Proteinaceous effector discovery and characterisation in filamentous plant pathogens

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Summary

The complicated interplay of plant-pathogen interactions occurs on multiple levels as pathogens evolve to constantly evade the immune responses of their hosts. Many economically important crops fall victim to filamentous pathogens which produce small proteins called effectors to manipulate the host and aid infection/colonisation. Understanding the effector repertoires of pathogens is facilitating an increased understanding of the molecular mechanisms underlying virulence as well as guiding the

development of disease control strategies. The purpose of this review is to give a chronological perspective on the evolution of the methodologies used in effector discovery from physical isolation and *in silico* predictions, to functional characterisation of the effectors of filamentous plant pathogens and identification of their host targets.

Key words: Effectors, fungal phytopathogens, oomycete phytopathogens, bioinformatic effector predictions, in planta methodologies, effector host-target interactions

Word Count: 9335

1. Introduction

"If people think nature is their friend then they sure don't need an enemy."
— Kurt Vonnegut

1.1 The threats from filamentous phytopathogens.

Our expanding global population forces us to intensify our crop production as we prepare to feed 2.2 billion more people by 2050. One of the main biotic challenges facing society to meeting these ever-growing demands are filamentous plant pathogens. Oomycetes and fungi are the causal agents of some of the most notorious plant diseases and are a true threat to our global food security and community structures. Plant disease outbreaks have occurred throughout human history, some of the most infamous include the Irish potato famine caused by the oomycete *Phytophthora infestans* (Turner, 2005), Panama disease caused by

Fusarium oxysporum f. sp. *cubense* (Gordon, 2017) and wheat stem rust caused by *Puccinia graminis* f. sp. *tritici* (Roelfs, 1985, Singh et al., 2011)

1.2 Effectors and the plant immune response

The elegantly described 'Zig-Zag' model by Jones and Dangl (2006) describes a two-tier immune response where pathogen associated molecular patterns (PAMPS) are first detected on host cell surfaces by pattern recognition receptors (PRRs) inducing pattern-triggered immunity (PTI). To evade this response, pathogens secrete effector proteins that manipulate the host and aid colonisation, yet in hosts that have the corresponding resistance (*R*) genes (Flor, 1971), these effectors are detected by receptors such as the intracellular Nod-like receptors (NLRs) that induce effector triggered immunty (ETI) resulting in a hypersenstive response HR and programmed cell death (de Wit, 2016, Zhang et al., 2017).

Just as with all models, the story is more complicated and not all features of the plant-microbe interactions are accommodated. Effectors can be highly conserved, thus not under selective pressure to evade host detection, such as the members of the oomycete Crinkler (CRN) effector family or the core fungal effector NIS1 (Depotter and Doehlemann, 2019, Irieda et al., 2019) whilst other effectors are detected extracellularly (van der Burgh and Joosten, 2019).

Recent studies suggest that, rather than a two-tier system of immunity, ETI and PTI activate different but interacting pathways leading to plant immunity. The activation of the paired *Arabidopsis* NLRs RRS1-R

and RPS4 by the bacterial effector AvrRps4 cannot induce HR without the presence of PAMPS (Ngou et al., 2020). Both co- and pre-delivery of AvrRps4 with PAMPS leads to an increased and prolonged expression of PTI associated defence genes such as BIK1, BAK1 and Rboh; the expression of these genes is not induced by effectors alone (Ngou et al., 2020). Similarly, ETI responses in *Arabidopsis* mutants lacking PRRs are greatly compromised, with the ETI induced ROS production being mediated by PRRs (Yuan et al., 2020). This suggests that PTI is a required component of ETI with mutual potentiation of immune mechanisms triggered by intracellular and cell-surface receptors.

1.3 The importance of effector research

Hundreds of small proteins, predicted to be effectors, are secreted by filamentous phytopathogens during host colonisation (Dean et al., 2005, Kämper et al., 2006, Yoshida et al., 2009, Duplessis et al., 2011). We have little understanding of the function of most of these putative effectors and each typically shares minimal or no sequence homology to proteins with previously defined functions. However, the effector repertoire of a pathogen is a major determinant of host specialisation and can greatly impact whether the plant-pathogen interaction is successful or not based on the genotype of the host (Raffaele et al., 2010, Sánchez-Vallet et al., 2018).

Molecular studies have characterised over 60 fungal effectors across multiple species, however this barely makes a dent in the candidate effector repertoire for each pathogenic species (Sperschneider et al., 2015). For example, the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* alone is suspected to have roughly 7% of its genome encoding candidate secreted effector proteins (CSEPs) (Pedersen et al., 2012).

Identifying and characterising the function of effector proteins will improve our understanding of their role in disease formation and influence our future strategies to combat them. Fundamental effector research is a key part of devising new plant disease control strategies and this is detailed further in **Sections 3.2** and **6** of this review. Effectors play an important role in crop breeding where, as well as being used to detect resistance genes in new cultivars, characterised effectors can be used to locate susceptibility loci in vulnerable crops (Vleeshouwers and Oliver, 2014). The development of mobile sequencing technology means that genes encoding effectors can also be used to detect the emergence of new strains of crop pathogens in the field and elude to the severity of future disease outbreaks (Radhakrishnan et al., 2019). Effectors function in multiple way including inhibiting host enzymes, modulating plant immune responses and targeting host gene-silencing mechanisms. All features of effectors described in this article are summarised in **Table 1** including their mode of action where known.

<table 1.>

2. The chronological perspective of finding effectors

"There is nothing like looking, if you want to find something."

— J.R.R. Tolkien, ***The Hobbit or There and Back Again***

2.1 The proteomics approach

Some of the most well characterised effector proteins come from the biotrophic fungal pathogen *Cladosporium fulvum*, the causal agent of tomato leaf mould and an early model system for fungal effector discovery. *C. fulvum* avirulence (Avr) effectors are a classic example of the gene-for-gene model. The detection of the Avr effector by the host carrying the cognate resistant (*R*) gene can induce a strong immune response in the plant and inhibit *C. fulvum* colonisation (Flor, 1971, De Wit et al., 1986).

Early *in planta* studies took advantage of the fact that *C. fulvum* only colonises the tomato leaf apoplast. Secreted proteins could be isolated by collecting apoplastic wash fluid from *Cf*-infected tomato leaves and studying the effects of this fluid on a range of tomato varieties (De Wit et al., 1985). When fluid collected from plants infected with *C. fulvum* races harbouring the *avr9* gene was infiltrated into the near isogenic tomato leaves carrying the *Cf-9* gene a strong hypersensitive response (HR) was triggered. Treating this fluid with proteases confirmed the Cf-9 mediated HR was triggered by proteinaceous entities (De Wit et al., 1986). The subsequent purification of the small Avr9 (**Figure 1**) then led to the first

fungal *Avr* gene to be cloned, whilst its low expression profile *in vitro* suggested for the first time that the host plant plays an important role in inducing *Avr* expression (Schottens-Toma and de Wit, 1988, Van den Ackerveken et al., 1992, van Kan et al., 1991, Van den Ackerveken et al., 1994). The mature Avr9 is a 28 amino acid protein with a high percentage of cysteines (n=6), features that become important in many subsequent effector identification stories (van Kan et al., 1991).

This apoplastic proteomics approach was successfully used to identify additional small cysteine rich *C. fulvum* effectors such as Avr4 (Schottens-Toma and de Wit, 1988, van den Burg et al., 2006) and was employed to identify Six1 (Avr3) and Six3 (Avr2), in *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) (Rep et al., 2004, Houterman et al., 2007, Houterman et al., 2009).

2.2 Homology searches

Once an effector has been cloned, the sequence can be used to identify homologous candidates in closely related species. Three elicitins were isolated from *Phytophthora* sp. using proteomics techniques; cryptogein (*P. cryptogea*), cinnamomin (*P. cinnamomi*) and capsicein (*P. capsici*) (Huet and Pernollet, 1989, Ricci et al., 1989). Primers were deigned based on conserved regions of the elicitin amino acid sequences and used to probe cDNA libraries from *P. parasitica* leading to the discovery of the host-specific elicitor protein PARA1 (Kamoun et al., 1993).

2.3 Genetic mapping

Prior to the genomics era, the isolation of Avr proteins from intracellular colonising fungal pathogens such as *Magnaporthe oryzae* and haustoria producing pathogens was unsuccessful using the proteomics approach. Instead, in the case of the rice blast fungus *M. oryzae*, map-based cloning techniques were used to clone Avrs such as Avr1-CO39 (Farman and Leong, 1998). Avr1-CO39 was mapped to a region on Chromosome 1 by a series of backcrosses of the progeny of the virulent isolate Guy11 and the avirulent isolate 2539 (Smith and Leong, 1994). Later, a chromosome walking strategy led to the physical mapping and identification of Avr1-CO39. The identity of the Avr1-CO39 locus was confirmed by transforming the virulent Guy11 strain with cosmids from the Avr1-CO39 genetic interval. This resulted in a loss of pathogenicity on rice cultivars containing the corresponding functional CO39 resistance gene (Farman and Leong, 1998).

2.4 Always lagging behind

By the end of the 20th century, over 30 bacterial *Avr* genes had been cloned and characterised by screening cosmid libraries, with almost all of these coming from two host-specific species of *Pseudomonas* and *Xanthomonas* (Leach and White, 1996, De Wit, 1997). In comparison using proteomics and genetic mapping, only eight fungal phytopathogen Avr genes had been successfully identified and confirmed to be effectors (Laugé and De Wit, 1998). But all this was about to change.

2.5 Sanger and Next Generation Sequencing (NGS) of pathogen genomes

In the early 2000s, the Fungal Genome Initiative (FGI) was established following the publication of a white paper (Birren, Fink and Lander, 2003) to promote the sequencing in the public domain of fungal genomes belonging to species important to human health, agriculture and industry. By 2017 a total of 191 genomes of fungal plant pathogens had been sequenced including the economically important *M. oryzae, Fusarium graminearum* and *Botrytis cinerea* (Dean et al., 2005, Cuomo et al., 2007, Amselem et al., 2011, Dean et al., 2012, Aylward et al., 2017). This, together with the publication of numerous oomycete genomes including the late potato blight pathogen *Phytophthora infestans* (Haas et al., 2009) as well as extensive *in planta* and *in vitro* transcriptome datasets, has led to an explosion in effector discovery. These techniques for effector discovery are summarised in table 2.

<table 2>

3. Refining effector prediction

"Truth, like gold, is to be obtained not by its growth, but by washing away from it all that is not gold."
— Leo Tolstoy

3.1 Secretion

As the de Wit et al. studies demonstrated, a key feature of effectors is secretion by the pathogen into the host (De Wit et al., 1985, Asai and Shirasu, 2015). Therefore, early studies in effector discovery using sequencing data focused on the predicted secretome.

In a bid to identify extracellular effector proteins, Torto et al. (2003) used their PEX-finder algorithm to mine transcript datasets of the potato pathogen *Phytophthora infestans*. The algorithm searched for a specific amino acid sequence known as a signal peptide followed by a cleavage site commonly found at the N- terminus of secreted proteins (Nielsen and Krogh, 1998, Torto et al., 2003). Of the 261 cDNAs predicted to code for secreted proteins, 78 had no matches to those found in the public databases, a feature common to candidate effectors. Using high-throughput functional expression assays this study led to the discovery of a large complex family of effectors called crinklers (CRNs) which are found throughout the pathogenic oomycetes (Schornack et al., 2010, Amaro et al., 2017).

However, some characterised secreted effectors lack a signal peptide. For example, the effectors, PsIsc1 and VdIsc1, produced by *Phytophthora sojae* and *Verticillium dahliae,* respectively, have been shown to be unconventionally secreted into the respective host to suppress salicylate (SA) -mediated defences *in planta* (Liu et al., 2014).

Another difficulty is that such broad criteria leaves a large pool of possible effector candidates that are demanding in both time and resources to functionally characterise with studies often having low

discovery rates. The *M. grisea* effector MC69, essential for appressoria formation (Motaung et al., 2017), was the only candidate from 1306 putative secreted proteins that was required for pathogenicity following large-scale gene disruptions (Yoshida et al., 2009, Saitoh et al., 2012).

3.2 Domains

The *C. fulvum* effector Ecp6 sequesters the fungal cell wall protein chitin, preventing chitin fragment detection by the host PRRs and thereby evades a host immune response (De Jonge et al., 2010). Ecp6 contains LysM domains which bind to chitin with ultra-high affinity therefore outcompeting host immune receptors (Sánchez-Vallet et al., 2013). The LysM domain found in Ecp6 has now been identified in over 302 putative effectors from 62 published fungal genomes, and is conserved among effectors targeting the chitin detection aspect of plant immunity (De Jonge and Thomma, 2009, Lee et al., 2014).

One the other hand, the Avr2 effector from *C. fulvum* and the EPIC1 and EPIC2 effectors from *P. infestans* both target the tomato defence protease Rcr3 (Song et al., 2009) yet are unrelated and share no sequence similarity; thus relying on the presence of conserved domains could cause many possible candidates to be overlooked.

3.3 Motifs

The first four oomycete Avr effectors cloned, ATR13 and ATR1^{NDWsB} from the downy mildew *Hyaloperonospora parasitica* (Allen et al., 2004, Rehmany et al., 2005), Avr3a from *Phytophthora infestans* (Armstrong et

al., 2005) and Avr1b-1 from *P. sojae* (Shan et al., 2004) showed no sequence similarity except for two conserved motifs at the N-terminus. These RxLR and DEER motifs have since been identified as N-terminal host targeting domains and, in *P. infestans,* the RxLR motif in the Avr3a effector is required for translocation into potato cells (Bos et al., 2010, Whisson et al., 2007).

RxLR effectors have been identified in multiple *Phytophthora*, *Albugo* and *Hyaloperonospora* species, with 568 RxLR genes being found in *P. infestans* alone, making this the largest oomycete effector family to date (Anderson et al., 2015). Rapid variation and host specialisation is attributed to the general lack of sequence similarity in filamentous pathogen effectors, yet this mostly contributes to the variation in the C-terminus of oomycete effector sequences, leaving the N- terminal motifs largely conserved (Win et al., 2007). Conserved motifs such as RxLR and the more downstream DEER are used as powerful bioinformatic tools to isolate putative effector repertoires from genomic sequences (Jiang et al., 2008, Raffaele and Kamoun, 2012).

Within pathogenic fungi there is limited evidence for conserved translocation motifs. One possible exception is the [YFC]xC motif found in *Blumeria graminis* f. sp. *hordei* and *Puccinia spp,* members of the phyla *Ascomycota* and *Basidiomycota*, respectively (Godfrey et al., 2010, Duplessis et al., 2011). The evolutionary distance between these two fungi suggest a deep homology in the conservation of this motif, linked to a biotrophic lifestyle that uses haustoria-based feeding.

The general lack of sequence similarity, however, or conserved domains, means that bioinformatic approaches to effector prediction needs to go beyond sequence homology.

3.4 Structure

The structural properties of proteins are more highly conserved than amino acid sequences (Illergård et al., 2009) and therefore, could be used as a tool for effector prediction. The structural similarities between the two sequenced *M. oryzae* effectors AVvr1-CO39 and Avr-Pia were found using two- and three dimensional NMR experiments (de Guillen et al., 2015) and led to the discovery of the *Magnaporthe* Avr and ToxB-like effector family (MAX) which contains half of all cloned *M. oryzae* Avrs despite sharing less than 25% sequence identity (de Guillen et al., 2015).

The structural analysis of four RxLR oomycete effectors showed the presence of a conserved C- terminus 3-α- helix fold (Boutemy et al., 2011, Yaeno et al., 2011). This 'WY' domain, named after the interacting tryptophan and tyrosine residues, hints to a core, stable protein scaffold as a source of protein function (Wirthmueller et al., 2013).

Resolving the structure of known effector proteins provides a useful tool for supporting the candidacy of putative effectors. One of the early effectors to be structurally resolved was ToxA produced by the tan spot fungus, *Pyrenophora tritici-repentis.* The ToxA crystal structure was resolved using X-ray crystallography (1.65Å) and revealed a novel β-sandwich fold (Sarma et al., 2005). Later, the resolution of the flax rust, *Melampspora lini,* effectors AvrL567-A and -D showed a similar β-sandwich

fold hinting at the structural homology of unrelated effector proteins (Wang et al., 2007).

Recently the structures of two candidate effectors in the poplar rust fungus*, Melampsora larici-populina,* were resolved using NMR. One, MLP124266, is the first fungal protein to present a knottin-like structure (Postic et al., 2017) whilst the other, MLP1124499, shares structural similarity with members of the Nuclear Transport Factor-2 (NTF2) superfamily. In both cases these candidate effectors show no sequence homology with structurally similar proteins and are the first examples of effectors with these structures (de Guillen et al., 2019).

3.5 Rich in cysteines but not in size

The additional criteria for candidate effector selection often requires secreted proteins to be small and cysteine-rich (Sperschneider et al., 2015). The presence of multiple cysteines enables the formation of stabilising disulphide bridges (De Wit et al., 1986, Doehlemann et al., 2009,).

Relying on such broad criteria can be problematic as despite many known effectors sharing these features, these are not universal requirements. NIS1, first described in the cucumber anthracnose fungus *Colletotrichum orbiculare* (Yoshino et al., 2012) is conserved across both *Basidiomycota* and *Ascomycota* (Irieda et al., 2019), but contains no cysteines.

Relying on the size of mature peptides as a parameter for effector identification can also be problematic. The maximum size of a ‘small’ protein in effector discovery can be anything from 150 to 400 amino acids

(Bowen et al., 2009, Saunders et al., 2012b). However, even the larger size limits would exclude the *P. graminis* f. sp. *tritici* effector AvrSr35 with a mature length of 578 amino acids (Salcedo et al., 2017).

With these issues in mind, bioinformatic pipelines have been developed to encompass multiple criteria to refine effector prediction.

3.6 Bespoke bioinformatic pipelines

Saunders et al. developed an *in silico* analysis pipeline that moved away from reliance on sequence similarity based methods for effector identification and included physiological functions such as expression profiles, taxonomic information and genomic features of potential candidates (Saunders et al., 2012b). To identify the repertoire of potential effectors within two rust fungus genomes, a clustering algorithm grouped candidates into families and ranked their likelihood of being effectors based on the knowledge that filamentous pathogen effectors have a least one of eight specific properties. These properties included; the absence of recognised Pfam domains, similarities to haustorial proteins and the presence of internal repeats. The number of candidates continued to functional analysis using this pipeline was greatly reduced (Saunders et al., 2012b). This approach has limitations as it is dependent on the thresholds based on *a priori* assumptions about effector properties; the number of missed effectors remains to be seen.

At each step of the general pipeline for effector prediction and subsequent characterisation *in silico* tools, whether bioinformatical software or web-based servers, have been developed to aid effector

refinement. The presences of signal peptides, transmembrane motifs or GPI anchors can all be predicted using tools such as SignalP (www.cbs.dtu.dk/services/SignalP/), TMHMM (www.cbs.dtu.dk/services/TMHMM/) and PredGPI (gpcr.biocomp.unibo.it/predgpi/pred.htm) which use neural networks or hidden Markov modelling to recognise motifs within protein sequences associated with these features (Pierleoni et al., 2008, Armenteros et al., 2019). The subcellular localisation of candidate effectors can also be predicted by searching for chloroplast or mitochondrial transit peptides or nuclear localisation signals using tools such as WoLF-PSORT (wolfpsort.hgc.jp/) or LOCALIZER (localizer.csiro.au/) (Horton et al., 2007, Sperschneider et al., 2017). Machine learning has also resulted in the development of web-based tools that can predict with 89% accuracy whether proteins in the predicted secretome are effectors or not. EffectorP2.0 (effectorp.csiro.au/) takes into account the net charge and serine/cysteine content of proteins to prioritise candidate effectors for further functional validation (Sperschneider et al., 2018).

3.7 Genomic landscape and transposable elements

Many fungal plant pathogens exhibit a ‘two-speed’ genome with distinct compartments within the genome evolving at different rates. Alongside ‘core’ stable regions, which are slow to evolve and often contain genes involved in metabolism, are hypervariable areas withhigh recombination and richness in repetitive sequences, including transposable elements (TEs). This genomic landscape and the presence of TEs serve to

drive adaptive evolution (Faino et al., 2016) and these hypervariable regions often are the location of genes associated with pathogenicity, including effectors (Fouché et al., 2018, Jones et al., 2018).

In *M. oryzae* and *Zymoseptoria tritici,* TEs are associated with pathogenicity clusters and are seen to flank the 1st characterised *Z. tritici* effector, AvrStb6 (Bao et al., 2017, Zhong et al., 2017). TEs have also been shown to interfere with effector gene expression via epigenetic control. For example, AvrLm1 in *Leptosphaeria maculans*, located in a TE rich genomic region, showed distinct histone methylation that acts to temporarily suppress expression during colonisation to evade host recognition (Soyer et al., 2014, Fouché et al., 2018). This suggests that the variability of the genomic region or the proximity to TEs maybe useful factors in refining the search for candidate effectors.

Following the sequencing, genome assembly and annotation of the tumour-forming maize smut fungus *Ustilago maydis*, ~18% of genes encoding secreted proteins were found to be arranged into twelve discrete clusters within the genome (Kämper et al., 2006). These clusters were co-regulated by a central pathogen-development regulator and expression induced in tumour tissue. Deletions of five clusters caused clear changes in virulence including the largest cluster, 19A, which caused a strong attenuation in virulence and reduced tumour formation upon deletion (Kämper et al., 2006, Brefort et al., 2014). Subsequent sub-deletions of 19A members led to the identification of the effector Tin2, required for anthocyanin production (Brefort et al., 2014, Tanaka et al., 2014).

3.8 Comparative Genomics

By comparing the genomes of *U. maydis* and *Sporisorium reilianum,* Schirawski et al. (2010) found that effector clusters and pathogenicity related regions were more highly diverged between the close relatives than the rest of the genome. This comparison led to the identification of the *pit* gene cluster involved in tumour formation in *U. maydis* (Doehlemann et al., 2011). Within this cluster the secreted effector Pit2, involved in plant defence suppression and cysteine protease inhibition, was found (Doehlemann et al., 2011, Mueller et al., 2013). This same comparison was used to locate gene clusters and candidate effectors in *S. reilianum*, and whilst genes that have a partial impact on disease severity have been identified, as of yet no candidates strongly attenuate virulence (Ghareeb et al., 2019).

3.9 Lineage specific elements

Novel effectors were identified in the asexual fungus *Verticillium dahliae*, where chromosome reshuffling has led to the formation of lineage specific (LS) regions of plasticity in the genome (de Jonge et al., 2013). These LS regions are enriched with retrotransposon and repetitive sequence elements, as well as being the location of many candidate effectors. Contrary to the ‘two-speed’ genome hypothesis, these LS regions show strong levels of conservation with little to no SNPs being identified, even within the intergenic regions (Depotter et al., 2019). In one such LS region, four putative effectors were identified including the LysM domain

413 containing effector Vd2LysM which was only found in the VdLs17 strain (de
414 Jonge et al., 2013).
415

416 **3.10 Sequence divergence**

417 Molecular variation in filamentous phytopathogen genes is known to
418 be essential for altering pathogen-host interaction outcome and can
419 provide insight into the evolution of virulence (Allen et al., 2008).
420 Polymorphisms in effector sequences among isolates can impact on
421 virulence and are involved in host adaptation; this makes them promising
422 targets for disease control strategies.
423 The genomes of four isolates of the wheat yellow stripe rust fungus
424 *Puccinia striiformis* f. sp. *tritici* (Pst), were re-sequenced and assessed for
425 single nucleotide polymorphisms (SNPs). Proteins that displayed non-
426 synonymous substitutions between Pst isolates that differed in virulence
427 on specific wheat cultivars were identified (Cantu et al., 2013). This led to
428 five secreted polymorphic candidate effectors being refined for further
429 characterisation from a predicted secretome of 2,999 proteins.
430 This sequence divergence has also proved useful in identifying
431 pathogens in the field. Using the Oxford Nanopore MinION sequencer, 242
432 highly variable genes were used to collect real-time population dynamics
433 data of Pst isolates in Ethiopia (Radhakrishnan et al., 2019). This Mobile
434 And Real-time PLant disEase (MARPLE) diagnostic system can be used to
435 monitor for the emergence of plant pathogen strains, but can also be
436 adapted to include newly characterised effectors within the panel of

genes. Going forward, MARPLE will allow for the monitoring of mutations and the detection of effector evolution that may be linked to gain of virulence of phytopathogens, all within the confines of the field.

3.11 Association mapping in the sequencing era.

In silico predictions of effectors, whilst allowing us to rapidly screen whole genomes for candidates, lack discriminatory power and often result in candidate effectors having no clear impact on pathogen virulence. Genome wide association studies (GWAS) and quantitative trait loci (QTL) mapping can identify loci associated with heritable phenotypic variation, such as virulence, therefore can complement techniques to identify and clone Avr effectors recognised by known host resistance proteins (Plissonneau et al., 2017).

The *Zymoseptoria tritici* effector AvrStb6 was isolated in this way (Zhong et al., 2017). Using crosses between two Swiss strains of *Z. tritici,* QTL mapping found a confidence interval containing nine candidates for *AvrStb6*. Combining this with a GWAS study from over 100 different natural isolates led to one candidate, a small cysteine-rich secreted protein that was not presentin the original *Z. tritici* genome annotation (Zhong et al., 2017).

An additional benefit of using GWAS in effector discovery is that the natural variation in SNP calling identified in wild populations can be used to quantify how each SNP contributes to pathogen virulence (Sánchez-Vallet et al., 2018). Integrating GWAS with transcriptome dataset, referred to as transcriptome-wide association studies (TWAS)

(Wainberg et al., 2019) identified the link between genes and traits across populations and has been used to discover *Blumeria graminis* f. sp. *hordei* Avr_a effectors including Avr_{a9} (Saur et al., 2019a).

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4. Functional characterisation.

"Make your work to be in keeping with your purpose"

— Leonardo da Vinci

4.1 Knock out or knock down - let's be disruptive.

One of the simplest ways to determine pathogenicity of a candidate effector is to disrupt the encoding gene and determine whether the virulence on a susceptible host or the Avr phenotype on a resistance genotype is compromised. Early transformation studies of the *C. fulvum* effectors relied on double homologous recombination (HR) to insert a selectable marker into the target gene encoding a knowneffector such as *ecp1* and *ecp2*, thus disrupting them (Laugé et al., 1997). Later sequencing technology allowed transformations without the need for cloning. Mutants of the corn smut fungus *Ustilago maydis* were made using PCR based protocols combined with protoplast transformation to create candidate effector knock-out mutants (Schulz et al., 1990, Kämper, 2004). This method is widely used and has successfully facilitated the functional characterisation of *U. maydis* effectors including Rsp3 and Cce1 (Ma et al., 2018a, Seitner et al., 2018).

Agrobacterium tumefaciens mediated transformation (ATMT) is another method to disrupt genes and is widely used in plant transformations. ATMT was first used in fungi in budding yeast in 1995 and then the technique was adapted for use in filamentous fungi, including *M. oryzae* (Bundock et al., 1995, Rho et al., 2001). This method relies on the targeted insertion of a selectable marker into the fungal genome from a disarmed Ti plasmid of transformed *Agrobacteria* to disrupt the gene of interest. The selectable marker is incorporated into the fungal genome via homologous recombination, a process that occurs easily in yeast. This mechanism, however, is highly variable in filamentous fungi, where non-homologous end-joining (NHEJ) appears to be the dominant DNA repair pathway over HR (Meyer et al., 2007, Villalba et al., 2008). The Ku70 protein is part of a complex that regulates the NHEJ pathway (Ninomiya et al., 2004), and its deletion has led to the increase of HR in *M. oryzae* from <25% to 80% (Kershaw and Talbot, 2009). Combining ATMT with the generation of Δ*Ku70* mutants led to the characterisation of the *Z. tritici* Avr effector AvrStb6 (Zhong et al., 2017).

Another, more recent, method of gene disruption is using the genome editing system CRISPR-Cas9. Originally identified as an immune mechanism in bacteria and archaea, the CRISPR-Cas9 system is used greatly as a genome-editing tool in plants, animals and was adapted by Nødvig et al. (2015) for use in filamentous fungi (Mali et al., 2013, Fauser et al., 2014, Nødvig et al. 2015). This technique has led to the targeted gene disruption and consequent characterisation of effectors in the oomycete *Phytophthora*

sojae and the fungal pathogen *U. maydis* (Fang and Tyler, 2016, Schuster et al., 2018).

There are, however, difficulties in producing stable transformants in phytopathogens that are obligate biotrophs (Thomas et al., 2001, Lorrain et al., 2019). In these cases, knock-down technologies such as host-induced gene silencing (HIGS) are more successful. The HIGS assay, detailed in **Figure 2** has led to the identification of many effectors including the barley powdery mildew *Blumeria graminis* f. sp. *hordei* ribonuclease-like effectors BEC1054 and BEC1011 (Nowara et al., 2010, Pliego et al., 2013, Pennington et al., 2019).

Gene disruption assays do have their limitations even iwhen successful transformants are produced. Many effector mutants display no associated phenotype. Genetic redundancies, where multiple effectors have the same function, or buffering, where the host compensates or interfers in signalling by using alternative pathways, may result in false negative results (Hillmer et al., 2017, Tyler, 2017).

4.2 *In planta* expression

When a candidate effector is heterologously expressed *in planta* various functional assays can be used to determine the virulence activities of the protein.

Necrosis assays monitor for the induction of hypersensitive response (HR)-like cell death which can be a result of Avr/R protein/guardee protein interactions or be directly induced by the candidate effector. These assays were first carried out using the model plant *Nicotiana tabacum,* which is

infiltrated with transformed *Agrobacteria* that delivers the effector gene expressed from a inducible promotor into the plant cell for transient protein production (Kamoun et al., 1999, Qutob et al., 2002, Ma et al., 2012,).

In 1999 the *P. infestans* and *C. fulvum* effectors *Inf1* and*Avr9* respectively were transformed into either wildtype or *Cf-9* transgenic *N. tabacum*using this method. The assay showed that INF1 was capable of inducing necrosis in wild-type tobacco whist Avr9 could only do so in transgenic tobacco expressing the corresponding R-gene *Cf-9 (*Kamoun et al., 1999*).* Later *Avr9* and *Cf-9* were transiently co-expressed in *N. tabacum* using agroinfiltration to confirm the induction of HR in the non-host plant following expression of the Av/R gene pairs (Van der Hoorn et al., 2000).

Effector characterisation in non-host dicotyledonous model plants maybe more suited to high-throughput screening than in cereal hosts. However, these highly artificial scenarios do have several limitations. A negative screen with no visible phenotype upon recombinant expression may indicate either the candidate is not an effector or the effector target/receptor is lacking in the model species. Whereas HR induced necrosis in an effector screen may not be caused by an specific effector/target interaction but by non-host resistance (NHR) triggered by detection of the candidate (Kettles et al., 2017). Though of interest, by definition the latter scenario would not occur in native host interactions. Therefore expression assays in the native host maybe the more useful for functional characterisation.

Candidate effectors can be transiently expressed in protoplast cells and cell death monitored via the reduction in expression of a co-transfected

556 reporter gene such as GUS (β-glucuronidase) or luciferase (Chen et al.,
557 2006, Lu et al., 2016). This approach was used to identify the cell death
558 inducing properties of five *M. oryzae* effectors including MoCDIP4 (*M.*
559 *oryzae* cell death inducing protein 4), in rice protoplasts (Chen et al., 2012)
560 and the NLR-meditated recognition of four newly identified barley powdery
561 mildew avirulence effectors, including $AVR_{a9,}$ in barley (Saur et al., 2019a).
562 Cell-death suppression assays are used to detect the alteration ofthe
563 plant immune response induced by a known cell death elicitor. The
564 overexpression of the stem rust candidate effector PSTha5a23 in *N.*
565 *benthamiana* suppresses *P. infestans* INF1 triggered cell death, indicating
566 that PSTha5a23 plays a role in controlling plant defence responses (Cheng
567 et al., 2017).
568 An alternative method of expressing effectors in plant cells uses the
569 bacterial type III secretion system (T3SS) derived from the tomato bacterial
570 speck pathogen *Pseudomonas syringe* pv *tomato* (DC3000) (He et al.,
571 2004). This system was first adapted for filamentous plant pathogens by
572 Sohn et al. (2007) to deliver oomycete effector proteins into *Arabidopsis*.
573 Sohn et al. showed that, by fusing the downy mildew (*H. parasitica*)
574 effectors ATR1 and ATR13 to the N-terminal secretion-translocation signals
575 of the *P. syringae* effectors AvrRpm1 and AvrRps4, the effectors could be
576 secreted into *Arabidopsis* plant cells and contribute to pathogen virulence.
577 Since then, the T3SS has been used to functionally characterise candidate
578 effectors from multiple oomycetes including *P. infestans* and *H.*
579 *arabidopsidis* (Whisson et al., 2007, Fabro et al., 2011). Despite T3SS being
580 used to screen candidate effectors of stem (*P. graminis* f. sp. *tritici)* and

bean rusts (*Uromyces appendiculatus)* , this system is rarely used for fungal effector characterisation and has limited success on cereals (Upadhyaya et al., 2014, Saur et al., 2019b, Qi et al., 2019). These problems are linked to the required unfolding and refolding of effectors prior to insertion, especially those rich in cysteine-cysteine bridges.

As well as monitoring for necrosis, or lack thereof, the *in planta* growth of another pathogenic species can be used as a proxy to determine the role in virulence candidate effectors play. Stable transformants of the non-host *Arabidopsis* that expressed ecandidate poplar rust fungus (*Melampsora larici-populina*) effectors were inoculated with the oomycete pathogen *H. arabidopsidis*. Eleven of sixteen effectors tested supported greater sporulation of this native *Arabidopsis* pathogen suggesting that the effectors had the capacity to interfere with processes in a non-host plant to favour pathogenesis (Germain et al., 2018).

4.3 The viral overexpression (VOX) system

Due to the limited effectiveness of both T3SS and Agrobacteria mediated transient expression in most cereal species, viruses have been developed as efficient vectors for heterologous protein expression (VOX) (Lee et al., 2012).

The barley stripe mosaic virus (BSMV) was first verified as a tool for protein expression when used to overexpress the luciferase reporter gene in protoplast cells and later to express GFP *in planta* (Joshi et al., 1990, Haupt et al., 2001, Lawrence and Jackson, 2001). The BSMV vector was adapted for use in the VOX system and used to characterise the function of

the fungal effector ToxA (Manning et al., 2010) (**Figure 3**). However, the compact nature of the virus results in a negative correlation between fragment size and stability of the viral vector (Avesani et al., 2007, Bruun-Rasmussen et al., 2007).. BSMV-VOX has been widely used for heterologous expression of proteins up to 150 amino acids, however as previously stated there is no agreed size limit for an effector (Bouton et al., 2018) (**Figure 3a**).

Another limitation of BSMV for use in effector discovery is that this virus has a tripartite RNA genome (**Figure 3b**). The heterologous protein is inserted into the RNAγ-genome yet all three sub-genomes are required to combine for successful expression *in planta* making BSMV-VOX unsuitable for high-throughput screening assays.

The foxtail mosaic virus (FoMV) has been adapted for use in VOX systems in cereals (Bouton et al., 2018). Vectors derived from FoMV such as PV101 avoid many of the caveats of those from BSMV. FoMV has a monopartite RNA genome and the PV101 vector can be used to successfully express proteins up to 600 amino acids in size. In addition, unlike BSMV vectors, PV101 allows for heterologous expression of proteins in their native form, including possible signal peptides, without the need for processing from proteases which may only be 90% efficient (Bouton et al., 2018). In situations where the effector expressed from the VOX vector rapidly triggers R protein mediated defences, virus spread is halted and therefore the phenotypic readout in the bioassay is the lack of systemic spread of the recombinant virus (Saintenac et al., 2018).

4.4 Where do they go?

Knowing the localisation of candidate effectors within host tissues not only demonstrates that the protein can be translocated from the pathogen to its host, but also suggests where the effector target(s) may be found. Traditionally *in situ* hybridisation assays were done where antibodies were raised against the effector or an added epitope tag and detected using transmission electron microscopy (TEM). Translocation of fungal effectors into the host call was first shown using an immunocytochemical approach in rusts. The gold- and fluorescence-labelling of four independently raised antibodies to the RTP1p protein in *Uromyces fabae* and its homolog in *Uromyces striatus* showed that in later stages of infection RTP1p translocated from the extra-haustorial matrix to inside the plant cell itself (Kemen et al., 2005).

For apoplastic effectors, localisation was often determined by means of their isolation. The *C. fulvum* effectors Avr2, Avr4 and Ecp6 were directly isolated from the apoplast fluid, whereas the *P. infestans* protease inhibitor EPIC1 was isolated from the apoplast after antibodies were raised (Bolton et al., 2008, Joosten et al., 1997, Rooney et al., 2005, Tian et al., 2007). Whilst successful, these approaches are laborious, expensive and not suited to high-throughput screening of either apoplastic or cytoplasmic effector candidates (Dalio et al., 2017).

The nuclear localisation of the *P. infestans* CRN effectors was determined using N-terminal GFP tagging and confocal microscopy. By overexpression five GFP-CRN (without the signal peptide) fusion proteins *in*

planta the effectors were shown to accumulate within plant cell nuclei (Schornack et al., 2010). High-throughput screening of 61 candidate effectors (ChECs) from the anthracnose fungus, *Colletotrichum higginsianum,* using this method found that whilst nine of the ChECs were imported into the nucleus, others localised to the Golgi bodies, microtubules and peroxisomes; all novel targets for fungal effectors (Robin et al., 2018).

The *U. maydis* effectors Cmu1 and Tin2 have been shown to localise to the maize cytoplasm however this could not be demonstrated when fluorescently tagged (Djamei et al., 2011, Tanaka et al., 2014, Tanaka et al., 2015) (). This may be due to the tags inhibiting the partial unfolding of the effectors, thereby preventing their translocation, or the incorrect refolding of the tag themselves upon entering the cytoplasm (Lo Presti et al., 2015).

Whilst investigating the translocation of *M. oryzae* effectors in to rice cells, fluorescent tagged cytoplasmic effectors were seen to first accumulate in the plant-membrane derived infection structure the BIC (biotrophic interfacial complex) prior to delivery into the cytoplasm, whereas tagged apoplastic effectors localised to the invasion hyphae (Mosquera et al., 2009, Khang et al., 2010). The BIC's role in effector translocation could only be confirmed by the addition of nuclear localisation signal (NLS) to cytoplasmic effectors causing artificial accumulation in the nucleus of the neighbouring rice cells. This approach concentrated the fluorescent signal into discrete foci observable using live cell imaging (Khang et al. 2010).

For apoplastic effectors it is difficult to distinguish between apoplastic or cytoplasmic localisation when the fluorescently tagged candidate effectors appear to localise to the plasma membrane or cell wall. Enlarging the apoplastic space by the stepwise addition of hypertonic solutions, a process known as plasmolysis, revealed that the *U. maydis* host-peroxidase inhibitor Pep1 was indeed apoplastic and was evenly distributed throughout the enlarged space (Oparka, 1994, Doehlemann et al., 2009,).

Alternatively, the BirA assay does not require the use of large fluorescent tags that may interfere with effector function or localisation. BirA, developed by Lo Presti et al, is based on the bacterial enzyme biotin ligase which biotinylates any protein that has a short (15 aa) peptide Avitag (Lo Presti et al., 2017). Maize lines that expressed the biotin ligase in the cytoplasm were infected with transformed *U. maydis* strains that had either the Cmu1 or the Tin2 effectors tagged with the Avitag. Biotinylation was detected via immunoprecipitation of extracted proteins using streptavidin-coated magnetic beads, thus confirming the tagged effectors had met the biotin ligase in the host cytoplasm (Lo Presti et al., 2017).

5. Effector interactions

"To manage a system effectively, you might focus on the interactions of the parts rather than their behaviour taken separately."

-Russel L. Ackoff

Arguably the Holy Grail of effector characterisation is to identify the exact molecular targets of each effector and/or the molecules used by the plant to bind to them. This can lead to defining the precise sequences and molecular interactions occurring at the point(s) of direct contact. The former is very challenging because the effector sequences do not give many clues as to their function(s).

5.1 A shot in the dark- unbiased screening

Unbiased "forward" screening to find protein – protein interactions (PPI) is a common technique used in many aspects of molecular biology. The yeast-two- hybrid system (Y2H), first developed 30 years ago, allows for the large scale screening of cDNA libraries derived from pathogen-infected plants for effector target identification (Fields and Song, 1989, Mukhtar et al., 2011). Interactions detected by Y2H screens must be validated by additional PPI assays as this approach is prone to false positives.

The most common Y2H validation technique is co-immunoprecipitation (Co-IP). Co-immunoprecipitation is used to screen effector interactors in heterologous systems. When 20 candidate poplar rust fungus (*Melampsora larici-populina*) effectors were tagged with GFP and expressed in *N. benthamiana,* five were found to specifically interact with plant proteins by pull down assays using anti-GFP followed by protein purification (**Figure 4a**) (Petre et al., 2015).

Biotinylation is also used for proximity labelling (PL) based on tools such as BioID (Li et al., 2017). A benefit of PL over co-immunoprecipitation is the possibility of identifying proteins that only weakly or transiently interact with the target (**Figure 4b**). Recently a new PL tool, TurboID had been shown to provide more efficient labelling *in planta* compared to BioID and can also reduce the biotin incubation time from 16 hours to 10 mins (Branon et al., 2018, Zhang et al., 2019). These new advances in PPI technology pave the way for higher-throughput effector interaction screening *in planta*.

5.2 Split-marker complementation (SMC)

The effector Pep1 is essential for the pathogenicity of the corn smut fungus *U. maydis* (Doehlemann et al., 2009). The direct interaction between Pep1 and the plant peroxidase POX12 was validated using the bimolecular fluorescence complementation (BiFC) assay (**Figure 4c**) which involves two parts of a fluorescence marker being fused to candidate interactors. Only when the interactors meet can the full length fluorescent marker assemble and be detected. Alternatively, the firefly derived enzyme luciferase can be used forSMC . This has the advantage over BiFC for *in planta* studies because luciferase does not require excitation by light for detection thereby eliminating auto-fluorescence interference(Li et al., 2011). However, using SMC for PPI validation is not infallible as heterologous overexpression of proteins in *N. benthamiana* can affect protein localisation and therefore interactors.

5.3 Structural interactions – pinpointing the surface contacts and their strengths

Knowledge of effector structures whilst in complex with their targets gives us a greater insight into the molecular basis of these cross-kingdom interactions.

The *C. fulvum* effector Avr4 was one of the first to be characterised from a family of effectors that bind to and protect fungal cell-wall chitin from host chitinase (Joosten et al., 1997, van den Burg et al., 2006). Recently the crystalline structure of Avr4 in complex with its chitin ligand (resolved to 1.95Å) has highlighted the residues required for this function (Hurlburt et al., 2018). Structural mutant studies have also shown that recognition of the Avr4 by the cognate Cf-4 immune receptor does not depend on the same ligand binding as previously thought (Hurlburt et al., 2018).

The crystal structure of the rice intracellular NLR immune receptor Pik in complex with the *M. oryzae effector* Avr-Pik (1.6Å resolution) reveals molecular details of the recognition event, that leads to HR-induced cell death (Maqbool et al., 2015). The effector surface involved in this interaction was also identified as being involved in the surface interactions between Avr-Pia and the NLR-RATX1 in *M. oryzae* (Ortiz et al., 2017).

In the past decade protein structures are increasingly being resolved without the need to form crystals or use damaging X-rays but by using cryo-electron microscopy (Cryo-EM). This technique is widely used to resolved proteins in complexes and has been used to show both inactive Arabidopsis

NLR complex ZAR1-RKS1 and the intermediate form when the complex interacts with a protein modified by the bacterial effector AvrAC (*Xanthomonas campestris* pv. *campestris)* (Wang et al., 2019). Cryo-EM, despite gaining popularity in structural biology, is unable to resolve proteins smaller than 65kDa, a size exclusion that would include many fungal and oomycete effectors (Muench et al., 2019).

The strength of effector/target interactions can determined by using isothermal titration calorimetry (ITC) whereby direct measurement of the heat that is either released or absorbed during the molecular binding event gives a complete thermodynamic picture of the reaction including affinity, enthalpy and stoichiometry (Duff Jr et al., 2011). For the conserved *M.oryzae* MAX effector Avr1-CO39, ITC was used to confirm that direct interaction with the heavy-metal associated (HMA) domain of the rice NLR RGA5 was required for effector binding. (Guo et al., 2018).

A greater understanding of how structural interactions aid the specificity of Avr recognition are vital for future work in developing sustainable disease resistance in important food crops.

6. Exploiting effector discoveries to control crop plant diseases.

“Knowing is not enough, we must apply. Willing is not enough, we must do.”

– Bruce Lee

The ultimate goal of effector discovery, from identification to characterisation to target interactions, is to apply this knowledge to the control of multiple pathogens that threaten our food security.

6.1 'Effectoromics'

For over 100 years disease resistance loci have been introduced into crops and subsequently shuffled through traditional breeding techniques, whether that be as individual genes or stacked to achieve often only short-lived resistance to pathogens (Vleeshouwers et al., 2011, Langner et al., 2018). Despite this, the search for novel resistance (*R*) genes with durable or broad-spectrum resistance remains ongoing.

The term 'effectoromics' is used to describe the use of effectors in high-throughput screening for R protein function in either the germplasms of crop cultivars or a sexually compatible species. Avr effectors can be harnessed to screen rapidly for HR phenotypes, a hallmark of an ETI response (Vleeshouwers and Oliver, 2014). Well established techniques of transient over-expression of Avrs using viral vectors such as PVX (potato virus X) in conjunction with agro-infiltration have been widely used for the identification and cloning of *R* genes in *Solanaceous* species such as potato, tomato and wild *Solanum* species (Takken et al., 2000, Du et al., 2014).

The search for broad-spectrum or more robust *R*- genes for breeding purposes maybe more nuanced than previously thought as multiple unrelated *R* genes can recognise the same pathogen effector (Aguilera-Galvez et al., 2018).

6.2 Screening with necrosis inducing effectors to remove host susceptibility loci

The necrosis inducing effector ToxA was isolated from the wheat tan spot fungus *P. tritici-repentis (Ptr)* in 1996. Infiltration of purified ToxA into the apoplastic space of a susceptible wheat cultivar containing the Tsn1 susceptibility (*S*) gene, is itself sufficient to induce tan spot symptoms (Tomas et al., 1990, Ballance et al., 1996, Ciuffetti et al., 1997, Welti and Wang, 2004). Wheat breeders routinely use the purified toxin to screen all new wheat germplasm to eliminate susceptible lines from their breeding programmes. This method is preferred over screening for molecular markers linked to the corresponding host susceptibility locus *Tsn1*, due to the ease of application and speed of results (Vleeshouwers and Oliver, 2014). *Tsn1* removal from all newly commercially released wheat varieties has improved resistance to tan spot disease and Australia has seen a 26% reduction in toxA-sensitive wheat grown in the ten years prior to 2016 (See et al., 2018).

7. Keeping track of effector discoveries in multiple species in an increasingly data rich world.

"A place for everything, and everything in its place"

– Mrs Beeton

In the past two decades effector discovery and characterisation has exploded with regards to crop pests and pathogens. This key information is found in multiple original research publications, review articles, in UniProt, individual pathogen genome browsers and species-specific website. However, to aid future research and guide the direction of work the genotype and fine phenotyping data surrounding these discoveries and new insights needs to be FAIR (Findable, Accessible, Interoperable and Reusable) to molecular plant pathologists as well as the wider life sciences communities.

Publicly available repositories of curated data regarding proteins with confirmed roles in pathogenicity and virulence are fundamental tool for effector study. The Pathogen-Host Interactions database (PHI-base, www.phi-base.org) is a manually curated database comprising of over 6,780 genes from 268 pathogens of over 210 hosts (September 2019), of which 60% are plants (Urban et al., 2020) . Within PHI-base (version 4.8), 799 interaction entries involve 731 distinct functionally characterised fungal or oomycete effectors, from over 40 species. Collectively, these

effector entries and their considerable metadata can be used for comparative studies, genome landscape explorations, the enrichment of transcriptome / proteome data sets, PPI network predictions, as well as the starting point for potentially novel artificial intelligence approaches.

8. Conclusions and outlook

'' Would you tell me, please, which way I ought to go from here?" "*That depends a good deal on where you want to get to.*"

- **Alice and The Cheshire Cat, *Alice in Wonderland.***

Effectors are the mysterious molecular tools evolved and utilised by plant pathogens in multiple ways. Effector studies are of vital importance in addressing the global food security challenge, yet the explosion in research efforts aimed at understanding effector biology over the last few decades has left us with a dichotomy in our knowledge. Due to early focus on a small number of pathosystems, whether due to experimental convenience or the economic impact of the disease, for some pathogens, such as *M. oryzae,* we have resolved 3D protein structures and know interacting surfaces of multiple effectors and their interactors. In other cases, important crop pathogens such as *Fusarium graminearum,* and the newly emerging pathogens *Ramularia collo-cygni* and *Corynespora cassiicola,* although several hundred candidate effectors have been

predicted, each lacks functional characterisation (McGrann et al., 2016, Lopez et al., 2018).

The arrival of full genome sequencing almost two decades ago has been a double-edged sword. Bioinformatic pipelines and the development of prediction software has sped up the refinement of putative effectors whilst simultaneous highlighting the vastness of the gene repertoires to be investigated. For effector characterisation, the future efficiency not only depends on the development of ultra-high-throughput functional assays but also their use in combination with lower throughput novel and well-established techniques such as QTL mapping and GWAS (Plissonneau et al., 2017).

Whilst multiple developments in effector discovery has increased our understanding of these enigmatic proteins arguably the explosion in effector research can be attributed to the development of three approaches: genome sequencing, bespoke bioinformatic pipelines and agrobacteria-mediated transient protein expression *in planta*. Armed with only an annotated genome, even in understudied conifer-infecting fungal pathogens can be screened for the presence of putative effector proteins (Raffaello and Asiegbu, 2017). With this in mind, genome re-annotations and improvements to prediction algorithms continuously widen the pool of effector candidates available, especially in well studied crop pathogens (Zhong et al., 2017, Frantzeskakis et al., 2018). Therefore, perhaps the greatest roadblock to effector discovery is the accuracy of genome assembly and annotation, an issue that will take at least 5- 10 years to resolve with the inclusion of pangenomes (Cissé and Stajich, 2019).

The genome annotation of multiple isolates through the construction of pathogen pangenomes allows for intraspecific genome analyse and will provide insight into the links between high polymorphisms and host-specificity. The use of pangenome analyses has already led to the differentiation between 'core' candidate effectors and 'novel' candidate effectors in *Z. tritici* and *M. oryzae* (Badet et al., 2019, Singh et al., 2019). Machine-learning based prediction tools as well as the robotic implementation of the practical molecular techniques, should help to fast track the progress from effector prediction to characterisation. This anticipated progress will undoubtedly erode some of the disparity in our interspecies knowledge and lift the veil on the enigmatic filamentous phytopathogen effector repertoire. Many novel functions, locations, interactions and generic underlying themes remain to be discovered.

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Figure Legends

Figure 1. A timeline showing the progression of filamentous plant pathogen effector prediction and identification from the pre-genomic era to the present day. The first effectors identified using these methods are included as well as the elicitins used for homology-based searches. Increasingly, pan-genome data is used to predict core and novel candidates but as yet none have been characterised by using this technique. For a recent review of pan-genomics see (Golicz et al., 2019). Details on individual effectors named are given in Table 1.

Figure 2. The HIGS construct encodes an inverted sequence that forms a hairpin dsRNA following transcription and is introduced into the host plant either by transient or stable transformation. The dsRNA is processed to form small interfering RNA (siRNA), either before or after delivery to the pathogen cell using the plants innate RNAi machinery. Once inside the fungal cells the siRNA silences the target effector genes by interfering with the target mRNA transcripts (Koch et al., 2018). The movement of small RNA between host and pathogen is detailed by Wang and Dean (2020).

Figure 3. The BSMV-VOX technology adapted from (Lee et al., 2012). A) virus-mediated overexpression (VOX) system. The heterologous protein coding sequence is inserted in the γ genome of BSMV, upstream of the in frame stop codon in the *γb* ORF. A gene for the autoproteolytic peptide 2A is also inserted between the 3′ terminus of the *γb* ORF and the gene of interest for processing the fusion protein during translation, thus releasing the heterologous protein of interest. B) The BSMV genome is composed of three RNAs that are capped at the 5′ end and form a tRNA-like hairpin secondary structure at the 3′ terminus. RNAα encodes the αa replicase protein containing methyl transferase and helicase domains. RNAβ encodes coat and movement proteins whilst RNAγ encodes the polymerase (POL) component of replicase, and the Cys-rich γb protein involved in viral pathogenicity.

Figure 4. Protein-Protein interaction techniques A) Co-Immunoprecipitation, effectors are tagged with a peptide sequences such as GFP and expressed *in planta*. Antibodies are used to pull down the protein complexes that can then be analysed using liquid chromatography and mass spectrometry (LC-MS/MS) (Petre et al., 2017). B) Biotinylation, effectors are fused to mutant biotin ligase enzymes and expressed *in vivo*. The fusion protein catalyses the biotinylation of interacting and proximal proteins in the presence of biotin. The biotinylated proteins are captured using streptavidin beads (Roux et al., 2012). C) Bimolecular fluorescence

complementation, the effector and putative interactors are tagged with non-fluorescent fragments of YFP. Direct interaction of the tagged effectors results in YFP reassembly visualised *in vivo* or quantified using flow cytometry (Graciet and Wellmer, 2010, Kerppola, 2008, Miller et al., 2015).