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"Order from disordered": Potential role of intrinsically disordered regions in phytopathogenic oomycete intracellular effector proteins

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Abstract

There is a continuous arms race between pathogens and their host plants. However, successful pathogens, such as phytopathogenic oomycetes, secrete effector proteins to manipulate host defense responses for disease development. Structural analyses of these effector proteins reveal the existence of regions that fail to fold into three-dimensional structures, intrinsically disordered regions (IDRs). Because of their flexibility, these regions are involved in important biological functions of effector proteins, such as effector–host protein interactions that perturb host immune responses. Despite their significance, the role of IDRs in phytopathogenic oomycete effector–host protein interactions is not clear. This review, therefore, searched the literature for functionally characterized oomycete intracellular effectors with known host interactors. We further classify regions that mediate effector–host protein interactions into globular or disordered binding sites in these proteins. To fully appreciate the potential role of IDRs, five effector proteins encoding potential disordered binding sites were used as case studies. We also propose a pipeline that can be used to identify, classify as well as characterize potential binding regions in effector proteins. Understanding the role of IDRs in these effector proteins can aid in the development of new disease-control strategies.

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Effectors, Intrinsically disordered regions oomycetes, Molecular mimicry, Short linear motifs, Molecular recognition features.

Introduction

Phytopathogenic oomycetes are devastating plant pathogens causing disease in a wide range of economically important crops, posing a threat to agriculture and global food security [1,2]. The success of these pathogens is largely attributed to their ability to secrete a plethora of virulence proteins, called effectors, that modulate host physiology or immunity and promote infection [3]. These effectors can either be secreted into the apoplastic space (apoplastic effectors) to inhibit non-specific defense mechanisms or are translocated into the interior of the host cell to exert their functions in different organelles (cytoplasmic/intracellular effectors) [4,5]. Crinkler (CRN) and RxLR (Arg-Xaa-Leu-Arg) effectors are two classes of oomycete cytoplastic/intracellular effectors [6]. RxLR effectors are largely implicated in the biotrophic phase of oomycete infection cycle; thus in some cases (not always), they inhibit cell death caused by recognition of non-self-components [7,8]. On the other hand, CRNs were initially identified through their ability to cause crinkling and necrosis upon expression in plant tissue, and thus, they were considered as a class of cell death inducing effectors [9]. However, studies show that this is not a universal feature of CRN proteins [10,11].

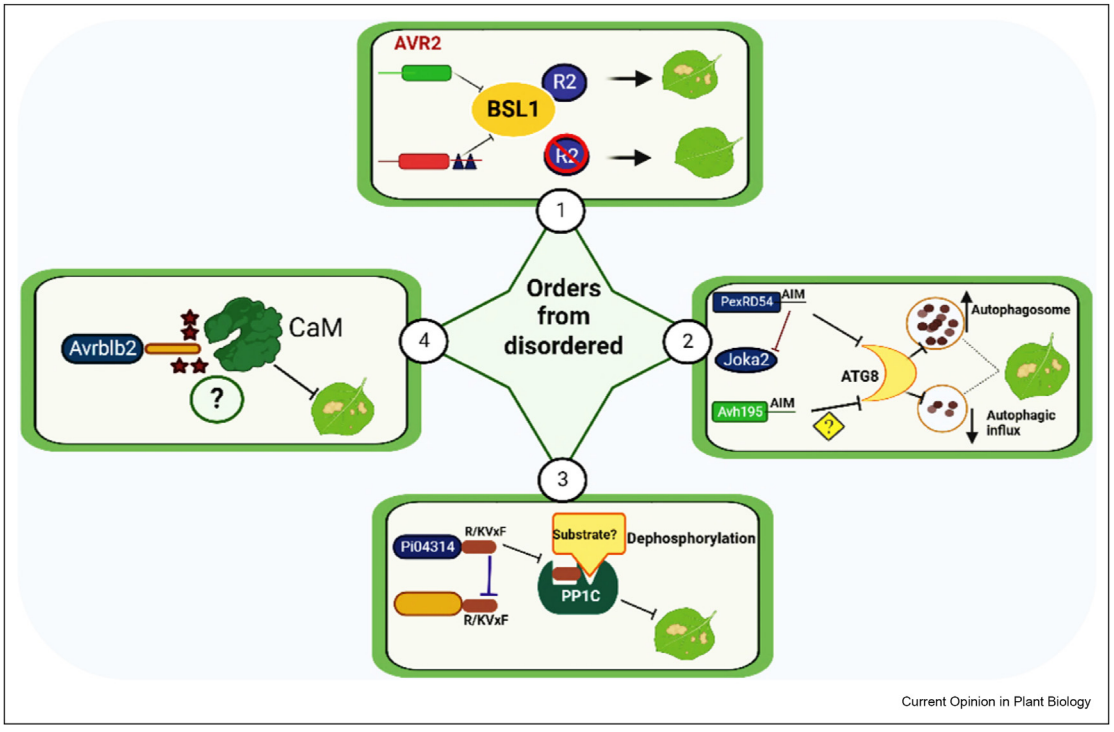
One of the mechanisms employed by these effectors to manipulate host immune responses is by interacting with crucial immunity-associated plant proteins [3,12]. Of importance to note is that the functionality of proteins, including effectors, lies not necessarily on the entire protein sequence/structure, rather within important regions or modules called domains or motifs. Most of these modules assume a stable three-dimensional (3D) structure conforming to the structure—function dogma, which states that a protein's structure determines its function. The last few decades have seen the discovery of hybrid proteins, protein with both well folded regions as well as intrinsically disordered regions (IDRs) [13–15]. One established function of IDRs is involvement in interactions with structured binding pockets [16,17]. The question that arises is: how do these regions mediate such crucial biological functions? Advances in biochemical and structural analyses of proteins reveal that IDRs usually contain more charged residues than hydrophobic amino acids [18], thus these charged molecules tend to

make larger interaction surfaces with a solvent instead of forming a folding core [19]. The binding sites within IDRs are often 3–8 amino acid long peptide for short linear motifs (SLiMs) and 10–70 amino acid residues for molecular recognition features (MoRFs) [20,21]. Intensive studies have been carried out on the role of IDR binding regions in viral proteins as well as bacterial effectors where they employ molecular mimicry by displaying similarities with SLiMs of the host, thus hijacking the host cellular machinery [22–26]. Although the occurrence of IDRs in oomycete effector proteins has been reported [8,27–29], to the best of our knowledge, there is no study that has provided a direct link between IDRs and host protein interaction in oomycete effector proteins. This lag could be attributed to the fact that there are few oomycete effector proteins with solved 3D structures, limiting the identification of motifs/domains that mediate effector–host protein interactions. This review, therefore, seeks to (i) collect data from the literature describing verified targets of oomycete RxLR and CRN effector proteins and further analyze for the presence of binding sites mediating protein interactions; (ii) use these characterized effectors to establish the potential role of IDR binding sites in mediating effector–host protein interactions; (iii) propose a pipeline for identifying, classifying, and characterizing potential binding sites within IDRs (Figure 1).

Targets of oomycete intracellular effectors: A decade of known preys

Since the discovery of E3 ligase CMPG1 as a host target protein of the well characterized AVR3 effector from *Phytophthora infestans* [30], various studies have been conducted in the past 10 years in search of phytopathogen oomycete effector preys [31]. This has been made

Figure 1



Potential role of intrinsically disordered regions (IDRs) in five oomycete effector proteins (grouped into four case studies). (1) AVR2 effector with avirulent (green) and virulent (red) phenotypes. The virulent phenotype is characterized by IDRs (straight line) at both N and C terminal regions. Both the avirulent and virulent AVR2 can interact with the target protein phosphatase BSU-LIKE PROTEIN1 (BSL1). The interaction between avirulent phenotype and BSL1 leads to recognition by the host resistance-protein (R2); however, the presence of IDRs in the virulent phenotype promotes mutations (blue triangle) leading to the effector escaping R2 recognition. (2) PexRD54 effector encodes an autophagy interacting motif (AIM), within an IDR, that mediates the interaction of the effector with a host autophagy-related protein 8 (ATG8) by outcompeting the autophagy cargo receptor Joka2 to activate an autophagic process for disease development. Similarly, *Phytophthora parasitica* Avh195 effector encodes a potential AIM motif that facilitates the association of the effector with ATG8 to lower autophagic influx, thus promoting virulence. However, the mechanism of action of this short linear motif (SLiM) is yet to be established. (3) *Phytophthora infestans*, Pi04314 RxLR effector, interacts with host protein phosphatase 1 catalytic (PP1c) via R/KVxF motif found within an IDR. It does this by mimicking a host regulatory subunit (yellow bar) to form a holoenzyme leading to dephosphorylation of an unknown target substrate for disease development. (4) Avrblb2 effector from the *P. infestans* encodes a calmodulin binding motif (yellow) that promotes binding to calmodulin (CaM) in the presence of calcium ions (brown stars) leading to immune response signaling and hypersensitive reactions. The mechanism of binding remains unknown.

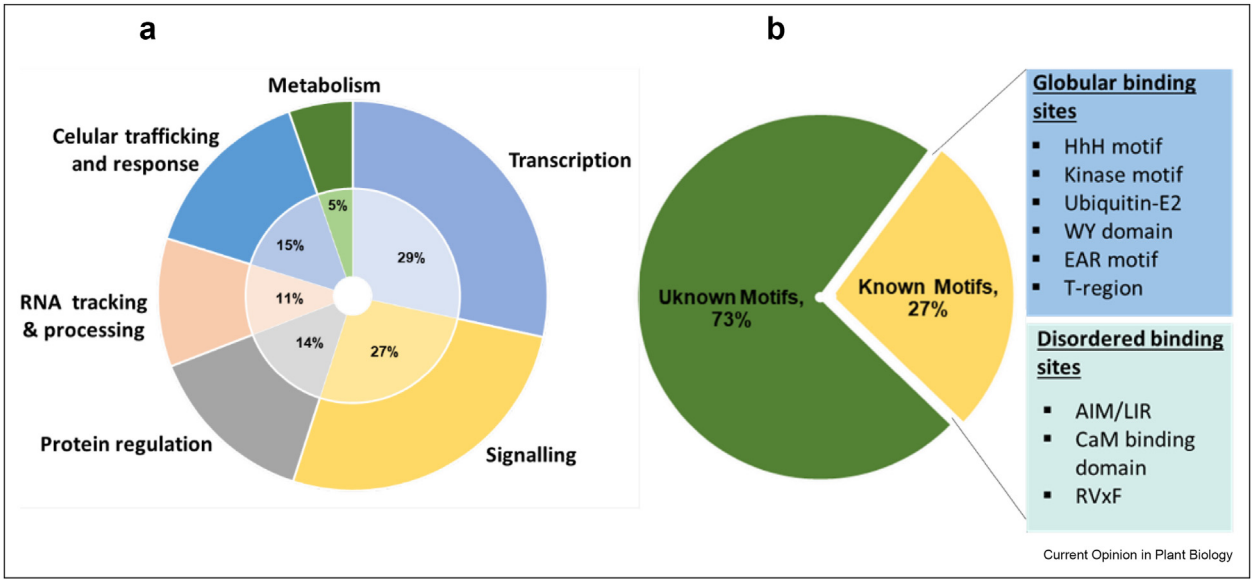
possible through recent developments in methods that enable mass screening of these targets using yeast two-hybrid (Y2H) or *in planta* coimmunoprecipitation/tandem mass spectrometry (coIP/MS) assays [32–34] as well as specific methods such as bimolecular fluorescence complementation, pairwise-Y2H, or fluorescence resonance energy transfer [35–37]. Effectors employ different mechanisms to target various host proteins/processes implicated in defense. To determine host proteins that are targeted by oomycetes, we manually collected data from the literature describing verified host targets of oomycete intracellular effectors and further classify these proteins manually based on their biological functions (Supplemental Table 1). Our data mining revealed 56 targets of oomycete effector proteins with the majority of these being involved in transcription and signaling. Other effector targets include proteins involved in metabolism, cellular trafficking, protein regulation, or RNA trafficking/processing (Figure 2a). It is important to note that these targets are positive as well as negative regulators/susceptibility factors (S factors) (Supplemental Table 1). Effectors from filamentous phytopathogens have been recently shown to target S factors for disease development [12]. Knowledge on S factors can help to develop new and effective strategies to control plant disease.

Although several oomycete effectors are known to interact with host proteins, specific domains/motifs that mediate the interactions have not been fully explored. From the few motifs that are known, we used the eukaryotic linear motif (ELM) resource to classify these motifs into globular and disordered binding sites (Figure 2b). Based on our analysis, WY motif is a prevalent globular motif in oomycete effectors followed by Helix–hairpin–helix motif. Other globular motifs include kinase and ubiquitin-associated motifs, ethylene-responsive element binding factor-associated amphiphilic repression motif as well as linker regions (T-regions). On the other hand, autophagy-associated motif, calmodulin (CaM)-binding motif, and protein phosphatase 1 (PP1) docking motifs can be classified as disordered binding sites (Figure 2b).

Playing both 'hide-seek' and 'copycat' games: Role of IDRs in evading host immune responses and molecular mimicry

The inability of IDRs to fold into a well-defined structure is portrayed as an advantage to effector proteins due to (i) increased interaction surface area; (ii) conformational flexibility to interact with several protein targets; (iii) the presence of molecular recognition elements that fold upon binding to facilitate their interaction with target substrates; (iv) accessibility of post-translational modification sites as described by Tompa [38]. These features promote molecular promiscuity of effectors such that they are able to interact with more than one host target. Of importance to note is that the N terminus of oomycete effectors is more enriched with intrinsically disordered content than the C terminus implying its role in effector translocation [27,28]. Intriguingly, the C terminal regions of these effectors is

Figure 2



(**a**) Percentage of host plant proteins targeted by phytopathogenic oomycete effector proteins. The proteins were classified based on their biological function in plants. (**b**) Percentage of known and unknown binding sites of 56 oomycete intracellular effectors. The identified binding sites can be categorized as either globular or intrinsically disordered motifs. The total numbers of motifs are indicated in brackets.

where the activity lies. Does this mean that IDRs in this region play a role in mediating the biological activity of these effectors? To answer this question, we conducted a case study of five oomycetes cytoplasmic effectors with known host targets as well as known motifs/domains mediating interactions. We first submitted the effectors' sequences to predictor of natural disordered regions (PONDR) tool http://www.pondr.com/to predict the presences of IDRs, followed by classification of binding regions within IDRs using the ELM http://elm.eu.org/.

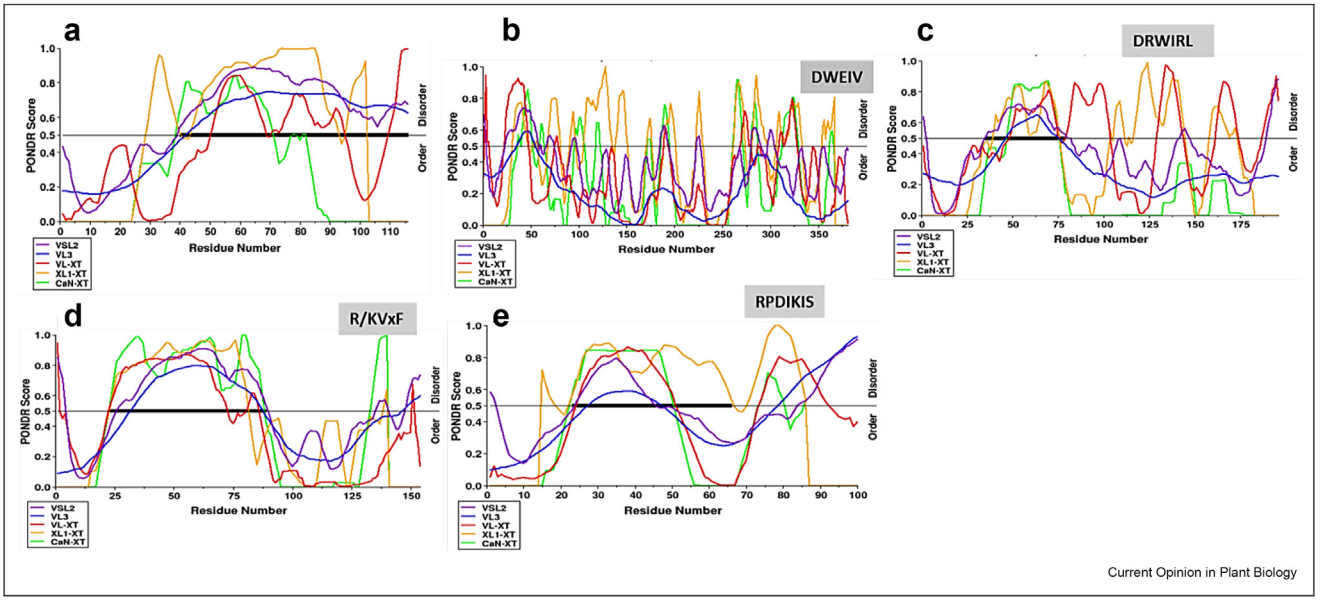
AVR2 effector: Role of IDRs in evasion of host recognition

Effector recognition is a central event in the activation of host immune responses that leads to taming of pathogen progression [39]. However, successful plant pathogens are constantly mutating their effectors to evade host recognition [40]. As an example, AVR2 effector can not only be perceived by the R2 protein (avirulent) inside plant cells, leading to an immunity reaction but it can also evade host recognition (virulent) by sequence and expression polymorphisms [41]. Both the avirulent and virulent AVR2 can interact with the target protein phosphatase BSU-LIKE PROTEIN1 (BSL1). However, only the avirulent type further promoted the interaction of BSL1 with R2 [42,43]. This suggests structural differences between the avirulent and virulent phenotypes [44,45]. A recent study showed that the virulent phenotype of this effector has a higher disorder content in both the N and C terminal regions than the avirulent phenotype [8]. The presence of IDRs at the C terminal of AVR2 effector domain (Figure 3a) allows the effector to be flexible and open to mutations such as deletion, insertion as well as intragenic recombination contributing to the evasion of resistance-protein detection in *P. infestans* [8]. Therefore, the structural flexibility of IDR regions provides functional advantages for effectors to maintain key features required for pathogen virulence activity but prevent recognition by host immunity systems.

PexRD54 and Avh195 effectors: Role of IDRs in manipulation of host autophagy

Plants employ autophagy to protect themselves against pathogens [46,47]. However, pathogens deploy effector proteins to interfere with autophagy-related processes for disease development. *P. infestans* PexRD54 effector has been shown to promote infection by interacting with plant autophagy-related protein ATG8CL, subsequently stimulating the formation of autophagosomes [48]. This allows PexRD54 to direct autophagic vesicles to the feeding sites of *P. infestans* so that the pathogen can potentially divert nutrients [49].

Figure 3



Prediction of IDRs in five oomycete intracellular effectors using PONDR® tool (Predictor of Natural Disordered Regions). The tool integrates five different types of predictors: VLXT, XLI_XT, CAN_XT, VL3-BA, and VSL2 (shown in different color lines). Identified binding sites within IDRs are shaded in gray. (**a**) AVR2 effector with IDRs largely at the C terminus. (**b**) PexRD54 effector with IDRs spanning the entire protein sequence. (**c**) Avh195 effector encoding IDRs both at the N as well as C terminal regions. (**d**) Pi04314 effector encodes IDRs largely at the N terminus with a conspicuous disordered region between 136 and 142 amino acids at the C terminal region. (**e**) Avrblb2 effector encodes IDRs at the N as well as C terminal regions. IDR, intrinsically disordered region.

Structural dissection of PexRD54 effector reveals five tandem WY domains with a disordered C terminal AIM from amino acid 378−381 that mediated the effector's activity [50]. Using the ELM resource, we classified this motif Asp-Trp-Glu-Ile-Val (DWEIV) as a ligand binding SLiM (LIG_LIR_Gen_1) (Figure 3b). How does a five amino acid motif take charge of the host autophagy machinery? The motif mimics autophagy adaptor/receptor (Joka2) by binding to host ATG8 protein to stimulate the formation of ATG8-marked autophagosome thus eliminating molecules implicated in defense [48]. Previous studies have shown that molecular SLiM mimicry is used by pathogenic bacteria effectors to hijack host cell machinery [24,51].

Similarly, *Phytophthora parasitica* RxLR effector Avh195 (not orthologous to PexRD54) interacts with ATG8 to manipulate the host immune responses [52]. Unlike PexRD54 effector that activates autophagy after its interaction with host ATG8, Avh195 was shown to slow down the autophagic influx [52]. The effector executes this activity with the help of three potential AIM motifs (DRWIRL, KSYDDI, and PIWREV) that are encoded in this effector. Among the three motifs, DRWIRL motif from amino acids 131−136 recorded the highest binding score. Interestingly, our prediction revealed that this region is within an IDR as depicted in Figure 3c. In addition, ELM database classified this motif as a ligand binding motif (LIG_LIR_Gen_1). Thus, Avh195 encodes a potential SLiM that is crucial in manipulating host autophagy. It remains to be determined whether its mechanism of action is like that of PexRD54 motif. From these findings, it is evident that effectors can encode more than one SLiM of the same class within IDRs. This could be a strategy the pathogen uses to increase its odds in manipulating host processes that are crucial for defense. Also, the same motif found in different effectors can employ different mechanisms to achieve a common goal.

Pi04314 effector: Role of IDRs in manipulating host protein regulation

Our structural analyses show that Pi04314 effector encodes IDRs at both the N and C terminal regions. Interestingly, SLiM classification using the ELM database revealed that the C terminal region amino acids 136−142 (Figure 3d) corresponds to a molecular docking motif (DOC_PP1_RVXF_1). The motif is found in regulatory subunits that either link PP1 with the substrates or inhibit it [53]. Remarkably, functional characterization of Pi04314 revealed that this effector interacts with three host PP1c isoforms via a conserved R/KVxF motif [54]. This is a typical example of molecular mimicry where the effector 'acts' as a regulatory subunit to co-opt host PP1c activity for the benefit of *P. infestans* pathogen. Previously, the regulation of PP1 has been mediated by intrinsically disordered proteins [55]. This shows that IDR binding sites are not only important for establishing interactions between catalytic and regulatory subunits but also contribute to the regulation of these interactions.

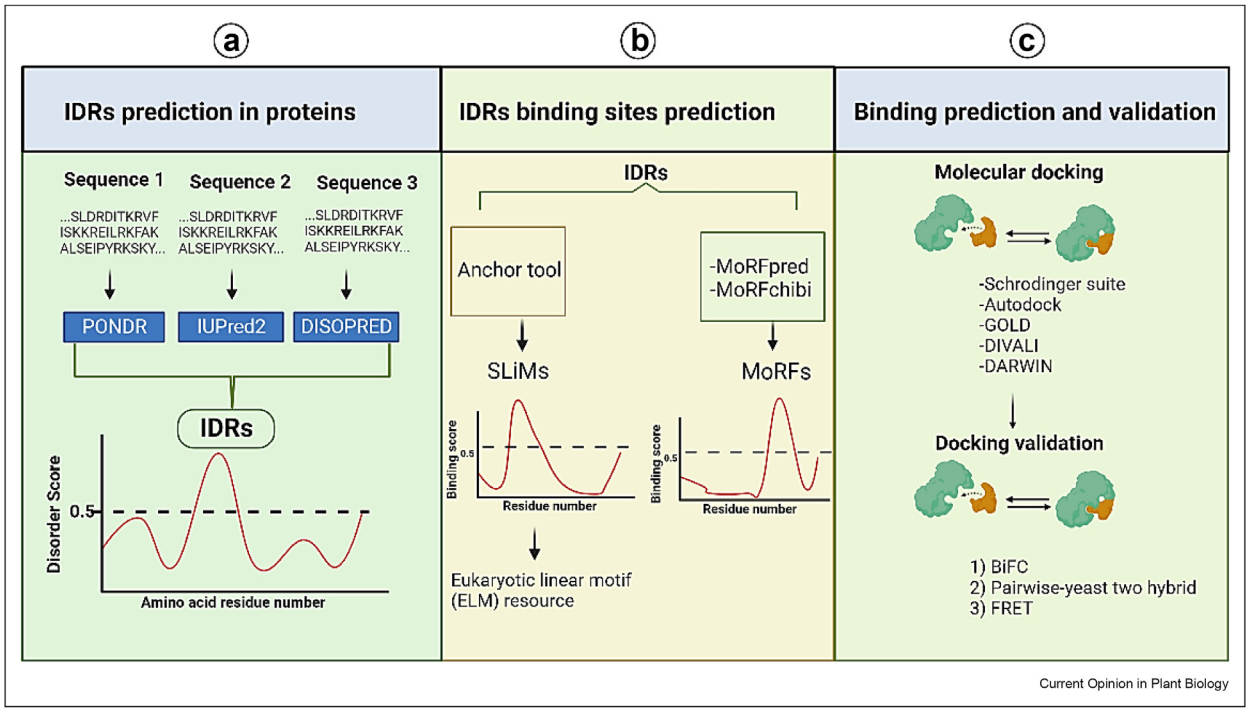
Avrblb2 effector: IDRs in perturbing host signaling pathways

P. infestans Avrblb2 effector encodes IDRs in both the N and C terminal regions (Figure 3e). Deletion of 1−87 aa and 77−100 aa in this effector impaired its activity. Specifically, amino acids 78−82 correspond to CaM-binding motif (CaM-binding site). Interestingly, ELM database classification revealed that amino acids 78−84 (RPDIKIS) correspond to a molecular docking motif (DOC_PP2B_PxIxI_1), classified as calcineurin substrate docking site implicated in dephosphorylation of serine/threonine phosphorylation sites. Furthermore, *in planta* functional analyses of this effector reveals that in the presence of Ca^{2+}, the effector binds to CaM via the CaM-binding site to perturb host signaling pathways [56]. These findings suggest IDR-associated motif (CaM-binding site) plays a critical role in determining the virulence activity of Avrblb2 in host cells. However, molecular and biochemical studies are needed to dissect the specific mode of action of this motif. In another *P. infestans* effector, SFI5 was reported to suppress host immune responses by interacting with CaM via CaM-binding motif [57]. However, this motif is unique with an alpha helical fold and amphipathic properties, thus it could not be classified under the ELM database.

Conclusion and future perspective

Significant progress has been made over the past two decades in the identification and functional characterization of oomycete intracellular effectors. However, many effectors await structural characterization to ascertain the occurrence of IDRs as well as motifs that mediate the activity of these effectors. What makes many oomycete effectors remain structurally uncharacterized? One significant challenge is that laboratory approaches for protein structure determination are expensive and cannot be used on all proteins. Therefore, there is a need to exploit existing *in silico* tools to predict the occurrence of both globular and disordered regions, identify as well as classify potential binding regions within IDRs. A typical pipeline used for identifying potential IDRs and motifs mediating effector−host protein interactions is presented in Figure 4. This starts with prediction of the presence of IDRs in effector protein sequences using web-based predictors such as PONDR, IUPred2 as well as DISOPRED (Figure 4a). This is followed by the identification and classification of potential binding sites such as SLiMs as well as MoRFs within the identified IDRs (Figure 4b). Finally, the potential IDR binding sites can be assessed for their role in binding to host targets using both *in silico* as well as *in planta* analyses (Figure 4c).

Figure 4



Proposed pipeline toward the identification and characterization of IDR binding sites. (**a**) Prediction of IDRs in protein sequences using Predictor of Natural Disordered Regions (PONDR), IUPred2, and DISOPRED predictors. Amino acids with a score of >0.50 are considered as disordered in all the three predictors. (**b**) Prediction of IDR binding sites (MoRFs and SLiMs) using MoRFchibi system and ANCHOR tools. This is followed by the classification of predicted SLiMs using the eukaryotic linear motif (ELM) resource. (**c**) Finally, the binding potential of MoRFs or SLiMs to potential targets is determined using molecular docking tools (Schrodinger suite, Autodock, Genetic Optimization for Ligand Docking (GOLD), Docking wIth eVolutionary AlgorIthms (DIVALI) as well as DARWIN). In addition, *in vivo* validation of the interactions can be performed using (1) bimolecular fluorescence complementation (BiFC) assays, (2) pairwise yeast two-hybrid (Y2H), or (3) fluorescence resonance energy transfer (FRET) assays. IDR, intrinsically disordered regions; MoRF, molecular recognition features; SLiM, short linear motif.

From the five effectors reviewed here, it is evident that IDRs are directly involved in oomycete effector activities such as escaping host recognition as well as protein–protein interactions. Therefore, more work is required on IDRs to provide a better understanding of their role in pathogenesis.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pbi.2023.102402.

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* of special interest
** of outstanding interest

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