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Review

Algorithms for Effector Prediction in Plant Pathogens and Pests: Achievements and Current Challenges

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Abstract: Effectors are key organism-associated molecules that aid in the establishment of interactions with other organisms. Effectoromics has become an important area of research in phytopathology. The lack of sequence conservation among effectors, even in closely related organisms, has led us to believe that effectors from organisms of different kingdoms are completely unrelated, which has fostered the independent development of effector identification strategies in bacteria, fungi, phytoplasmas, etc. This review focuses on the different algorithms available for effector identification in different plant pathogens and pests, using the following classification: (1) translocated effectors (bacteria, oomycete) and (2) secreted effectors (phytoplasmas, fungi, insects, nematodes). The objective of this type of classification is to identify, for the first time, the common features that exist among these organisms to streamline future effectoromics identification strategies. Among the organisms' commonalities, certain bacteria, fungi, phytoplasmas, and nematodes may cause similar symptoms, and some of their effectors may target the same proteins or biological processes in the plant hosts. The integration of effector analyses of organisms of different living kingdoms, through the identification of common short linear motifs, domains, and three-dimensional structures, may aid in the development of novel algorithms for effectoromics. Future algorithms may contemplate these highlighted features and will be better equipped to identify not only canonical effectors but highly elusive non-canonical effectors as well.

Keywords: algorithms; bacteria; effectors; effector prediction; effectoromics; fungi; insects; nematodes; oomycete; phytoplasmas

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1. Introduction

Effectors were first termed "avirulence factors" (Avr), as described by the botanist Flor in the 1940s in his research on the fungus *Melampsora lini* and the plant host, the flax plant, *Linum usitatissimum* L. [1]; pathogen Avr genes were recognized by products of the cognate "R" or resistance gene present in the resistant plants. While they were first discovered in pathogens, effectors have since been discovered in non-pathogenic microbes as well. They govern positive (symbionts, mutualists) and negative (antagonists) microbial interactions with other organisms [2]. Proteins are the largest class of known effectors, but carbohydrates, secondary metabolites, and RNA effectors also exist [3,4]. In this review, we focus on effector proteins. Some features such as the composition of amino acids may vary among effectors of organisms in different living kingdoms: for example, fungal effector proteins are rich in cysteine residues [5,6], while phytoplasma effector proteins are poor or lacking in cysteine [7]. However, effector proteins of different organisms (fungi, insects,

nematodes) are described as small secreted proteins (SSP), usually without transmembrane domains [5,8,9].

Some plant disease symptoms, such as witches´ broom, a structural alteration commonly observed in trees or woody plants, may be caused by nematodes, insects, oomycetes, fungi, viruses, and phytoplasmas [10]. Therefore, it is not surprising that, although extensive homology does not exist at the sequence level, effectors of different living kingdoms may target the same proteins or the same processes in the host. Salicylic acid (SA) is an important defense hormone as well as other plant hormones such as jasmonic acid (JA), abscisic acid (ABA), ethylene (ET), and auxin [11,12]. The fungal pathogen *Ustilago maydis* secretes the effector Cmu1 (chorismate mutase, CM), which likely extracts chorismate from the host plastids, interfering with plastidic SA biosynthesis. The effector Cmu1 is required for full virulence of this pathogen [12,13]. Similarly, the root-knot nematodes *Meloidogyne javanica* and *M. incognita*, and the migratory nematode *Hirschmanniella oryzae*, also secrete CM to target host SA biosynthesis and lower SA content during pathogen infection, increasing host susceptibility [14]. Other pathogens target the SA biosynthesis pathway by secreting effectors with isochorismatase activity (ICM). ICM catalyzes the conversion of isochorismate to 2,3-dihydroxybenzoate and pyruvate. The fungus *Verticillium dahliae* and the oomycete *Phytophthora sojae* secrete the effectors VdIsc1 and PsIsc1, respectively; these effectors with ICM activity decrease host SA content, affecting host immunity [15].

Another mechanism targeting SA was described for the bacterium *Pseudomonas syringae*, which secretes the HopI1 effector that localizes to the chloroplast, disturbing the thylakoid structure and finally reducing the SA content [16]. When the effector $SAP11_{AYWB}$ from "*Candidatus* Phytoplasma asteris" was stably overexpressed in *Arabidopsis* plants, the overexpression resulted in a total of 59 upregulated and 104 downregulated genes. Among the downregulated genes, Lipoxygenase2 (LOX2), a gene encoding an enzyme involved in JA biosynthesis, and two SA responsive genes, were found [17,18]. Genes involved in JA and SA synthesis are downregulated in phytoplasma-infected plants with witches' brooms symptoms [19]. These reports evidence that SA y JA are targeted by many pathogen effectors from different living kingdoms [20,21]. Common targets in the host that attract many effectors have been denominated "hubs". Apart from plant hormones such as SA and JA, other plant hubs have been identified [22].

Although effectors may have common targets, they have been difficult to identify and classify since effectors have a limited taxonomic distribution, with little to no sequence similarity to proteins of other organisms. As such, effectoromics strategies for different classes of organisms have developed independently of each other. This article presents a brief review of the algorithms for effector identification in plant pathogens, categorizing them into two large groups of effectors: translocated effectors (bacteria and oomycetes) and secreted effectors (phytoplasmas, fungi, insects, and nematodes).

2. Algorithms for the Identification of Plant Pathogen and Pest Effectors

2.1. Algorithms for the Identification of Translocated Effectors: Bacteria and Oomycetes

2.1.1. Bacterial Effector Predictors

Bacteria have evolved complex machines to transfer effector proteins into eukaryotic cells. At least nine secretion systems have been identified in these microorganisms. T1SS, T3SS, T4SS, and T6SS are one-step systems that directly deliver proteins to the cytosol of a target cell, while T2SS, T5SS, T7SS, T8SS, and T9SS are two-step systems. In these systems, the proteins cross the inner membrane with the help of Sec (secretion) or Tat (twin arginine transportation) pathways before going through the outer membrane. For details about bacterial secretion systems, see [23–25]. Bacterial secretion systems T3, T4, and T6 nanomachines have primarily evolved to deliver effectors into plant cells; T3 and T6 are widespread in Gram-negative bacteria, while the T4 system can also be found in Gram-positive bacteria [26]. Most pathogenic bacteria make use of various protein secretion systems to successfully infect the host [26]. Oomycetes also translocate the effectors to the

host cell [27,28], but the delivery system has not been characterized. Figure 1 shows the delivery of effectors of bacteria and oomycetes to the plant cell.

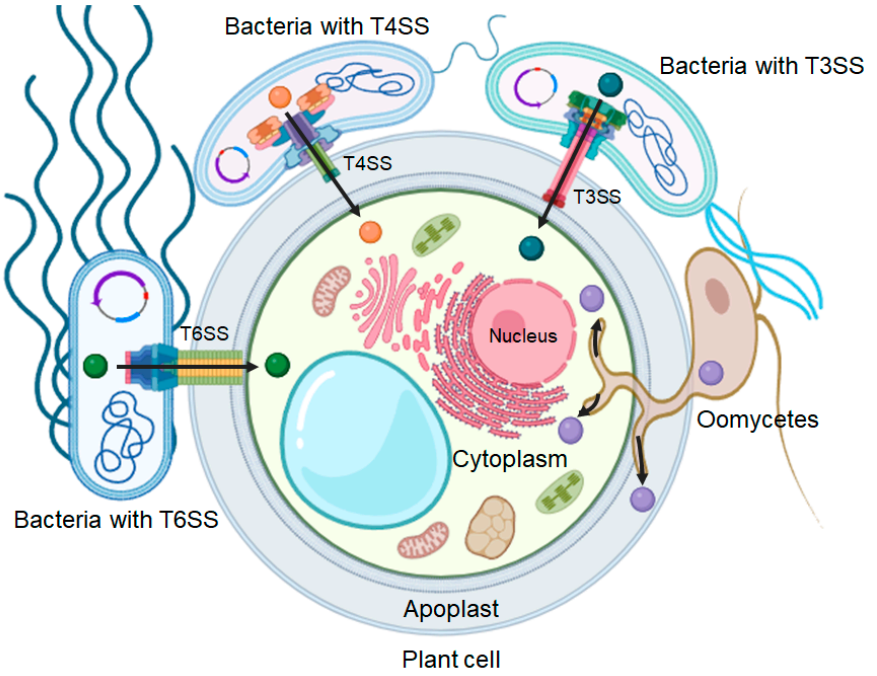

Figure 1. Bacteria and oomycetes translocate effectors directly into plant cells. In bacteria, effector transfer occurs mainly through T3, T4E, or T6 secretion systems; in oomycetes, the delivery system for effectors has not been characterized. Special algorithms for oomycetes, T3E, T4E, or T6E identification have been independently created based on motifs or domains that serve as the signals for effector translocation. The green, orange, blue, and purple circles represent the effectors.

Predictors of Effectors Delivered Through T3 Secretion System (T3SS)

T3SSs are encoded by hrp (for hypersensitive response and pathogenicity) genes; they were named hrp as they are required to cause a disease in susceptible plants and to elicit the hypersensitive response in resistant plants. The T3SS is also termed "the injectisome" because of its needle-like structure that secretes proteins directly from the cytosol of the bacterial pathogen into the host cell. Although the first T3SS-associated filamentous structure was discovered in the plant pathogen *Pseudomonas syringae* [29], most of the characterization of the T3SS was carried out in the mammalian pathogen *Salmonella enterica* [30]. Effectors delivered by the T3SS have been described in the following human and animal pathogens: *Yersinia* species (e.g., Yop effectors), *S. enterica* (e.g., SPI1, AvrA, SipA/B/C/D, SlrP, SseK, SopA/B/D/E/E, and SptP; SPI2, SpiC, SseF/G/I/J, SspH1/H2, SifA, SifB, PipB/B2, SseK1/K2, GogB, and SopD2 effectors), *Shigella* species (e.g., IpaA/B, C terminus of IpaC, VirA, IpaH, Osp's, IpgB1 effectors), *Pseudomonas aeruginosa* (ExoS, ExoT, ExoU, ExoY effectors) and *Chlamydia* species (e.g., Inc proteins, Cpn0909 and Cpn1020 effectors) [31], and the plant bacterial pathogens *Pseudomonas syringae* (e.g., HopAI1, Hop52, HopAU1, AvrPto1Psy, AvrB, and AvrRps4) [32], *Xanthomonas* spp. (e.g., AvrBs3, Xops effectors) [33], *Ralstonia solanacearum* (e.g., RipAY, RipR, RipAL, RipG1, RipG3, RipAR, RipAW, RipA5, RipTPS, and RipTAL) [34], and *Erwinia* spp. (e.g., Eop1, DspE) [35]. In addition to Gammaproteobacteria, T3SS is present in some *Bradyrhizobium* strains that nodulate plant legumes such as *Aeschynomene evenia* (e.g., ErnA and Sup3 effectors) [36] and the human pathogen *Chlamydia trachomatis* (e.g., CT622 effector) [37]. Readers can observe differences in the secretion apparatus of the T3SS between plant and animal pathogenic bacteria in Büttner and He [38].

Characteristics of T3 Effectors (T3Es) include the amphipathic nature of the N-terminal region that is enriched in Ser residues and coiled regions, but lacking Leu residues; there

is also a lack of Asp or Glu residues in the first 12 amino acids and an enrichment of polar residues in the first 50 amino acids [38]. The first algorithm to identify T3E was EffectiveT3, a machine learning approach trained using the amino acid composition and secondary structure of N-termini of 100 experimentally verified effector proteins; authors reported sensitivity of ∼71% and selectivity of ∼85% for EffectiveT3. Unfortunately, when applied to 739 bacterial and archaeal proteomes, EffectiveT3 retrieved a high number of false positives [39]. SIEVE (SVM-based Identification and Evaluation of Virulence Effectors) was also created in the same year (2009) and trained on a set of known secreted effectors to detect the presence of a protein-encoded secretion signal in the first 16–20 amino acid residues. The specificity of SIEVE was 88% when used to predict *Salmonella enterica* serovar Typhimurium effectors and 87% when applied to *P. syringae* effectors [40]. Table 1 presents a list of algorithms which have been created to identify T3E in silico.

T3E prediction has presented a great computational challenge: many algorithms retrieve high numbers of false positives. Interestingly, Hobb et al. [41] compared the algorithm GenSET with EffectiveT3, SIEVE, BPBAac, Meta-analytic, T3SEpre, and T3MM; they used these algorithms in the prediction of T3E in *Shigella dysenteriae*, *Escherichia coli*, *Pseudomonas syringae*, and *S. enterica* serovar Typhimurium and found that GenSET had the best sensitivity (0.938) and specificity (0.979) of all the algorithms.

Additional predictors currently available for T3E are T3SEpp, DeepT3 2.0, and MolPhase. The T3SEpp pipeline combines the identification of T3E signal sequences (as many other algorithms do) with the identification of features in regions outside the T3E signal sequences. This pipeline integrates the identification of homology using full-length effector proteins, the presence of a signal sequence, chaperone binding, the presence of effector domains, and the promoter region of the effector genes. When this pipeline was used on 519 verified T3Es and a negative dataset of 310 sequences, the results were compared with seven other machine learning algorithms, and the best performance (accuracy 0.941) was found for T3SEpp [42]. The most recent algorithm created to identify T3E is MolPhase [43]. This algorithm is based on the prediction of protein phase separation and assesses electrostatic pi-interactions, disorder, and prion-like domains in the search for T3Es. In vivo and in vitro analyses of T3Es showed a significantly higher propensity for phase separation compared to other proteins, supporting the functionality of the algorithm; however, non-T3Es such as DNA and RNA regulatory proteins also exhibit a propensity for phase separation, which has to be considered in the analysis of the prediction results. Identification of T3Es is still a challenging task. Users may choose their favorite algorithms based on performance as well as how user-friendly the algorithm is. The combination of multiple algorithms is also recommended to strengthen the prediction of the T3SS-effectorome.

Table 1. Algorithms available for the identification of bacterial effectors delivered through the T3SS.

Algorithms	Description	Sensitivity/Specificity/Accuracy	Reference
EffectiveT3	Machine learning; trained with amino acid composition and secondary structure of N-termini of 100 experimentally verified effector proteins	0.75/0.85/0.86	[39]
SIEVE	Machine learning; trained on a set of known effectors to detect the secretion signal in the first 16–20 amino acids	0.90/0.88/0.96 for *P. syringae*	[40]
BPBAac	Support vector machine (SVM)-based classifier. Trained with a set of experimentally validated T3E from animal pathogens, plant pathogens, and symbiotic bacteria	0.91/0.97/0.95	[44]
Meta-analytic	Vector machine-based discriminant analysis followed by a simple criteria-based filtering. Trained with known effectors of *S. enterica*	0.90/0.90/-- for *S. enterica*	[45]

Table 1. *Cont.*

Algorithms	Description	Sensitivity/Specificity/Accuracy	Reference
T3SEpre	Mathematical model based on amino acid composition, secondary structure, and solvent accessibility in the N-termini of type III secreted proteins	0.60/0.96/0.80	[46]
T3MM	A Markov model based on the amino acid composition within the N-terminal 100 amino acids from T3E	0.84/0.90/0.90	[47]
GenSET	Based on 21 genomic and proteomic attributes such as peptide properties, molecular weight, charge, A280 molar extinction coefficient, probability of expression in inclusion bodies, isoelectric point, instability index, aliphatic index, and G + C content, among others	094/0.98/--	[41]
T3SEpp	Deep learning to identify the atypical features in signal sequences of T3E, and then integration of the results of individual modules	0.93/0.71/0.83	[42]
DeepT3 2.0	Combines multiple deep learning architectures including convolutional, recurrent, convolutional-recurrent, and multilayer neural networks to learn N-terminal representations of T3E	----	[48]
MolPhase	Prediction of protein phase separation	0.90/0.75/--	[43]

Predictors of Effectors Delivered Through the T4 Secretion System (T4SS)

The T4SS is related to bacterial conjugation systems [49]. Type IV pili (T4p) are formed with peptidoglycan polymers and the major pilin protein on the surfaces of many Gram-negative and Gram-positive bacteria. T4p is involved in many functions such as locomotion, adherence to eukaryotic cells, microcolony formation, DNA uptake, carrying electric current, and protein secretion [50]. The T4SS allows bacterial proteins or DNA to traverse both membranes and the periplasm and translocate these molecules into host cells.

The T4SS is classified as type 4A (T4ASS; the structural components resemble the VirB/D4 complex of *Agrobacterium tumefaciens*) or 4B (T4BSS, for the conjugal transfer system of the self-transmissible IncI plasmid). Both sub-systems translocate effectors to the host cytosol via a central pore [49]. Both sub-systems are very complex, with 12 and 22 structural proteins for the T4ASS and T4BSS, respectively; for details, see Costa et al., 2021 [51]. Examples of bacteria with the T4ASS are *Agrobacterium tumefaciens* (e.g., VirB and VirD4 effectors), *Brucella* spp. (e.g., VirB, BspB, BPE123 effectors), *Bartonella* spp. (e.g., VirB, VirD4, Beps, YopJ effectors), *Rickettsia* spp. (e.g., rvh, RalF, RARP-2 effectors), and *Anaplasma phagocytophilum* (e.g., AteA, AptA effectors). Although the most studied bacteria have been Alphaproteobacteria, the T4ASS is not restricted to this bacterial class; it was recently reported in the beneficial plant rhizosphere *Lysobacter enzymogenes* (Gammaproteobacteria) [52]. Examples of bacteria with the T4BSS are *Legionella pneumophila* (e.g., Lvh, DenR, LpdA effectors) and *Coxiella burnetii* (e.g., AnkF and AnkG effectors) [49]. To date, the T4BSS has only been described in Gammaproteobacteria. Further investigations will uncover whether the T4BSS is present in other classes of bacteria, too.

Due to the serious nature of the infectious diseases that these pathogens cause, the T4SS has been the focus of intense research efforts. To date, few algorithms have been created to identify T4Es at a genome-wide level. The first algorithms for genome-wide identification of T4Es were T4EffPred and S4TE. T4EffPred is a vector machine classifier based on amino acid composition, dipeptide composition, position-specific scoring matrix composition, and auto covariance transformation of a position-specific scoring matrix; it was trained with the databases "AtlasT4SS" and "SecRet4". T4EffPred was able to identify both T4A and T4B effectors with 95.9% accuracy with a false-positive rate of 4% [53]. S4TE combines 13 sequence characteristics, such as homology to validated effectors, homology to eukaryotic domains, subcellular localization signals or secretion signals, among others [54].

The positive dataset was composed of all the T4Es reported until 2013, but this list was not provided or described in detail.

Later, a second version of S4TE was created, S4TE 2.0, a graphical interface (website) of S4TE 1.0 for genome and proteome analysis. This algorithm was tested on *Legionella* and *Coxiella* species, both T4BSS-type organisms [55]. Similarly, Esna-Ashari et al. [56] created OPT4e, another graphical user interface that integrates previously developed tools to identify T4Es. This software works well in the prediction of T4Es of *Anaplasma phagocytophilum* [56]. The last two software do not require previous knowledge of a specific programming language, which is an advantage over other effector predictors only available for use on Linux operating systems.

The two latest algorithms, which have been constructed for identification of T4Es, are T4SE-XGB and iT4SE-EP; the T4SE-XGB prediction is based on 20 different types of features of T4Es such as secondary structure information, peptide sequence, sequence length, molecular weight, total hydropathy, global properties, terminal properties, motifs, etc. [57], similar to previous algorithms. With iT4SE-EP, the protein sequences are transformed into fixed-length feature vectors, then evolutionary features from their PSI-BLAST profiles are selected based on the random forest algorithm. When compared with other machine learning algorithms, iT4SE-EP showed a reduced false positive rate [58]. Table 2 summarizes a list of algorithms available for the identification of T4Es.

Table 2. Algorithms available for the identification of bacterial effectors delivered through the T4SS.

Algorithms	Description	Sensitivity/Specificity/Accuracy	Reference
T4EffPred	Machine learning trained with AtlasT4SS and SecRet4. This algorithm is able to identify T4ASS and T4BSS effectors	0.70/0.98/0.93	[53]
S4TE	Machine learning based on 13 sequence characteristics	0.80/0.65/0.55 for *Legionella and Coxiella* species	[54]
S4TE 2.0	Web interface version of S4TE 1.0	0.75/0.91/0.90 for *Legionella and Coxiella* species	[55]
OPT4e	Graphical user interface that integrates previously developed bioinformatics tools	--/--/0.94 for *Anaplasma phagocytophilum*	[56]
T4SE-XGB	Algorithms based on 20 different types of features of T4Es	0.82/--/0.94	[57]
iT4SE-EP	Uses PSI-BLAST Profiles	0.89/0.96/0.96	[58]

Predictors of Effectors Delivered Through the T6 Secretion System (T6SS)

The type VI secretion system (T6SS) is a multiprotein transmembrane nanomachine that works as a contractile tail. It is composed of at least 13 conserved proteins and other less conserved accessory proteins (chaperones/adaptors) such as Eag, Tec, and Tap proteins that are required for loading effectors onto the system. Allsopp and Bernal [59] recently reviewed the assembly and recycling of the T6 secretion system [59]; readers interested in the T6SS are invited to read this review. The T6SS is present in Gram-negative bacteria and allows them to translocate, in a contact-dependent manner, effector proteins into the host cells. One of the main functions of T6SS machinery is the transfer of toxic proteins into rival bacterial or eukaryotic cells to kill competitors [60].

Examples of bacteria with the T6SS are *Vibrio cholerae* (e.g., VgrG-1 and VasX effectors), *E. coli* (e.g., KatN and VgrG1 effectors), *Pseudomona aeruginosa* (e.g., Tse1, Tse2, Tse3, PldA, PldB, VgrG2b, and TpIE effectors), *Klebsiella pneumonia* (Pld1 and VgrG4 effectors), *Francisella turalensis* (e.g., PdpC, PdpD, OpiA, and OpiB effectors), *Edwardsiella tarda* (e.g., EvpP effector), *Burkholderia* sp. (e.g., TecA and VgrG5 effectors), *Serratia marcescens* (e.g., Tfe1 and Tfe2 effectors), and *Yersinia pseudotuberculosis* (e.g., YezP effector) [60]. In addition to Gammaproteobacteria and Betaproteobacteria, the T6SS is also present in Bacteroidetes (Bacteroidia class) [59] and *Agrobacterium tumefaciens* (Alphaproteobacteria) [61].

Bastion6 was the first tool for T6 effector protein prediction, and it was based on sequence profile, evolutionary information, and physicochemical properties of T6Es. A set of SVM-based models was then developed and integrated into ensemble learning models. The algorithm was further tested on a dataset of 20 newly discovered T6Es and had an accuracy of 0.943 [62]. The second T6Es predictor was PyPredT6, a Python-based algorithm that uses 837 unique features extracted from the peptide and nucleotide sequences of T6Es. The applicability of PyPredT6 was tested on *Vibrio cholerae* and *Yersinia pestis* proteomes [63]. Recently, a novel algorithm, Foldseek, was created for T6E identification. This is a protein structure-based algorithm that can search millions of predicted protein structures. It works like BLAST, but for 3D protein structures. Foldseek uses Alphafold-Multimer to quantify the interaction between putative T6Es and their putative cognate immunity proteins [64]. Table 3 summarizes the algorithms available for the identification of T6Es.

Table 3. Algorithms available for the identification of bacterial effectors delivered though the T6SS.

Algorithms	Description	Sensitivity/Specificity/Accuracy	Reference
Bastion6	Machine learning. SVM classifier	--/--/0.94	[62]
PyPredT6	Python-based algorithm using 837 protein features	0.91/0.90/0.89	[63]
Foldseek	Protein structure-based algorithm	--/--/0.90	[64]

Effectors from Gram-positive bacteria have been scarcely studied. Some examples are *Clavibacter michiganensis*, e.g., effector Pat-1Cm delivered by T2SS [65], and *Bacillus cereus*, e.g., effector EsxA secreted by T7SS [66]. Effectors of Gram-positive bacteria are not discussed in this review.

2.1.2. Predictors of Oomycete Effectors

Oomycetes are Protists (eukaryotic organisms that are not animals, land plants, or fungi) [67,68]. Oomycetes share some physiological and morphological features with fungi such as the formation of hyphae but are phylogenetically related to algae [69]; oomycete cell walls have β-1,3-glucans, β-1,6-glucans, and cellulose as the major microfibrillar component while fungi possess chitin [70]. Oomycetes are a large group with about 600 species contained in 90 genera. Plant pathogens are found within the orders Rhipidiales, Albuginales, Peronosporales, and Pythiales, while animal pathogens are found mostly in the Saprolegniales order [28]. The Top 10 oomycete phytopathogens and examples of their effectors are *Phytophthora infestans* (e.g., Avr3a, PiSFI3, PITG06478, and Pi04314 effectors), *Hyaloperonospora arabidopsidis* (e.g., ATR1, ATR13, and HaRxL77 effectors), *Phytophthora ramorum* (e.g., Avh120 and Avh121 effectors), *Phytophthora sojae* (e.g., Avr1b, Avr1d, PsAvh113, and PsAvh262 effectors), *Phytophthora capsici* (e.g., CRISIS2, RXLR25, PcAvr3a12, and PcAvh1 effectors), *Plasmopara viticola* (e.g., PvRXLR111 and RxLR50253 effectors), *Phytophthora cinnamomi* (e.g., PcAvh87 and PHYCI_587572), *Phytophthora parasitica* (e.g., PpRxLR2 effector), *Pythium ultimum* (e.g., effectors with RXLR-dEER motifs), and *Albugo candida* (e.g., ECFG14 and CCG effectors).

In oomycetes, effectors are also translocated from the pathogen cell to the host cell [27,28]. However, while the literature related to effector identification is extensive, the secretory machinery in these microorganisms is largely ignored. Effector identification in oomycetes has been based on the detection of motifs such as RXLR [71,72], LXLFLAK [73], and crinkler (CRN) [74,75], among others. Currently, only a few existing bioinformatics predictors can identify effectors in oomycetes. The first algorithm for this purpose was EffectR, which combined regular expressions and hidden Markov model statements to identify RXLR and crinkler effectors [76]. Interestingly, the next bioinformatics tool for oomycete effector identification, EffectorO, did not use the characteristic motifs widely used in oomycete effector prediction. Instead, this machine learning approach predicts effector probability based on the biochemical properties of the N-terminal amino acid

sequence of the protein. Subsequently, it applies a pipeline for lineage-specificity to find proteins that are unique to the species or the genus. EffectorO was tested on *Bremia lactucae* and *Phytophthora infestans*, and it retrieved the majority of known effector candidates, as well as many novel effector candidates [77]. The latest algorithm created for oomycete effector identification is POOE, an algorithm that uses the sequence embedding from a pre-trained large protein language model (ProtTrans) as input and develops a support vector machine. Researchers have shown that POOE is competitive, with a performance similar to existing predictors, and represents a complementary tool to accelerate the identification of oomycete effectors [78]. Table 4 is a summary of the algorithms available for oomycete effector identification.

Table 4. Algorithms available for the identification of oomycete effectors.

Algorithms	Description	Sensitivity/Specificity/Accuracy	Reference
EffectR	Hidden Markov model to identify characteristic motifs of oomycete effectors	-----	[76]
EffectorO	Machine learning based on biochemical properties of the effector N-terminal combined with lineage-specific distribution	--/0.82/0.84	[77]
POOE	ProtTrans-based support vector machine learning algorithm	--/0.94/0.89	[78]

In summary, the algorithms that identify translocated effectors are based on different biochemical (e.g., protein length, composition), physicochemical (e.g., isoelectric point, hydropathy), and genomic (e.g., association with particular genomic regions, grouping in clusters) features of the effectors. Figure 2 corresponds to the general pathway that has been followed for the creation of algorithms for effector identification of translocated effectors in bacteria and oomycetes.

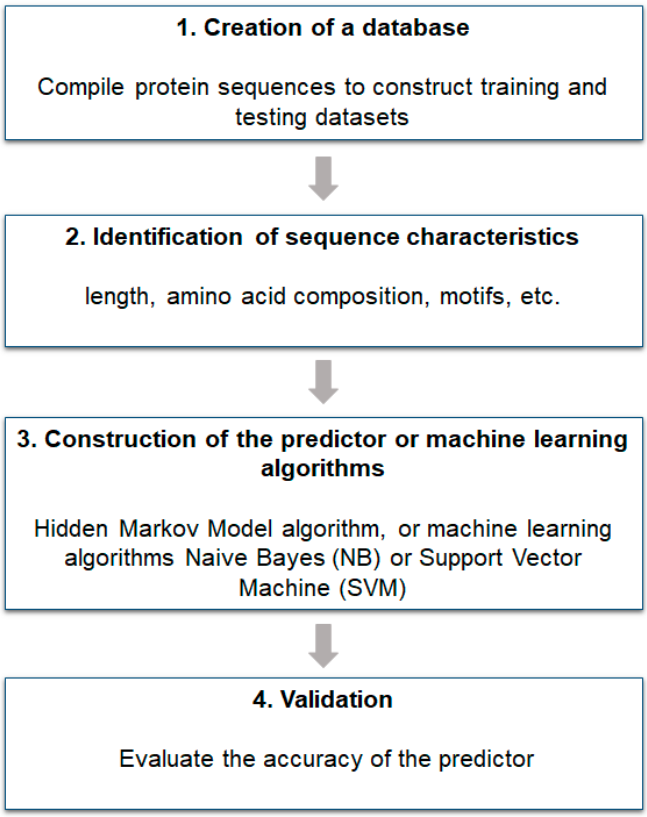

Figure 2. Schematic representation of the steps followed in the creation of algorithms for effector identification in bacteria and oomycetes.

2.2. Algorithms for the Identification of Secreted Effectors: Phytoplasmas, Fungi, Nematodes, Insects

The effectors of phytoplasmas, fungi, nematodes, and insects are delivered to the plant apoplast through the Sec-dependent secretion system that recognizes a signal peptide at the N-terminal of the protein (Figure 3). This secretion system transports enzymes, toxins, and effectors through the outer membrane of these organisms into the extracellular space.

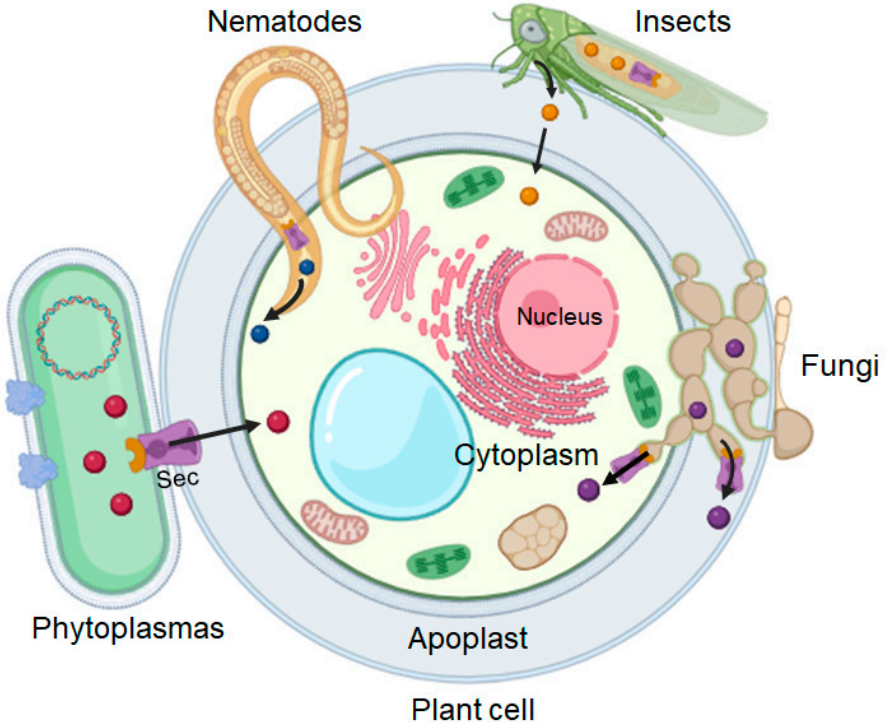

Figure 3. The Sec-dependent system delivers effectors in phytoplasmas, fungi, nematodes, and insects. During phytoplasma infection, the effectors are secreted to the plant phloem, while during fungal infections, the effectors are usually secreted to the plant apoplast. In nematodes, the effectors are secreted to the dorsal gland and then travel to the nematode's mouth. In insects, effectors are secreted from salivary gland cells to reach the insect saliva and get into the plant phloem during insect feeding. The Sec-dependent machinery is represented as a purple rectangle in each kind of organism. The orange, blue, red, and purple circles represent the effectors. Effectors in these biological kingdoms usually have characteristic signal peptides at the N-peptide terminal and lack transmembrane domains.

2.2.1. Predictors for Effector Identification in Phytoplasmas

Phytoplasmas are Mollicutes and have evolved from a Gram-positive *Clostridium*-like ancestor through genome reductions and a loss of the cell wall. They are associated with diseases in thousands of plant species around the world, including agriculturally important plants and horticultural crops, where they cause minor to extensive damage [79]. They are obligate symbionts of plants and insects, as in vitro culture has not been achieved in any cell-free medium [80].

Phytoplasmas are mostly dependent on insect transmission for their spread and survival. Insect vectors of phytoplasmas are phloem feeders of the order Hemiptera, mostly leafhoppers (Cicadellidae), planthoppers (Fulgoromorpha), and psyllids (Psyllidae). Inside the insects, phytoplasmas travel from the gut and salivary gland cells to reach the insect saliva; during insect feeding, the phytoplasma enters the plant host´s phloem [81]. Once infected, phytoplasmas interfere with plant development and cause symptoms such as witches' broom (proliferation of shoots), phyllody (transformation of floral organs into

tissues similar to leaves), virescence (green coloration in floral organs), yellowing (chlorosis), among others [82].

This group of Mollicutes is classified into 14 major "16Sr groups" based on RFLP profiles [79], and to date, only 21 phytoplasma effectors have been validated: TENGU, SAP05, SAP11, SAP54, PHYL1, SWP1, SWP11, SWP12, SWP21, Zaofeng3, Zaofen8, IdpA, Imp, VmpA, Amp, ncSecP3, ncSecP9, ncSecP12, ncSecP14, ncSecP16, ncSecP22 [83]. Phytoplasma possess two secretion systems, the YidC system for the integration of membrane proteins, and the Sec system for the secretion of effectors into the plant or insect cell cytoplasm [84]. For the identification of effectors from phytoplasmas, different versions of SignalP have been used for signal peptide prediction, in combination with the TMHMM v2.0 program to identify and eliminate proteins with transmembrane domains [85–87]. Currently, there exists only one algorithm for effector identification in phytoplasmas, PhyEffector.

For PhyEffector construction, SignalP 3.0, SignalP 4.0, and SignalP 5.0 were evaluated in Gram-positive and Gram-negative modes on the positive training dataset; the best retrieval of phytoplasma effectors was achieved by SignalP 4.0 in the Gram-positive mode, consistent with the Gram-positive bacterial origin of phytoplasmas. PhyEffector integrates SignalP 4.0, TMHMM v2.0, Phobius, and SecretomeP programs and the search for homologs of known effectors. All retrieved proteins are pooled, and redundant proteins are eliminated. False positives are excluded by eliminating proteins that share annotations of conserved core proteins, while effector candidates are supported by annotations of phytoplasma effectors. Annotations such as hypothetical proteins, unknown function, predicted proteins, and "no hits" are also considered as potential effectors, since these unknown proteins are not part of the "core" proteins, which have well-known essential functions. Since many phytoplasma genomes have many proteins annotated as "hypothetical", for example, 257 hypothetical proteins in "*Candidatus* Phytoplasma solani" SA-1, and 337 in "*Ca*. Phytoplasma australiense" PAa [88], PhyEffector anticipates that phytoplasma effectoromes have been underestimated.

2.2.2. Predictors for Effector Identification in Fungi

The most economically devastating fungi and examples of their effectors are as follows: *Magnaporthe oryzae* (e.g., MoSPAB1, AvrPik-D, Rgs1, MoHEG13, and MoHEG16 effectors), which affects rice and wheat; *Botrytis cinerea* (e.g., BcCrh1, BcSSP2, BcXYG1, and BcCDI1 effectors), which has a broad host range; and the wheat pathogen *Puccinia* spp. (e.g., PSEC2, PSEC17, and PSEC45 effectors). Other fungi ranked in the top 10 list of fungal phytopathogens are *Fusarium graminearum* (e.g., effectors FgNls1, FgNls1, and Fg62,), *Fusarium oxysporum* (e.g., effectors SIX1, SIX2, SIX3, SIX4, SIX5, SIX6, SIX7, SIX8, SIX9, SIX10, SIX11, SIX12, SIX13, and SIX14), *Blumeria graminis* (e.g., effectors BEC1019, CSEP0027, and CSEP0064), *Mycosphaerella graminicola* (e.g., effectors Mg1LysM and Mg3LysM), *Colletotrichum* spp. (e.g., effectors CEC3, CfEC92, CgEP1, CgNLP1, SIB1, and SIB2), *Ustilago maydis* (e.g., effectors Jsi1, Vp1, Pep1, and See1), and *Melampsora lini* (e.g., effectors AvrP, AvrP4, AvrM14, and CPGH1) [89]. The most frequently studied fungal phylum is Ascomycota, followed by Basidiomycota and Glomeromycota. Other fungal phyla, such as Blastocladiomycota and Chytridiomycota, have still not been studied in effectoromics.

In contrast to oomycetes effectors, where the presence of conserved amino acid motifs (e.g., RxLR, LFLAK, CRN) has supported the creation of regular expressions or HMM algorithms, the prediction of fungal effectors is more challenging since most fungal effectors do not share significant sequence similarity with each other. However, fungal effectors share structural properties such as a signal peptide for secretion, absence of transmembrane domains, small–medium molecular weight sizes, and cysteine-rich content [90–92]; various predictors combine available bioinformatics tools that identify these characteristics, such as SignalP, which detects signal peptides and TMHMM for the detection of transmembrane domains (proteins with transmembrane domains are then excluded). The first pipeline for fungal effector prediction was Secretool [93], which retrieves the secretome from the total proteome using SignalP, TargetP, and PredGPI and pools the results together. This total

list is then filtered by TMHMM and WolfPsort. Effectors are identified from the secretome by homology to known effectors (BLAST) or the presence of functional domains (PFAM). Secretool uses 300 amino acids as the length cutoff and when applied on 150 true fungal effectors, Secretool identified 72 effectors (~50%).

A bit later, the first machine learning classifiers for fungal effectors, EffectorP 1.0 [94] and 2.0 [95], were created and have been the most preferred fungal effector prediction tools used to date. The second version of EffectorP (EffectorP 2.0) was improved in its identification of pathogen effectors since the negative dataset contained proteins from non-pathogens (e.g., saprophytes, endophytes); however, this negative dataset included both non-effectors and also undiscovered effectors. EffectorP 1.0 performs better than EffectorP 2.0 in the prediction of effectors in non-pathogenic fungi [96]. Its negative dataset was constructed with the total set of secreted proteins of 16 fungal species, filtering the known effectors and homologs. Recently, the third algorithm of this series, EffectorP 3.0, was reported, which classifies effectors according to their localization in the apoplast or cytoplast [97]. While EffectorP algorithms were trained with imbalanced positive and negative datasets, another machine learning algorithm, FunEffector-Pred, overcame this bias by training its algorithm with a similar number of proteins in both datasets [98]. The last machine learning algorithm dedicated to fungal effectoromes is Predector, created for the predictive ranking of candidate effector proteins [99].

One of the most recent predictors, EffHunter, is a pipeline that integrates SignalP 4.1, Phobius, TMHMM 2.0, and WoLFPSORT, with Perl/Bioperl scripts for filtering protein size (cutoff 400 amino acids) and cysteine content (at least four cysteines). EffHunter was compared with Secretool, EffectorP 1.0, and EffectorP 2.0 and had the best performance in the identification of canonical (classical) effectors; the accuracy value for EffHunter was 99% compared to 97% for EffectorP 2.0 [100]. Table 5 summarizes the algorithms for fungal effector identification.

Table 5. Algorithms available for the identification of fungal effectors.

Algorithms	Description	Sensitivity/Specificity/Accuracy	Reference
Secretool	Pipeline integrating SinalP, TargetP, PredGPI, TMHMM, and WolfPsort. This pipeline retrieves the secretome from the total proteome	----	[93]
EffectorP 1.0	Machine learning algorithm suitable for effector identification in non-pathogenic fungi	0.84/0.83/0.86	[94]
EffectorP 2.0	Machine learning algorithm suitable for effector identification in pathogenic fungi	0.87/0.81/0.90	[95]
EffectorP 3.0	Machine learning algorithm for effector subcellular localization	--/--/85	[97]
FunEffector-Pred	Similar to EffectorP 1.0 but trained with balanced positive and negative datasets	0.86/--/0.92	[98]
Predector	Machine learning algorithm that ranks candidate effector proteins	--/--/0.59	[99]
EffHunter	Pipeline integrating SignalP 4.1, Phobius, TMHMM, and WoLFPSORT. Suitable for identification of fungal canonical effectors (<400 amino acids, at least four cysteines)	0.7/1.0/0.99	[100]

2.2.3. Predictors for Effector Identification in Insects

Insect pests are a major constraint to agricultural production and productivity. Insect pests cause direct damage to crops during feeding, indirectly by transmitting plant pathogens (e.g., viruses, phytoplasmas, fungi), or by contaminating agricultural products with body parts and insect eggs, leading to major yield losses [101,102]. They are estimated to reduce the annual worldwide crop yield by 20%, with crop losses valued at more than 470 billion USD [103]. Insects are mainly controlled by pesticides; however, the rising

incidence of pesticide resistance and the negative environmental impacts and progression of a number of health problems in humans and animals have fostered the use of integrated pest management for their control [103].

There are more than six million species of insects, but around thirty of them are important pests of major crops [104]. The top 10 most important pests that threaten global agriculture and examples of their effectors are as follows [105]: (1) *Helicoverpa armigera* (cotton bollworm) (e.g., effector HARP1); (2) *Bemisia tabaci* (tobacco whitefly) (e.g., effector G4); (3) *Tetranychus urticae* (two-spotted spider mite) (e.g., effector SHOT); (4) *Plutella xylostella* (diamondback moth) (e.g., PxABCC2); (5) *Spodoptera litura* (taro caterpillar) (no reports on specific effectors was found); (6) *Tribolium castaneum* (red flour beetle) (e.g., effectors Tc-Piwi/Aub and Tc-Ago3); (7) *Myzus persicae* (green peach aphid) (e.g., Mp10 and Mp42); (8) *Spodoptera frugiperda* (fall armyworm) (no reports of specific effectors); (9) *Aphis gossypii* (cotton aphid) (e.g., effectors C002, ACE1, ACE2, and ACE3), and (10) *Nilaparvata lugens* (brown planthopper) (e.g., EF-hand calcium-binding protein).

The identification of insect effectors is mainly conducted using salivary gland transcriptomes or proteomes [106–108]. To the best of our knowledge, there is no specific algorithm available for public use that can identify effectors in insects. Manual identification of insect effectors involves the use of individual programs such as SignalP (prediction of signal peptides), TMHMM v. 2.0 (prediction of transmembrane domains), PredGPI (prediction of GPI-anchors), and TargetP, WolfPSort, and DeepLoc (for prediction of subcellular localization). Predicted proteins with a signal peptide and no transmembrane domains or GPI-anchors are considered to be secretory proteins [109–112]. The list of true, validated effectors in insects is still limited, but the number is slowly increasing. Experiences from phytoplasma and fungal effectoromics may support the advancement of insect effectoromics.

2.2.4. Predictors for Effector Identification in Nematodes

Plant-parasitic soil nematodes are ubiquitous, attacking all agricultural crops. The nematode damage to crops generally remains unrecognized to farmers because of their microscopic size and the absence of specific symptoms. Plant nematodes damage plant roots and also contribute to aggravating infections caused by soil-borne plant pathogenic fungi and bacteria [113,114]. In addition, nematodes also act as vectors for plant pathogens. Plant nematodes exhibit 5–20% yield loss corresponding to around USD 175.0 billion [115]. Taking into consideration their economic importance, wide distribution, and wide host range, the top 10 most important threats are the following nematodes [116]: (1) the root-knot nematodes (*Meloidogyne* spp.) (e.g., effectors Minc03329, Minc00344, MjShKT, MjPUT3, MeMSP1, MaMsp4, MilSE6, and Mj-NULG1); (2) the cyst nematodes *Heterodera* spp. (e.g., effectors GLAND5, G16B09, and HsGPx) and *Globodera* spp. (e.g., effectors Gr29D09, GpSPRY-414-2, and SPRYSEC proteins); (3) the root lesion nematode *Pratylenchus* spp. (e.g., Ppen10370 and Pp-EXPBeffectors); (4) the burrowing nematode *Radopholus similis* (e.g., RsVAP, Rs-eng, Rs-cb-1, and RsCM); (5) *Ditylenchus dipsaci* (e.g., effector DdVAP2); (6) the pine wilt nematode *Bursaphelenchus xylophilus* (e.g., BxML1, BxICD1, BxKU1, BxKU2, BxSCD3, BxLip3, and BxNMP1); (7) the reniform sedentary nematode *Rotylenchulus reniformis* (e.g., effectors CLE and CEP); (8) the virus vector *Xiphinema index* (no report of a validated effector was found); (9) *Nacobbus aberrans* (no report of a validated effector was found); and (10) *Aphelenchoides besseyi* (e.g., effectors AbSCP1, AbFAR1, Ab-atps, and ACE).

In nematodes, the effectors are identified by transcriptomics or proteomics of dorsal glands, identifying proteins containing a predicted N-terminal signal peptide and lacking transmembrane domains; SignalP and TMHMM are usually used [117–119]. Interestingly, in *Meloidogyne javanica*, Macharia et al. (2023) [120] applied the recently identified core motif Mel-DOG (*Meloidogyne* DOrsal Gland; TGCMCTT), associated with the expression of dorsal gland effectors [120,121]. They searched for this motif in the genes of the predicted *M. javanica* secretome within 2 kb upstream of the coding region. They detected the core motif in 1608 secretory proteins, suggesting that this motif may aid in effector screening

directly from the *Meloidogyne* spp. genomes [118]. Its occurrence in other nematodes remains to be determined. Currently, there is no integrative algorithm available for the identification of nematode effectors. For improved nematode effector prediction, it may prove beneficial to identify motifs associated with dorsal gland expression in different nematodes. As proposed above for insect effectoromics, the experiences from other plant pathogens and pests may help to unravel effectoromics in nematodes.

In summary, one strategy for in silico identification of secreted (apoplastic) effectors has been the development of machine learning algorithms (as seen in Figure 2 for translocated effectors); the other frequently used strategy has been the integration of various tools into pipelines; these include the programs SignalP, TMHMM, SecretomeP, Phobius, LOCALIZER, and WolfPsort, sometimes in combination with the use of regular expressions for the identification of motifs and domains. Figure 4 displays the most commonly used effector prediction tools and a description of their applications.

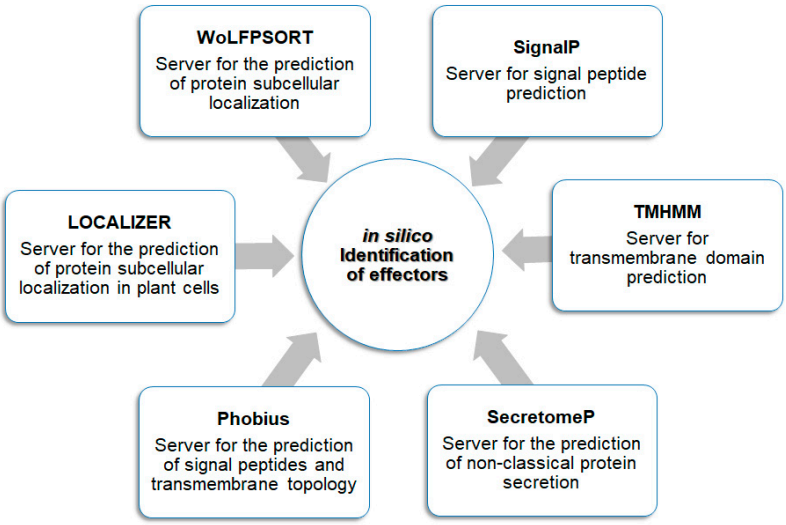

Figure 4. Programs used for the identification of secreted effectors. Similar combinations of programs have been used for effector identification in phytoplasmas, fungi, insects, and nematodes.

3. Effectoromics: Beyond Canonical Effectors

While effectoromics has advanced through the discovery of canonical (classical) effectors, i.e., proteins that are small (less than 200–400 amino acid residues), secreted, extracellular, lacking transmembrane domains, and rich in content of cysteine, there are many examples of effectors that do not meet these canonical characteristics among the different living kingdoms. In fungi, for example, the *Blumeria graminis* effector called CSEP0064 contains a "RNase-like" domain denominated "RALPH" and has only two cysteines [122]. Similarly, VdIsc1 of *Verticillium dahliae* with isochorismate synthase activity has no signal peptide [15]. Other effectors are larger than the 300 or 400 amino acid residues limit of canonical effectors. For example, the *Sporisorium reilianum* effector SAD1, which induces the loss of apical dominance in maize and Arabidopsis, is composed of 626 amino acid residues [123], while the effector AvrSr35 of *Puccinia graminis* f. sp. *tritici* is 578 amino acid residues in length [124]. Recently, the positive dataset of the predictor WideEffHunter, Carreón-Anguiano et al. [96], was compiled using 314 true effectors (228 from fungi and 86 from oomycetes), from which 172 were non-canonical, evidencing the necessity of the inclusion of non-canonical effectors in identification strategies.

Previously, motifs such as RxLR-dEER and Y/F/WxC were believed to be exclusive to oomycetes and were, therefore, excluded in the identification of fungal effectors. However, these motifs were found in 35 and 107 candidates, respectively, in effectors of the fungus *Blumeria graminis* f.sp. *hordei* [125]. Likewise, 244 cysteine-rich small, extracellular proteins of the basidiomycete fungus *Puccinia triticina* had the [Y/F/W]xC motif, 24 had RxLR, 5 had G[I/F/Y][A/L/S/T]R, 64 had [L/I]xAR, and 2 had the YxSL[R/K] motif [126]. In

contrast, Nur et al. [67] used a new approach to identify oomycete effectors, which focused on seven biochemical characteristics of the N-terminus of the protein sequence instead of the classical oomycete effector motifs. With this novel approach, they predicted 5814 candidates in the effectorome of *Phytophthora infestans*, which is two times larger than the previously estimated effectorome of this pathogen, showing that going beyond the canonical criteria permits the discovery of novel effectors.

The algorithm, WideEffHunter, was created to elucidate fungal and oomycete complete effectoromes, including non-canonical effectors [96]. This algorithm integrates the identification of domains and motifs associated with fungal/oomycete effectors, as well as the search for homology to potential orthologs of known fungal/oomycete effectors. False positives that contain motifs exclusively found in the sequences from the negative dataset were eliminated, resulting in an accuracy of 0.96. Prediction by WideEffHunter of fungal and oomycete effectoromes suggests that previous prediction strategies had uncovered just the tip of the iceberg, since canonical effectors were estimated to represent 10% or less of the effectoromes. In addition, the results of WideEffhunter on seven genomes suggest that evolution has shaped similar effectorome patterns in fungi and oomycetes, contrary to the current literature.

In other microorganisms, novel non-canonical effectors have been identified. In the *Vibrionaceae* family, Kanarek et al. [127] identified a protein domain, RIX, that defines a new class of polymorphic T6 effectors; RIX is located at the N-termini of proteins containing diverse antibacterial and anti-eukaryotic toxic domains. This domain is not only necessary for T6SS-mediated secretion but can also enable the T6SS-mediated delivery of other cargo effectors by unknown mechanisms. Similarly, in the bacterium *Acinetobacter baumannii*, a non-canonical T6 secretion system membrane complex was recently identified. The *Acinetobacter baumannii* membrane complex lacks the essential TssJ lipoprotein, which usually anchors the T6 membrane complex to the outer membrane. Instead, three *Acinetobacter*-specific envelope-associated proteins form an intricate network leading to the assembly of the membrane complex [128]. All these novel insights on effectors and their delivery systems reveal the constant evolution of our knowledge in effectoromics.

In phytoplasmas, as mentioned above, effectors are usually secreted through the Sec-dependent secretion system. Recently, six non-canonical effectors were identified in "*Ca*. Phytoplasma ziziphi"; these effectors lack signal peptides but are secreted through a Sec-independent secretion pathway. Agroinfiltration of these effectors in *Nicotiana benthamiana* suppressed the hypersensitive response (HR) by enhancing the expression of the cell death suppressor genes PR-1 and PR-5 [129]. In addition, some phytoplasma effectors are also transmembrane proteins, such as Imp (immunodominant membrane protein) [130] and Amp (antigenic membrane protein) [131]. Based on the fact that phytoplasmas have some genuine transmembrane proteins acting as effectors, Debonneville et al. [132] recently identified effectors in the Flavescence dorée phytoplasma using SignalP v5.0 and Phobius. They found seven effector candidates with transmembrane domains.

The algorithm PhyEffector was able to retrieve both classes of effectors (canonical and non-canonical) from 20 phytoplasma genomes [83] with an accuracy value of 0.90, supporting PhyEffector as a suitable pipeline for the identification of effectors in phytoplasma genomes. This shows that going beyond the canonical criteria allows for the expansion of effectoromes and the discovery of novel effectors. New algorithms may consider these novel discoveries surrounding non-canonical effectors for improved performance and accuracy.

4. Breaking the Box: Opportunities to Revolutionize Effectoromics

Current effectoromics strategies have been developed independently of each other for the organisms of different kingdoms; as shown before, the majority of the algorithms that have been developed to identify effectors are dedicated to retrieving candidates from only a particular kingdom; fungal effector predictors are not usually compatible with bacteria, for example.

In fact, some common concepts have received different terminologies in the different areas: the effectors that have no homologs in other organisms are termed "pioneer effectors" in nematodes [133,134], "orphan effectors" in insects [135], "species-specific effectors" in fungi [136], and "lineage specific effectors" in oomycetes [77]. The lack of sequence conservation among the effectors, even among organisms of the same kingdom, has led us to believe that effectors from organisms of different kingdoms are completely unrelated. However, fungal effectors contain motifs previously believed "exclusive" to oomycetes, such as RxLR and LFLAK [73]; conversely, oomycetes also share some domains previously associated specifically with fungi, such as the WY domain [137]. When new or "out-of-box" strategies have been used, novel insights about effectoromics have been discovered.

Effectors from organisms of different kingdoms may target the same protein in the plant host (hub proteins), which play key roles in the plant host cell. Maybe there are similarities between those effectors that are not easy to identify due to the lack of sequence conservation. Screening for other features such as short linear motifs (SLiMs) or eukaryotic linear motifs (ELMs), involved in key transient interactions with proteins, DNA, or RNA, may help uncover common avenues among effectors. Many eukaryotic, bacterial, and viral pathogens mimic SLiMs present in host cell proteins, developing mimicry peptides (mimitopes) that hijack and sabotage cellular processes as part of the infection cycle. Motif screening in phytoplasma effectors recently revealed the SliM LIG_GBD_Chelix_1 motif in eight effectors [7]; this motif allows for the recruitment of the actin-regulatory proteins that initiate actin polymerization. Interestingly, actin polymerization is a common molecular mechanism found in infections by both plant and human bacterial and virus pathogens [138]. SLiMs/ELMs are becoming increasingly studied in pathogens and may help to unravel currently unknown functional relationships.

Likewise, effectors with similar three-dimensional structures may share similar functions and may be termed "functional orthologs" even though they do not share significant sequence identity [139,140]. Some analyses found conservation in 3D protein structure in effectors of the same organism such the MAX effectors from the blast fungus *Magnaporthe oryzae*; MAX effectors have high sequence divergence but share a common fold characterized by a ß-sandwich core stabilized by a conserved disulfide bond [134,135]. In *Leptosphaeria maculans*, the causal agent of oilseed rape stem canker, crystal structure analysis found that AvrLm3 and AvrLm5-9 are structural analogues of AvrLm4-7. These three effectors are not conserved in sequence and are recognized in oilseed rape by different resistance proteins [141]. Recently, two independent studies performed 3D protein structure analyses of effectors from the Pathogen–Host interaction database (PHI-base). Rozano et al. [142] used the program RaptorX for modeling ToxA-like effectors, MAX-like effectors, and virulence proteins obtained from PHI-base and observed the conservation of these ancestral structural folds amongst cytotoxic peptides from a diverse range of distant species. Seong and Krasileva [136] used AlphaFold 2 and modeled the structures of 26,653 secreted proteins from 14 agriculturally important fungal phytopathogens taken from the PHI-base. These authors found evidence that many effector families could have originated from ancestral folds conserved across fungi. Similar to Foldseek, an algorithm created for bacterial T6Es that works like BLAST but for 3D protein structures [64], future algorithms for effector identification in other organisms should consider the comparison of a three-dimensional structure of proteins. The 3D protein structures of effector proteins would not only be important for effector identification but also for the prediction of their interactions with their plant targets through molecular docking. As such, it would be necessary to develop a comprehensive database of host targets.

In addition, interesting findings may result from comprehensive in silico characterization of effectors collected from organisms of the different living kingdoms (Figure 5), including novel characterizations such as post-translational modifications that may be critical for final cell localization and function. This is an exciting era of frequent novel discoveries in effectoromics.

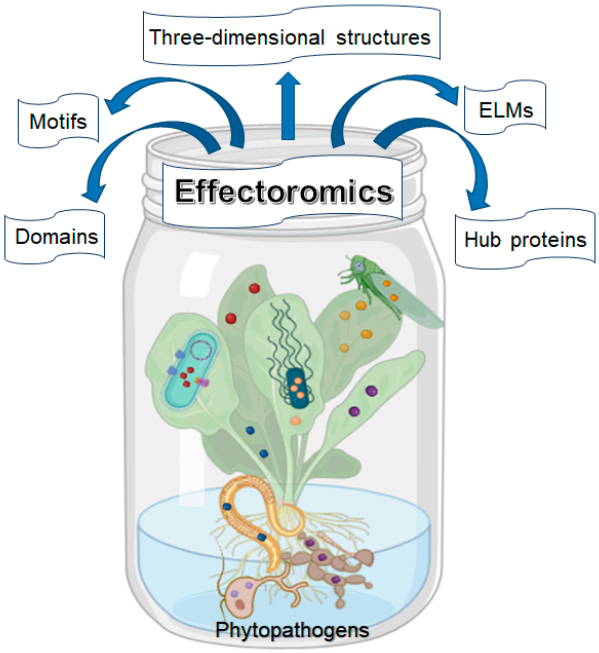

Figure 5. Proposal for effectoromics integration. Pooling of effectors of different biological kingdoms and cross-kingdoms in silico characterization. The orange, blue, red, and purple circles represent the effectors.

5. Conclusions

Effectoromics is an area in constant evolution. Its history has evolved, in large part through canonical effectors that are captured by identification strategies that often fail to identify non-canonical effectors. New perspectives such as the routine searches for conserved and de novo domains, motifs such as SliMs/ELMs, and routine identifications involving three-dimensional structural predictions and post-translational modifications may help to uncover similarities between unrelated effectors. These new perspectives could also foster the creation of algorithms that elucidate the complete effectorome, including the highly evasive non-canonical effectors. Lastly, efforts should be made to integrate effector identification strategies by pooling true effector databases from different classes of organisms to create comprehensive effectorome algorithms that function for pathogens and pests of different living kingdoms.

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