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James R. Alfano

University of Nebraska-Lincoln, jalfano2@unl.edu

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Roadmap for future research on plant pathogen effectors

James R. Alfano*

The Center for Plant Science Innovation and the Department of Plant Pathology, University of Nebraska, Lincoln, NE 68588-0660, USA

SUMMARY

Bacterial and eukaryotic plant pathogens deliver effector proteins into plant cells to promote pathogenesis. Bacterial pathogens containing type III protein secretion systems are known to inject many of these effectors into plant cells. More recently, oomycete pathogens have been shown to possess a large family of effectors containing the RXLR motif, and many effectors are also being discovered in fungal pathogens. Although effector activities are largely unknown, at least a subset suppress plant immunity. A plethora of new plant pathogen genomes that will soon be available thanks to next-generation sequencing technologies will allow the identification of many more effectors. This article summarizes the key approaches used to identify plant pathogen effectors, many of which will continue to be useful for future effector discovery. Thus, it can be viewed as a ‘roadmap’ for effector and effector target identification. Because effectors can be used as tools to elucidate components of innate immunity, advances in our understanding of effectors and their targets should lead to improvements in agriculture.

INTRODUCTION

Plant pathogens, including bacteria, fungi, oomycetes and nematodes, secrete proteins, referred to as effectors, into plant cells to favour parasitism (Block *et al.*, 2008; Davis *et al.*, 2008; Ellis *et al.*, 2009; Tyler, 2009; Zhou and Chai, 2008). Although the vast majority of effector activities and host targets remain unknown, at least a subset suppress innate immune responses (Abramovitch *et al.*, 2006; Boller and He, 2009; Espinosa and Alfano, 2004; Hein *et al.*, 2009). This suppression activity means that effectors can be used as tools to identify important components of innate immunity and can potentially lead to innovative strategies for crop improvement. For this article, the term ‘effector’ will be limited to proteins secreted by pathogens and translocated into plant cells where they exert specific functions, usually in collusion with other effectors exported by the same secretion/export system. Many effectors only subtly modify the host cell to sway the pathogen–plant interaction to the pathogen’s advantage. They are often functionally redundant with other co-secreted effectors. This narrow definition is similar to that recently used in a review on bacterial animal pathogen effectors (Galan, 2009) and is distinct from a more inclusive effector definition used in a recent review on plant pathogen effectors (Hogenhout *et al.*, 2009). The inclusive effector definition includes all pathogen proteins and small molecules that alter host cell structure and function. Although both definitions are appropriate, the narrower effector definition allows for a more focused discussion on the future research on plant pathogen effectors in this article.

A myriad of important discoveries regarding plant pathogen effectors have been made in the 25 years since the first plant pathogen effector was discovered by Staskawicz *et al.* (1984). Many of these discoveries are associated with bacterial pathogen type III protein secretion

systems (T3SSs): for example, the seminal discovery of *hrp* genes (Lindgren *et al.*, 1986), which were identified because they were required for both the defence-related hypersensitive response (HR) and pathogenicity, and were later shown to encode a T3SS present in many eukaryotic-associated bacteria (Van Gijsegem *et al.*, 1993). Another critical advance was the discovery that type III effectors were injected into plant cells, because it revealed that their plant targets were intracellular (Alfano and Collmer, 1996). More recently, the RXLR (arginine, any amino acid, leucine and arginine) motif in the N-terminus of some oomycete effectors was discovered, allowing for many more oomycete effectors to be identified (Birch *et al.*, 2008; Tyler, 2009).

What new discoveries will be made in the next 25 years and what technologies and/or approaches will allow them to be realized? One thing is for sure—next-generation sequencing technologies will provide the genome sequences of multiple strains of many plant pathogen species, greatly accelerating effector discovery. For newly sequenced pathogens, the initial focus will be to identify effector inventories and the translocation strategies that allow effector delivery into plant cells. For plant pathogens for which this is already established, the next decade promises to reveal plant targets of these effectors and the strategies that pathogens use to attack plants. We are therefore entering an exciting period for molecular plant pathology as we may learn the fundamental reasons why some organisms are pathogens, as well as important factors controlling host specificity and nonhost resistance.

This article summarizes the techniques and approaches likely to be used to identify plant pathogen effectors and their targets. New technologies or discoveries will of course also have an impact on effector research. However, effector and effector target discovery and confirmation will require multiple approaches. Figure 1 shows a flowchart summarizing these approaches and is referred to throughout this article. It represents a ‘roadmap’ for future research on plant pathogen effectors. The best starting point on this roadmap is at the genome sequence of a pathogen, which allows the most approaches to be employed. However, several approaches can be used without the benefit of sequence data. Important stops along the way are the identification of effector targets and how effector–target interactions promote the pathogenesis of plants. The final destination of this road trip is to exploit the information gained to engineer plants with increased pathogen resistance.

EFFECTOR DISCOVERY

The inundation of plant pathogen genome sequences is expected to result in the identification of a large number of plant pathogen effectors over the next decade. Considering that bacterial plant pathogen strains can have greater than 30 effectors each and oomycete strains several hundred (Lindeberg *et al.*, 2006; Tyler *et al.*, 2006), thousands of novel effectors may exist in bacteria, fungi, oomycetes and nematodes. The majority of bacterial effectors appear to suppress innate immunity (Block *et al.*, 2008; Boller and He, 2009; Guo *et al.*, 2009; Zhou and Chai, 2008). A clear exception is the large family of transcription activator-like (TAL) effectors found mostly in *Xanthomonas* pathogens. These effectors induce the transcription of susceptibility genes in the host plant (Kay and Bonas, 2009). Plant innate immunity can also be suppressed by several eukaryotic effectors (Bos *et al.*, 2006; Dou *et al.*, 2008a; Houterman *et al.*, 2008; Sohn *et al.*, 2007), but it is too early to know whether this is a common effector activity. The techniques currently employed to identify effectors are summarized in the top portion of Fig. 1. Additional bioinformatics approaches are greatly needed to more efficiently identify effectors, their activities and targets. Select approaches are discussed in more detail below.

The tried and true approach

A classic strategy to identify effector genes is to determine whether they induce effector-triggered immunity (ETI) when expressed in a virulent pathogen strain (Chisholm *et al.*, 2006). Indeed, it is this approach, pioneered by Staskawicz and colleagues, that was used to identify the first plant pathogen effector that this issue is commemorating (Staskawicz *et al.*, 1984). This tried and true approach is particularly successful because the main ETI response tracked, production of an HR, is easily and rapidly evaluated in the laboratory. A protein capable of inducing ETI was classically referred to as an avirulence (Avr) protein. It has become clear that many Avr proteins are effector proteins that plants have evolved the ability to recognize with resistance (R) proteins (Jones and Dangl, 2006). Map-based cloning based on ETI induction is an effective strategy to isolate *avr* genes from eukaryotic pathogens (Ellis *et al.*, 2009; O'Connell & Panstruga, 2006; Panstruga and Dodds, 2009). Another useful variation is to screen a collection of candidate effectors for their ability to induce ETI, as performed by Catanzariti *et al.* (2006). These authors identified a set of *Melampsora lini* genes induced in the haustoria (specialized pathogen structures that invaginate into plant cells) and found that several elicited the HR and co-segregated with independent *avr* loci. This classic approach will probably continue to be included in various effector hunting strategies.

Location, location, location

Comparative genomics can allow the identification of effector genes based on their location. For example, effector genes in bacterial pathogens are often clustered in pathogenicity islands (PAIs) (Kim and Alfano, 2002). These PAIs can be on plasmids or integrated into the chromosome. One common site for PAI integration is within or adjacent to tRNA genes. In addition, PAIs are often associated with mobile DNA elements (Hacker and Kaper, 2000). Thus, new effector genes can be identified by scanning genomes for regions of atypical GC content, codon usage and other relevant nucleotide statistics (Juhas *et al.*, 2009). Although this approach is less well established in eukaryotic pathogens, similar approaches are becoming important. For example, the *AvrLm1* effector gene from the fungal pathogen *Leptosphaeria maculans* is located in a gene-poor heterochromatin-like region of the genome (Fudal *et al.*, 2007; Gout *et al.*, 2006; Kamoun, 2007), suggesting that effector genes may be flanked by long intergenic regions. In addition, there are also examples in which fungal and oomycete effector genes are clustered (Kamper *et al.*, 2006; Win *et al.*, 2007). Therefore, genomes could be searched for both of these characteristics to identify or at least enrich for novel effectors.

It has to get out before it can get in

Type III effectors from the bacterial pathogen *Pseudomonas syringae* have secretion signals that share common biochemical characteristics in their N-terminal 50 amino acids (Collmer *et al.*, 2002; Guttman *et al.*, 2002; Petnicki-Ocwieja *et al.*, 2002). These characteristics allowed a bioinformatics search of the *P. syringae* pv. *tomato* DC3000 genome, yielding candidate effectors that were directly tested for their ability to be secreted and/or trans-located (Guttman *et al.*, 2002; Petnicki-Ocwieja *et al.*, 2002). More recently, other sequence-based bioinformatics tools have been developed to identify type III effector genes across bacterial genera (Arnold *et al.*, 2009; Samudrala *et al.*, 2009).

Researchers identifying effectors in fungal and oomycete pathogens have screened expressed sequence tags (ESTs) and genomes for genes encoding proteins with putative N-terminal type II secretion signals (Ellis *et al.*, 2009; Panstruga and Dodds, 2009). This has been extremely useful in conjunction with other approaches to enrich for effector genes. For example, Kemen *et al.* (2005) identified the Rust transferred protein 1 (*Uf-RTP1p*) by isolating *Uromyces fabae* haustorium-specific cDNAs using differential hybridization, which they then screened for putative type II secretion signals. A similar approach was used to identify a group of effectors from *Phytophthora infestans*, called crinklers, based on the presence of a putative

secretion signal and a leaf-criinkling and cell death phenotype when they were transiently expressed in plants (Kamoun, 2007; Torto *et al.*, 2003). However, fungal effectors have been identified that do not have N-terminal type II secretion signals (Ridout *et al.*, 2006), highlighting the need for additional effector search criteria.

A major advance in effector research, as noted above, was the recognition that many oomycete effectors possess an RXLR motif (Birch *et al.*, 2006; Rehmany *et al.*, 2005; Tyler *et al.*, 2006). This allowed the genomes of *Phytophthora* species and *Hyaloperonospora parasitica* to be searched for both a putative N-terminal secretion signal and an RXLR motif, facilitating the identification of oomycete effectors. Importantly, the RXLR motif is related to the 'Pexel' motif present in the N-termini of effectors from the malaria parasite *Plasmodium* (Hiller *et al.*, 2004; Marti *et al.*, 2004), which implicated the RXLR motif in translocation into plant cells. Oomycete researchers have recently confirmed that the RXLR motif is required for translocation into plant cells (Dou *et al.*, 2008b; Whisson *et al.*, 2007). The identification of additional searchable secretion/translocation signals or motifs will be important in identifying other types of eukaryotic effector.

Looking for known unknowns

There are many examples of the identification of effectors based on their similarity to known proteins or the presence of known domains or motifs. This includes similarities to effectors (usually Avr proteins) from other pathogens, to enzymes or to the presence of eukaryote-like domains and motifs (Armstrong *et al.*, 2005; Collmer *et al.*, 2002; Fouts *et al.*, 2002; Poueymiro and Genin, 2009). One of the first clues that type III effectors from bacterial plant pathogens were injected into plant cells was the recognition that the *Xanthomonas* AvrBs3 effector family possesses functional nuclear localization signals (NLSs) (Van den Ackerveken *et al.*, 1996; Yang and Gabriel, 1995). Although members of this effector family were already identified, the recognition of NLS motifs led to the discovery that they were injected into plant cells (Van den Ackerveken *et al.*, 1996) and acted as transcriptional activators (Zhu *et al.*, 1999). In addition, a family of *Ralstonia solanacearum* type III effectors was identified, in part, because of their plant-specific leucine-rich repeat (LRR) motifs (Cunnac *et al.*, 2004). These effectors, named GALAs after conserved amino acids within the LRRs, were later shown to contain putative F-box domains and, therefore, potentially to interfere with the plant ubiquitin–proteasome pathway (Angot *et al.*, 2006, 2007).

Searching for eukaryote-like motifs in candidate effector genes in prokaryote genomes is obviously easier than searching for them in eukaryotic genomes. However, it is still possible to employ this strategy to search for eukaryotic effectors if combined with other search criteria. For example, Mueller *et al.* (2008) first searched the *Ustilago maydis* genome for putative secretion signals and then winnowed down the candidates by searching for domains and motifs needed for proteins to function in the apoplast or inside eukaryotic cells. From the latter, they identified putative effectors possessing NLSs, zinc finger domains or RING domains, which suggested that they act inside plant cells.

Turn on only when needed

It is no wonder that energy-efficient microbes have evolved 'green' strategies to tightly regulate genes that are needed for specific purposes, such as survival and growth in plant tissue. Effector genes have been identified as a result of their *in planta* induction. Bacterial T3SSs (including effectors) are induced in plants by known regulatory proteins (Tang *et al.*, 2006). One strategy to identify type III effector genes is to identify promoters and/or genes dependent on these regulatory proteins (Buttner *et al.*, 2003; Collmer *et al.*, 2002; Cunnac *et al.*, 2004; Furutani *et al.*, 2009). In *P. syringae*, the alternate sigma factor HrpL binds to a conserved sequence in type III promoters, called the Hrp box. Searching for Hrp boxes in *P. syringae* genomes allowed

for the identification of many *P. syringae* effectors (Fouts *et al.*, 2002; Guttman *et al.*, 2002; Zwiesler-Vollick *et al.*, 2002).

Essentially, the identification of type III effector genes from bacterial genome data has become fairly straightforward. This usually involves searching for type III-related promoters, N-terminal secretion signal characteristics and/or determining whether their expression is dependent on a specific regulatory system. However, one lingering complication is that type III-secreted extracellular accessory proteins are also identified by these criteria, and therefore, if the protein is not similar to known accessory proteins or if it does not have similarity to eukaryote-like domains, further consideration is needed to determine whether it is an injected effector or an extracellular accessory protein.

Eukaryotic pathogens also appear to induce their effectors during infection (Dodds *et al.*, 2009). Various subtractive hybridization approaches will continue to be attractive for the identification of eukaryotic effectors. With next-generation sequencing technologies becoming available and more affordable, one approach will probably be the sequencing of cDNA libraries from pathogens grown on plants (or plant-mimicking conditions) versus a control medium.

TARGET DISCOVERY

The next critical juncture for effector research is target identification. To date, the only plant targets identified have been for bacterial effectors (Block *et al.*, 2008; Boller and He, 2009; Zhou and Chai, 2008). However, several oomycete effectors have been shown to suppress innate immunity, and the enzymatic activities for two fungal effectors are known (Dodds *et al.*, 2009). With the current and rapid identification of effectors from oomycetes and fungi, it is likely that eukaryotic effector targets will soon be found and targets for bacterial effectors should continue to be identified. Several proven strategies for the identification of plant targets are discussed below and are included with other strategies in the middle portion of Fig. 1.

Guilt by association

The most productive approach for the identification of effector targets has been protein–protein interaction assays. The *Arabidopsis* RIN4 protein, a plant pathogen effector target, was identified on the basis of its interaction with the *P. syringae* AvrB type III effector in a yeast two-hybrid (Y2H) system (Mackey *et al.*, 2002). RIN4 was subsequently shown to interact with the effectors AvrRpm1 and AvrRpt2 (Axtell and Staskawicz, 2003; Mackey *et al.*, 2002, 2003) and the R proteins RPS2, RPM1 and RPS2 (Axtell and Staskawicz, 2003; Mackey *et al.*, 2002, 2003). Y2H approaches have also been successful in showing that the *P. syringae* AvrPto and AvrPtoB effectors interact with the tomato Pto kinase (Kim *et al.*, 2002; Tang *et al.*, 1996). More recently, co-immunoprecipitation approaches have shown that these effectors interact with and disable pathogen-associated molecular pattern (PAMP) receptors (Gimenez-Ibanez *et al.*, 2009; Gohre *et al.*, 2008; Shan *et al.*, 2008; Zhang *et al.*, 2007). Targets identified in this manner require confirmation of the identified interactions using complementary approaches. In this regard, a new *in planta* split luciferase system shows promise (Chen *et al.*, 2008).

In the right place at the right time

Some effectors interact too weakly or transiently with their targets for protein–protein interaction approaches to be useful. The *in planta* subcellular localization of effectors can provide clues to their function and target identity. Bacterial, oomycete and fungal effectors have been localized to the plant nucleus (Deslandes *et al.*, 2003; Kanneganti *et al.*, 2007; Kay and Bonas, 2009; Kemen *et al.*, 2005), suggesting potential functions such as transcriptional regulation and nucleocytoplasmic trafficking. Many bacterial effectors localize to the plasma

membrane (Dowen *et al.*, 2009; Nimchuk *et al.*, 2000; Robert-Seilaniantz *et al.*, 2006; Shan *et al.*, 2000). The co-localization of potential targets with their effectors increases the likelihood that they are bona fide targets. The importance of determining an effector's site of action is also illustrated by the *P. syringae* HopI1 effector, which localizes to chloroplasts (Jelenska *et al.*, 2007). HopI1 suppresses innate immunity, alters chloroplast thylakoid structure and reduces salicylic acid accumulation—all plausible effects considering HopI1's subcellular localization. It is apparent that microscopy and biochemical fractionation approaches will continue to be an important aid for the determination of effector functions and targets.

Change is for the best

There is a fascinating evolutionary struggle between pathogens and plants in which effectors are under selection to evade detection by plant R protein-based surveillance systems, whilst maintaining their virulence activities, and R proteins adapt to acquire and maintain effector recognition (Ma and Guttman, 2008). Several examples exist which indicate that eukaryotic effectors change to avoid direct recognition by R proteins. For example, Dodds *et al.* (2006) have shown that selected sites within the AvrL567 effector from *M. lini* allow it to evade direct recognition by R proteins. The selection pressure is less clear for the bacterial effectors. Bacterial effectors are under selection to avoid 'guarded' plant targets or possible direct recognition by R proteins. Both eukaryotic and bacterial effectors may also be under selection to acquire the ability to recognize novel virulence targets. Whatever the case, next-generation sequencing technologies will provide the sequence for many effector gene alleles, making it possible to identify polymorphisms within effector families. These polymorphisms are likely to help us hone in on important regions within effectors.

TARGET FUNCTION DISCOVERY

In some cases, effector targets have turned out to be proteins already known to be involved in plant immunity. This includes the RIN4 protein targeted by the type III effectors AvrRpt2, AvrRpm1 and AvrB (Axtell and Staskawicz, 2003; Mackey *et al.*, 2002, 2003), and the PAMP receptor kinases targeted by AvrPto and AvrPtoB (Gimenez-Ibanez *et al.*, 2009; Gohre *et al.*, 2008; Shan *et al.*, 2008; Xiang *et al.*, 2008). AvrB has also been reported to target the RAR1 protein (Shang *et al.*, 2006), which is a protein found in R protein complexes. In other cases, the identification of effector targets has revealed a novel component of innate immunity. For example, the *P. syringae* HopM1 and HopU1 effectors target AtMin7 (Nomura *et al.*, 2006), involved in vesicle trafficking, and AtGRP7 (Fu *et al.*, 2007), a glycine-rich RNA-binding (GR-RBP) protein, respectively. These targets essentially identified vesicle trafficking and the activities of GR-RBP as important components of innate immunity. Once novel effector targets are discovered, a similar battery of approaches can be used to determine their function in pathogen–plant interactions. Many of these are listed in the bottom portion of Fig. 1.

The identification of pathogenicity phenotypes in plants lacking and over-expressing the target may determine the extent to which the target is involved in innate immunity or other plant processes co-opted by pathogens. Plants over-expressing the target or expressing a target that no longer interacts with the effector may lead to greater resistance to biotic stress. Studies of target protein polymorphisms and their relationship to effector efficacy will indicate whether the target is under evolutionary pressure to diversify. This will not only validate the target's importance, but also illustrate the co-evolutionary dynamic between the pathogen and its host.

CONCLUDING REMARKS

Molecular plant pathologists are on the verge of the identification of thousands, perhaps tens of thousands, of pathogen effectors. Improvements in bioinformatics approaches will be critical for handling and analysing this deluge of information. This information promises to answer

some of the long-standing questions of plant pathology—Why can some organisms infect plants? What factors control host specificity and nonhost resistance? At least part of the answer to these questions will probably be found in the effector repertoires possessed by different plant pathogens. However, given that some effectors induce ETI, whereas others suppress it, and that these activities may be altered by effector polymorphisms, it is probably unlikely that the effector repertoire alone will answer these questions. Instead, extensive molecular characterization will be needed to determine the activity of each effector and, from this, patterns should emerge that will shed important light on the above questions.

The other fascinating question that will soon be explored is what host processes are targeted by these newly discovered effectors. Will they primarily target plant innate immunity, as do the majority of bacterial type III effectors (Boller and He, 2009; Guo *et al.*, 2009)? Do effectors target host processes that allow for water and nutrient acquisition? Bioinformatics approaches will be important for the identification of effector function and targets. It is satisfying to realize that effector target identification will probably result in novel biotechnology applications and improvements in agriculture. Research advances in molecular plant–microbe interactions over the past 25 years, together with important technological advances, have paved an excellent road, which should allow for major research progress in the near future. And to paraphrase the songwriter Willie Nelson—I just can't wait to get on the effector road again.

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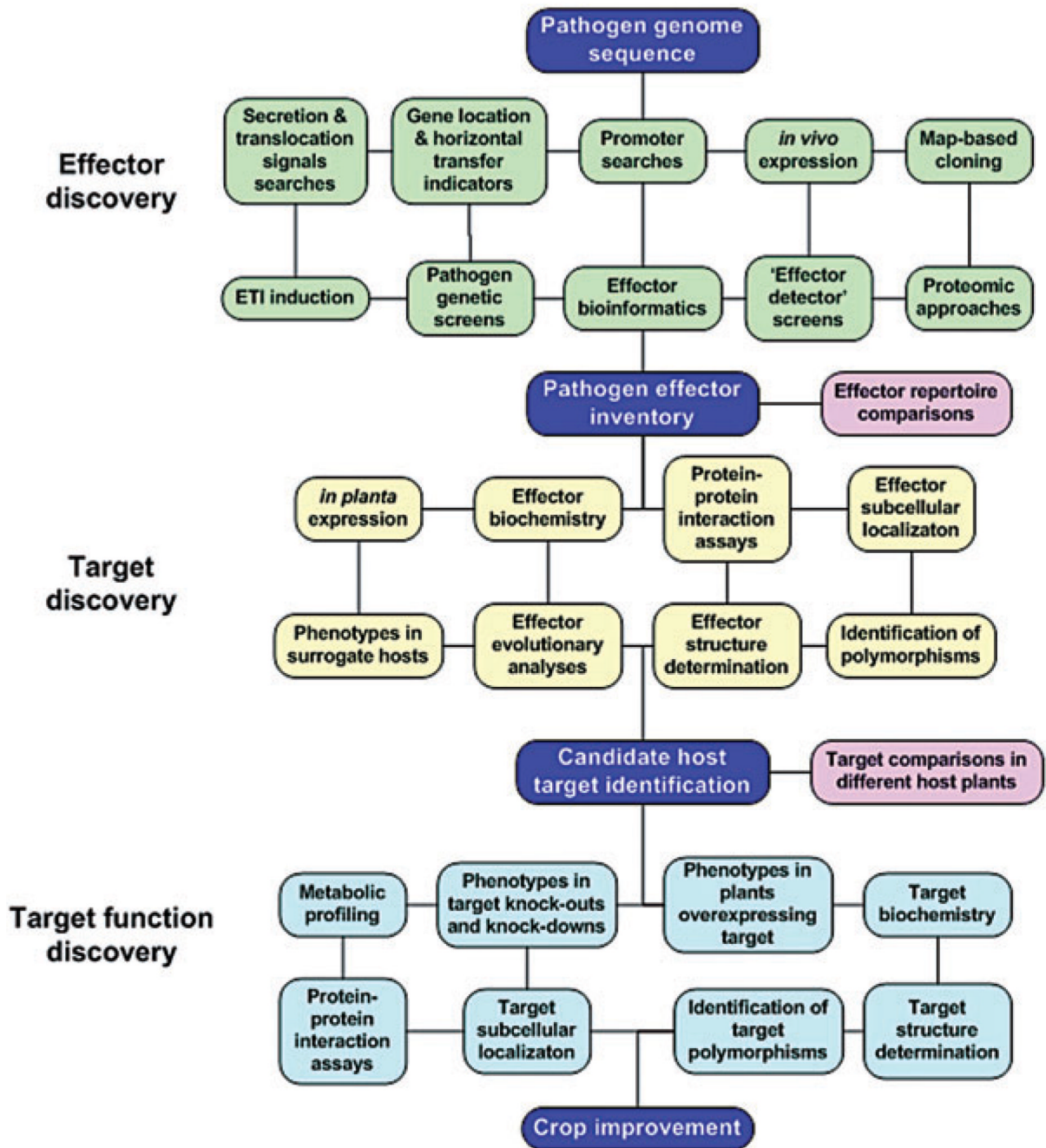


Fig. 1. 'Roadmap' for discovery of plant pathogen effectors and their targets. The flowchart shows the approaches used for effector discovery (green boxes), target discovery (yellow boxes) and target function discovery (light blue boxes). Starting, intermediate and stopping points in the flowchart are depicted as dark blue boxes. Pink boxes depict comparisons that can be carried out after effector repertoires and targets have been isolated that may give insights to virulence and nonhost resistance. The lines connecting each box indicate that it may be used in combination with other approaches in the network, not necessarily just the adjacent boxes. Useful tools for studying eukaryotic effectors are the 'effector detector' screens that fuse candidate effectors to type III secretion signals, such that they are injected into plant cells by

a bacterial strain containing a type III protein secretion system (Rentel *et al.*, 2008; Sohn *et al.*, 2007). Effectors can be expressed transiently or transgenically *in planta* to determine whether they produce phenotypes, such as enhanced susceptibility to pathogens, or whether they suppress plant innate immunity outputs. T-DNA knock-out and RNA interference knock-down plants or plants over-expressing effector targets can be used to determine target function and to confirm that they are effector targets. See text for additional details.