibility of rice to *Magnaporthe grisea* [316] and Arabidopsis to powdery mildew (*ROP6*) [317], proteins involved in amino acid metabolism [318] SWEET transporters that transport glucose and sucrose across cell during infection [319, 320] and negative regulators of immunity [314]. S-genes are interesting targets for plant breeding because the loss or mutation will limit or abolish pathogen infection. It is expected that S-gene based resistance is more durable than R-gene

based resistance because the pathogens has to overcome a dependency on a host factor rather than evading recognition by an R-gene [308]. For that, a pathogen has to acquire a new function which is more difficult than to lose one [296]. This is especially evident in biotrophic species that depend on host factors, such as particular metabolites that they cannot produce themselves [314, 315].

Combining inter- and intra-specific comparative effector data

The above-mentioned complementary approaches to asses intra-species variation to identify potential recognized effectors and inter-species conservation to identify Achilles heel effectors can be combined to build a further framework of effector conservation. Apart from the polymorphisms in different *Pfs* races, 41 candidate effectors were found to be conserved in all sequenced *Pfs* isolates [207]. That is, the protein sequence of those 41 effector proteins had no homozygous amino acid changes between *Pfs* isolates. When we overlay these data with the comparative study described in **chapter 3**, we observe that fifteen of these conserved effector proteins are unique to *Pfs* and are possibly important in the compatible interaction between *Pfs* and spinach. The other proteins were conserved in orthogroups that have proteins of *Pfs* and other downy mildews (6), other Peronosporales (4) or proteins from more distantly related species used in the comparison (16) (*Albugo*, *Pythium*, Peronosporales). Their conservation within *Pfs* races points to an important virulence function within *Pfs* while effector conservation between species points to targeting of conserved plant proteins in multiple plant species. Studying plant proteins targeted by conserved effectors may reveal molecular mechanisms employed in a broader range of plant species. **Table 2** provides a shortlist of interesting candidate effectors, conserved within *Pfs* and between *Pfs* and other species for future study on their role in the infection process. Their putative translocation domains, WY-folds, transmembrane helices and conservation with proteins of other species and identified interactors as described in **chapter 3** are indicated.

Table 2. Candidate host-translocated effectors of *Pfs* that are non-polymorphic between *Pfs* races and have orthologs in other oomycete species.

Gene name	Orthogroup	Conservation	gene name	WY-fold (#)	TM helix (#)	RxLR	EER	CRN	HVL	
Pfs1 10282	OG0010880	DM specific	NAC014, USP1,At3g13800, NAC078 NAGK, TCP14, At5g03300, TCP21, RNA- ligase, PTB	0	1	RMLR	EER	AFTAK		
Pfs1 10648	OG0010880	DM specific		0		RMLR				
Pfs1 3172	OG0010838	DM specific		0		RRLR				
Pfs1 3869	OG0010260	DM specific		0		RNLR				
Pfs1 4214	OG0010838	DM specific		0	1	RRLR	EER			
Pfs1 4980	OG0013057	DM specific		0		RFLR	EER			
Pfs1 10123	OG0011230	Peronosporales		0		RDLL	EEK			HVL
Pfs1 12474	OG0009969	Peronosporales		0		RYLK	EER			
Pfs1 439	OG0009312	Peronosporales	0	RTLRL						
Pfs1 8208a	OG0010393	Peronosporales	1	RFLR	EER					
Pfs1 10382	OG0007491	Broad	SCN5a, TCP14	0	1	SALR	EER	LFLAK	HVL	
Pfs1 10418	OG0000255	Broad		0		KTLR	DEK			
Pfs1 10814	OG0000132	Broad		0		RGLR				
Pfs1 13378	OG0005883	Broad		0		RSLR				
Pfs1 3487	OG0000437	Broad		0		RKLR	DNK			
Pfs1 3927	OG0008595	Broad		0		RFLR				
Pfs1 4560	OG0000033	Broad		0		RSLQ				

Five (Pfs1|3172, Pfs1|3869, Pfs1|3487, Pfs1|9071 and Pfs1|9976) of the eight candidate effectors studied in this thesis are included in the list of conserved effectors in table 2 (**Chapter 3 and 4**). For example, Pfs1|3172 was found to interact with two membrane-anchored NAC transcription factors, AtNAC078 and AtNAC014. Pfs1|3172 is conserved between *H. arabidopsidis*, *P. belbahrii* and *Pfs*. The proteins have a conserved RxLR-EER motif and a C-terminal transmembrane domain. It is likely that these downy mildew proteins have similar functions in their respective host plants.

An interesting candidate identified using this shortlist is candidate RxLR effector Pfs1|439. It is conserved between *P. parasitica*, *P. capsici*, *P. sojae*, *P. belbahrii*, *Plasmopara halstedii* and *Plasmopara viticola* (**Fig. 1**). All proteins except Phal02046 are predicted to be secreted and the RxLR domain, located in the N-terminus, is strongly conserved. The C-terminal effector domain is more diversified although some residues are conserved, which is a common pattern for effector proteins [321]. The intra and inter species conservation of Pfs1|439 makes the effector an interesting candidate for further study of its virulence function. Studying the interaction between these conserved effectors and their interactors may aid the identification of conserved S-genes that may be employed for resistance breeding.

Signal peptide RxLR

Figure 1. Interspecies conservation of candidate RxLR effector Pfs1439.

Candidate RxLR effector Pfs1439 is conserved between species in the Peronosporales order; *P. infestans* (PITG_11947), *P. parasitica* (PPTG_03152, PPTG_21216), *P. sojae* (PHYSODRAFT_246483), *P. capsici* (Pcap_19600, PPTG_22005), *Pl. halstedii* (Phal02046), *Pl. viticola* (PVIT_0005941), *P. belbahrii* (PBEL_07925). Only one representative protein of each species is included in the alignment and PVIT_0005941 was left out because it has a ~200 amino acid insert that creates a gap in the alignment.

Outlook

The sequencing and assembly of the reference genome of *Pfs1* gave way to the identification and characterization of RxLR and CRN effectors. In this thesis I describe the identification of the potential virulence targets of the first 27 candidate host-translocated effector proteins. Eight candidate effectors and fourteen interacting plant proteins were further characterized to provide a first insight into the possible role of individual effectors during *Pfs*-infection. This study confirmed co-localization of the majority of the studied plant proteins with the candidate effectors within *Benthamiana nicotiana* cells. In some cases, co-expression of the interactor and effector induced subcellular relocalization of either protein, an additional indication of a physical interaction between the proteins. After confirmation of the interaction by co-IP or FRET-FLIM the question that remains is how the interaction with the effector alters the function of the plant protein during infection. A quick way to mimic the inhibition or stabilization of a plant protein by its interaction with the effector is to study plants in which the interactor proteins are mutated, silenced or overexpressed. Disease assays on Arabidopsis T-DNA insertion lines, mutated in genes encoding interactors identified in **chapter 3** and further studied in **chapter 4**, using disease assays with *H. arabidopsidis* could quickly reveal differences in susceptibility to downy mildew infection. The advantage of using *H. arabidopsidis* is that the T-DNA insertion lines are available, and the finding of altered susceptibility would suggest a role of the plant protein in susceptibility that is not restricted to the *Pfs* – spinach interaction. Ideally, the assays should be performed in spinach because the effector interactors may be specific for *Pfs* effectors and hence may not have an effect on the susceptibility of Arabidopsis to *H. arabidopsidis*. Silencing of the interactor genes in spinach could be achieved by RNAi mutagenesis.

Developing reliable and high-throughput effector screening assays for application in spinach is of crucial importance to improve the understanding of *Pfs* effector functions. Especially the delivery of proteins into spinach tissue should be optimized. We found that expression of

fluorescent effector-fusions in spinach leaves yielded few transformed cells and lower levels of expression compared to fusion proteins expressed in *N. benthamiana* using the same vector (CaMV 35S promotor) (**Chapter 4**). Perhaps the expression levels could be improved by using a different promotor.

To improve effector delivery, other systems of protein delivery systems could be explored. Previously the expression of *Hpa* RxLR29 fused to a type III secretion signal in *Pseudomonas syringae* pv. *tomato* (*Pst*) was shown to suppress activation of the plant immune response, indicating the potential for the use of bacteria for delivery of oomycete effectors in planta [321]. Plant transformation methods independent of *Agrobacterium* T-DNA transfer, like particle bombardment, may also be tested and optimized for spinach plants [322]. (Stable) transformation or genome editing of spinach plants would aid the functional analysis of interactor genes in disease susceptibility in future experiments.

Finally, research on obligate biotrophic pathogens like *Pfs* would greatly benefit from the ability to manipulate and transform the pathogen. Systematic introduction or knock-out of host-translocated effectors in different *Pfs* races would greatly enhance the understanding of the contribution of individual effectors to virulence. Transformation of *Phytophthora* protoplasts or zoospores with plasmids containing effectors fused to fluorescent proteins is now quite well established, although not easy [323, 324]. Additional challenges include wrong localization of proteins, formation of insoluble aggregates and instability of transgene expression [325]. Transformation protocols have been adjusted for specific species and sometimes races to improve expression, hopefully this will be accomplished for obligate biotrophic oomycete species in the near future as well.

Revealing the function of conserved effectors will greatly aid resistance breeding in crops. Most forms of resistance used in breeding, so far, are based on recognition of effectors by R-loci. Due to redundancy in the effector repertoire, oomycetes can easily lose effectors that are recognized, hence avoiding their recognition. However, conserved effectors may not be dispensable as they form the Achilles heel of the pathogen, so R-genes against those effectors may be more durable.

Additionally, employing natural or induced variation in the plant proteins that interact with conserved effectors to avoid interaction possibly results in more durable resistance of the plant. When the interaction between effector and plant protein is abolished the effector will no longer be able to manipulate the underlying plant process resulting in reduced fitness of the pathogen or elimination of the infection all together. If the interacting plant protein is a conserved effector target, variation in its sequence may be applicable to a wider range of crop species and pathogens [308]. Taken together, the study of conserved effectors and their interacting plant proteins provides a great opportunity to understand the molecular interaction between plants and pathogens and translate that knowledge to resistance breeding.

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References

Summary

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About the author

List of publications

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Summary

Peronospora effusa (*Pfs*)* is the most important pathogen of cultivated spinach worldwide. It is an obligate biotrophic oomycete that has a filamentous appearance and grows solely on spinach plants. The visible symptoms include irregular chlorotic spots and gray to brown sporangial masses, primarily on the underside of the leaf. Specialized hyphae form 'haustoria' that penetrate the plant cell walls and form the site of interaction between pathogen and plant.

During infection, oomycete pathogens are known to secrete proteins called effectors. Effectors promote infection by suppressing the plant immune system and manipulation of other plant cell processes. Secreted effectors can either function in the plant tissue (apoplast) or be translocated into plant cells and function there. These host-translocated effectors manipulate processes as broad as immune- and hormone signaling, transcription/translation, production of ROS, and vesicular trafficking (**Chapter 1**).

To get a better understanding of the molecular interaction between *Pfs* and its spinach host the genome of the first reference strain, *Pfs1*, was sequenced, assembled and annotated (**Chapter 2**). The sampling of *Pfs1* material from leaves resulted in co-sampling of other foliar microbes and plant DNA. Hence the retrieved sequences are derived from multiple organisms and can be considered a metagenome. We used the CAT tool to filter sequences that were not derived from *Pfs* from the genome pre-assembly. The CAT tool calls all ORFs on the contigs of the pre-assembly and assigns taxonomy based on the annotation of the ORFs. The reads assigned to contigs that preliminary had ORFs that were not derived from *Pfs* were removed from the assembly. The filtered reads were used to create a clean and improved assembly. The CAT tool can thus be successfully used to filter eukaryotic contigs. The obtained *Pfs1* genome assembly was 32.4 Mb consisting of 8,635 scaffolds (*k*-mer prediction 36.2Mb). The BUSCO analysis estimated an 88.9% genome completeness. Transcript sequencing of a range of infection time points aided the prediction of a total of 13,277 gene models.

We compared the translated gene models to those of other plant-infecting oomycete species that have different lifestyles. Specifically, we looked into the proteins that are predicted to be secreted (secretome) by the oomycete and may hence be involved in the infection process. We identified common features in the predicted secretomes of different obligate biotrophic oomycetes, regardless of their phylogenetic distance. Their secretomes are generally smaller, compared to hemi-biotrophic and necrotrophic oomycete species. Based on the presence and absence of functional domains, we observe a reduction in proteins involved in cell wall degradation, in Nep1-like proteins (NLPs), proteins with PAN/apple domains, and host-translocated effectors.

Downy mildew species have two known types of effectors that are translocated into the plant cells during infection, the RxLR and CRN effectors. We found that the genome of *Pfs1* encodes 99 candidate RxLR and 14 CRN effector proteins. By comparing them to the proteins of other species we found that 39 candidate effectors are specific to *Pfs*, 10 to downy mildew species, 23 to both downy mildew and *Phytophthora* species, and 40 are found in more distantly related species like *Pythium* and/or *Albugo*. Generally, effector repertoires of different species are highly variable, possibly as a result of positive selection imposed by the plant immune system. Especially, the C-terminal effector domain, that is thought to be responsible for effector function inside plant cells, is often highly variable. Nevertheless, a small number of RxLR effectors are conserved among phytopathogenic oomycetes, possibly because they contribute significantly to virulence. These effectors may target conserved interacting plant proteins that are involved in the infection process and contribute to plant disease susceptibility. The identification of Arabidopsis proteins that interact with *Pfs* effectors supports the idea of conserved plant targets across species (**Chapter 3**).

One of the more conserved *Pfs* RxLR proteins showed similarity to the *Hyaloperonospora arabidopsidis* (*Hpa*) HaRxL44 effector. HaRxL44 is known to interact and degrade Mediator Subunit 19a, thereby enhancing ethylene and jasmonic acid signaling and ultimately suppressing salicylic acid-triggered immunity. Also, we found that seven of the CRN proteins group into one family that has expanded within the *Pfs* genome. These proteins are phylogenetically related to a group of *Hpa* CRN effectors that are remarkable because they have a RxLRLFLAK motif, that shows characteristics of both the RxLR and LFLAK putative translocation domains.

A total of 33 candidate RxLR and CRN effectors of *Pfs1* were cloned and used as baits in a yeast two-hybrid screen to identify interacting plant proteins from an Arabidopsis oligo-dT primed cDNA library. In these assays the first 46 plant proteins that interact with RxLR and CRN effectors of *Pfs* were identified. A protein interaction network was created with the identified proteins and supplemented with reported protein-protein interactions from the plant-pathogen literature. We found that nine interactors of *Pfs* effectors were targeted by effectors from other bacterial, fungal and/or oomycete pathogens. These highly connected plant proteins like CSN5A, TCP14, and FBN1A likely play important regulatory roles in the plant cell during infection. Other *Pfs* effectors interact with less connected proteins. Some of these, like HBI1, two NAC transcription factors and two prenylated Rab acceptor (PRA1) proteins, have been linked to disease susceptibility in other plant species before but for most of them their biological function is unknown.

Eight candidate effectors and their fourteen interacting plant proteins were selected to further study their interaction in planta (**Chapter 4**). The spinach orthologs of the Arabidopsis proteins were identified and the subcellular localization of the candidate effectors, interacting proteins and spinach orthologs was assessed using confocal microscopy. The effector proteins were found to localize to the nucleus, cytoplasm, ER and punctate structures. All but two of the 14 tested Arabidopsis interactors and their spinach orthologs localized with the candidate effector in the same subcellular compartment. The subcellular localization of six plant

interactors and two effector proteins was altered upon co-expression of the effector and interactor proteins. Furthermore, seven plant proteins showed a more stable signal when co-expressed with the effector. Further studies are needed to confirm the interaction between the proteins and assess the role of the plant proteins in disease susceptibility or resistance.

The general discussion is focused on the genetic differences between saprotrophic and plant pathogenic species and explores the dynamics behind the remarkable small genomes of downy mildew species (including *Pfs*) (**Chapter 5**). Also, the unresolved taxonomic issues in the large and diverse taxa of *Pythiaceae* and *Peronosporales* are discussed. Finally, we supply the interspecies effector conservation data (**Chapter 3**) with data of the variation of effectors between races of *Pfs* to identify interesting targets for further research and breeding. Taken together, the study of conserved effectors and their interacting plant proteins provides a great opportunity to understand the molecular interaction between plants and pathogens and to translate that knowledge to resistance breeding.

**Pfs* was previously known as *Peronospora farinosa forma specialis spinaciae*

Samenvatting

Peronospora effusa (*Pfs*)*, of valse meeldauw, is wereldwijd de meest voorkomende ziekte van het spinazie. *Pfs* is een oomyceet met een schimmelachtig voorkomen die uitsluitend groeit op levende planten. De zichtbare symptomen van de infectie zijn onregelmatige lichte vlekken op de bladeren en een bruine tot grijze ‘vacht’ van sporendragers en sporen dat voornamelijk zichtbaar is aan de onderkant van de bladeren.

De hyfen groeien in het plantenweefsel tussen de cellen, waar gespecialiseerde structuren door de celwanden van de planten heen breken en haustoria vormen. Haustoria doen dienst als platform waar uitwisseling van moleculaire componenten tussen de oomyceet en plant plaatsvindt. Oomyceten scheiden tijdens de infectie eiwitten uit die effectoren genoemd worden. Effectoren kunnen actief zijn in het plantenweefsel tussen de cellen of over de celmembraan van de plantencel worden getransporteerd. Effectoren dragen bij aan de infectie door het immuunsysteem van de plant te onderdrukken en door diverse celprocessen te beïnvloeden. Voorbeelden hiervan zijn beïnvloeding van transcriptie/translatie-gerelateerde processen, het verstoren van de hormoonbalans, de productie van reactieve zuurstof die een rol spelen bij de afweerreactie en het transport van eiwitten (**Hoofdstuk 1**).

Om een beter beeld te krijgen van de moleculaire interactie tussen *Pfs* en spinazie hebben we het genoom van het referentie-isolaat *Pfs1* in kaart gebracht (**Hoofdstuk 2**). Het verkrijgen van DNA van *Pfs* werd bemoeilijkt doordat *Pfs* uitsluitend op levende planten kan groeien. Het verzamelde materiaal bevatte daarom naast DNA van *Pfs* ook DNA van andere micro-organismen die op het blad voorkomen. Er werd een filteringstap toegepast om de verkregen ‘DNA reads’ en daaruit samengestelde ‘DNA contigs’ te selecteren op basis van hun herkomst. De filtering werd gedaan met behulp van de CAT tool. Deze methode identificeert de open leesramen (ORFs), of genen, op elk contig en bepaald de taxonomische herkomst hiervan. De contigs met overwegend ORFs die niet afkomstig zijn van *Pfs* werden geïdentificeerd en uit de DNA set gefilterd. Deze studie is de eerste die laat zien dat de CAT tool

succesvol eukaryote genomen kan filteren. Het verkregen *Pfs1* genoom had een grootte van 32.4 Mb en bestond uit 8,635 scaffolds (*k*-mer voorspelling 36.2Mb). Naar schatting is het genoom voor 88.9% compleet (BUSCO). Met behulp van RNA, geïsoleerd van samples verzameld op verschillende infectietijdspunten, werden 13,277 genmodellen voorspeld op het genoom.

De eiwitten die gecodeerd worden door bovengenoemde genen werden vergeleken met de eiwitten van andere plant-infecterende oomyceten met uiteenlopende levensstijlen. Hierbij lag de nadruk op de eiwitten die mogelijk door de oomyceten worden uitgescheiden, het secretoom, omdat deze mogelijk een rol spelen in het infectieproces. We ontdekten dat de secretomen van soorten die uitsluitend op levende planten kunnen groeien opmerkelijke overeenkomsten hadden, onafhankelijk van hun fylogenetische afstand tot elkaar. De secretomen van obligaat-biotrofe soorten waren over het algemeen kleiner dan die van hemi-biotrofe of necrotrofe soorten. Gebaseerd op de aanwezigheid van bekende functionele eiwitdomeinen vonden we een lagere abundantie van gesecreteerde eiwitten die betrokken zijn bij celwandaafbraak, van Nep1-like proteins (NLPs) en van eiwitten met PAN/apple domeinen alsmede van effectoren die functioneren in plantencellen.

Valse meeldauw soorten hebben twee typen effectoren die in de plantencel terechtkomen tijdens de infectie. Deze RxLR en CRN effectoren interacteren met planteneiwitten om celprocessen te beïnvloeden. In het genoom van *Pfs1* hebben we 99 RxLR en 14 CRN kandidaat effectoren gevonden. In een vergelijking met andere soorten zagen we dat 39 effectoren specifiek zijn voor *Pfs*. Voor de andere effectoren zijn verwante eiwitten gevonden in andere soorten. Voor 10 effectoren zijn er alleen verwante eiwitten in andere valse meeldauwsoorten, voor 23 zijn er verwanten in zowel andere valse meeldauwsoorten als in *Phytophthora* soorten en de laatste 40 effectoren zijn daarnaast ook geconserveerd in de minder verwante *Pythium* en/of *Albugo* soorten (**Hoofdstuk 3**).

In de regel zijn er weinig overeenkomsten tussen de effectorrepertoires van verschillende soorten, mogelijk doordat de effectoren onder evolutionaire druk staan om herkenning van het

plantenimmuunsysteem te voorkomen. Met name het effector domein dat verantwoordelijk is voor de functie van effectoreiwitten is vaak sterk variabel. Ondanks deze trend zijn er een aantal RxLR effectoren bekend die voorkomen in meerdere soorten en sterke overeenkomsten vertonen. Deze effectoren zijn mogelijk behouden gebleven doordat ze een belangrijke rol hebben in het infectieproces. Mogelijk interacteren deze effectoren met geconserveerde planteneiwitten die een rol spelen in de vatbaarheid van de plant voor de ziekte.

Zo hebben we in *Pfs* een effector gevonden die vergelijkbaar is met *Hyaloperonospora arabidopsidis* effector HaRxL44. HaRxL44 induceert de afbraak van Mediator Subunit 19a. De afbraak leidt tot een toename in ethyleen en jasmonzuur en daarmee indirect tot een onderdrukking van de immune reactie die gereguleerd wordt door salicylzuur. Daarnaast liet het vergelijkingsonderzoek zien dat zeven van de CRN effectoren van *Pfs* aan elkaar verwant zijn en zijn ontstaan binnen *Pfs*. Fylogenetisch zijn ze onder anderen verwant aan een opmerkelijke groep CRN effectoren van *Hpa* die zowel een mogelijk RxLR als een LFLAK (CRN) motief hebben. Deze motieven zijn karakteristiek voor de RxLR en CRN effectoren en spelen mogelijk een rol in het transport van de eiwitten over het membraan van de plantencel.

Drieëndertig kandidaat *Pfs* effectoren werden gekloneerd en gebruikt als 'bait' in een 'yeast two-hybrid' studie. De effectoren werden gebruikt om interacterende eiwitten uit een verzameling van Arabidopsis eiwitten te selecteren (vanuit een "oligo-dT primed yeast two-hybrid cDNA library"). In totaal werden er 46 interacties gevonden tussen planteneiwitten en 18 effectoren. De gevonden planteneiwitten gelden als de eerste *Pfs*-interacterende planteneiwitten ooit geïdentificeerd. De interacties werden weergegeven in een interactienetwerk met daarin ook interacterende planteneiwitten geïdentificeerd met effectoren van andere soorten. Het netwerk liet zien dat negen van de eiwitten die interacteerde met *Pfs* effectoren ook interacties aangingen met effectoren van andere (bacteriële, schimmel, of oomyceet) ziekteverwekkers. Deze sterk verbonden eiwitten, zoals CSN5a, TFP14 en FBN1A, spelen mogelijk een belangrijke regulerende rol in de plantencel tijdens de infectie. Een aantal van de gevonden eiwitten zoals HBI1 en twee NAC transcriptiefactoren,

werden al eerder in verband gebracht met ziektegevoeligheid in andere soorten maar voor de meeste van de 46 eiwitten is de biologische functie nog niet bekend.

Acht effectoren en veertien interacterende *Arabidopsis* eiwitten werden uitgekozen voor verder onderzoek (**Hoofdstuk 4**). Voor deze *Arabidopsis* eiwitten werden de spinazie orthologen geïdentificeerd en meegenomen in het onderzoek. De effectoren en planteneiwitten werden gefuseerd met fluorescente eiwitten en de locatie in de plantencel werd in kaart gebracht met behulp van confocale microscopie. De effectoreiwitten waren zichtbaar in verschillende cellulaire locaties zoals de celkern, het cytoplasma, het ER en als kleine spikkels verspreid door de cel. Twaalf van de veertien *Arabidopsis* eiwitten en hun spinazie orthologen bevonden zich in dezelfde cellulaire compartimenten als de corresponderende effectoren. De locatie van zes planteneiwitten en twee effectoren veranderde wanneer ze tegelijk tot expressie werden gebracht. Daarnaast waren er zeven planteneiwitten waarvan het signaal beter zichtbaar werd in aanwezigheid van de effector. De interactie tussen de eiwitten en de rol van de interacties tijdens de infectie zullen worden bevestigd in toekomstige experimenten.

In de algemene discussie ligt de nadruk op de genetische verschillen tussen saprotrofe en pathogene oomyceten en ga ik verder in op de opmerkelijk kleine genomen van de valse meeldauwsoorten (waaronder *Pfs*) (**Hoofdstuk 5**). Daarnaast ga ik in op de taxonomie van de plant infecterende oomyceten en de problemen in de fylogenetische indeling van met name de soorten in de *Pythiaceae* en *Peronosporales*. Daarnaast integreer ik de gegevens over de conservering van effectoren tussen soorten (**Hoofdstuk 3**) met informatie over conservering van effectoren binnen *Pfs* isolaten. De gecombineerde data leveren een lijst op van interessante kandidaten voor verder onderzoek en voor toepassing bij het veredelen van resistente gewassen.

Het bestuderen van geconserveerde effectoren en de eiwitten waarmee ze interacteren is een unieke kans om inzicht te krijgen in de moleculaire interactie tussen planten en ziekteverwekkers en levert waardevolle informatie op voor de resistentieveredeling.

**Pfs* stond oorspronkelijk bekend als *P. farinosa forma specialis spinaciae*

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About the author

Manon Neilen was born on 14 March 1990 in Utrecht, the Netherlands. She completed her secondary education, VWO, at Openbare Regionale Scholengemeenschap (ORS) Lek en Linge in Culemborg in 2008. She started her Biology Bachelor at Utrecht University during the same year and obtained her Bachelor of Science degree in 2011. She continued her studies by enrolling in the Environmental Biology Master

at the same University. During her Master studies, Manon carried out two research projects. The first one was at Naturalis Biodiversity Centre within the Fungal Biology group under supervision of Dr. József Geml. In her project she worked on the environmental sequencing of the *Salix*-associated ectomycorrhizal fungal community in the arctic tundra and Dutch sand dune ecosystems. In her second project she studied the diversity of bacterial and fungal decomposers in agricultural soils in a collaborative project between the institute of risk assessment (IRAS, UU, Dr. Heike Schmitt) and Netherlands institute of ecology (NIOO, Prof. dr. Eiko Kuramae). After her graduation in 2014, Manon worked as an assistant researcher Phytopathology at Enza Zaden in Enkhuizen. In February 2015, she started as a PhD candidate at the Plant-Microbe Interactions group at Utrecht University under the supervision of Prof. dr. Guido van den Ackerveken. During her PhD Manon worked on comparative genomics and host-translocated effectors of the spinach downy mildew, *Peronospora effusa*.

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