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REVIEW ARTICLE

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Nuclear Effectors in Plant Pathogenic Fungi

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ABSTRACT

The nuclear import of proteins is a fundamental process in the eukaryotes including plant. It has become evident that such basic process is exploited by nuclear effectors that contain nuclear localization signal (NLS) and are secreted into host cells by fungal pathogens of plants. However, only a handful of nuclear effectors have been known and characterized to date. Here, we first summarize the types of NLSs and prediction tools available, and then delineate examples of fungal nuclear effectors and their roles in pathogenesis. Based on the knowledge on NLSs and what has been gleaned from the known nuclear effectors, we point out the gaps in our understanding of fungal nuclear effectors that need to be filled in the future researches.

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1. Introduction

The nuclear import of proteins is a pivotal process for nearly all aspects of eukaryotic cells. Proteins as diverse as transcription factors, core histone, cell cycle regulators and ribosomal proteins, are transported from cytoplasm to the nucleus. Such import occurs *via* a cylindrical proteinaceous, ring-like structure called nuclear pore complexes (NPCs) [1]. These structures are composed of approximately 500 proteins (nucleoporins, Nups) that consist of 30 different polypeptides [2,3]. While NPCs allow passive diffusion of small molecules (<40–60 kDa), the trafficking of larger proteins requires a specific sorting signal called nuclear localization signals (NLS) [4]. This NLS-mediated nuclear localization is a highly regulated process and has been associated with multiple physiological activities of the cell.

Studies on the host-pathogen interactions are vital to understanding the disease biology and subsequent development of the preventive strategy. At the heart of the interaction are fungal effectors that are secreted into the host cells. The fungal effector proteins generally contain a signal peptide for secretion, no trans-membrane domains, no similarity with other known protein domains, and are fairly small in size and mostly species-specific. They have usually been associated with the suppression of the host defense and induction of susceptibility through different mechanisms including interference of the host RNA silencing, hypersensitive response, PTI process, and immune signaling pathways [5].

Effector proteins from plant pathogenic microorganisms such as bacteria, viruses, and nematodes have been widely reported to localize into the plant nucleus and target plant components to survive against plant defense response and induce the pathogenicity [6]. Similarly, phytopathogenic fungi have been shown to secrete different effector proteins that can migrate to the apoplastic region or various intracellular compartments (chloroplast, mitochondria, nucleus, etc.) of the plant cell. Once localized into the nucleus, the effector protein appears to target plant components associated with the defense responses. Several previous reviews summarized the evolution, secretion, and function of the effector protein in different pathogenic fungi and oomycetes [7–13].

A few studies showed translocation of effector proteins secreted by fungal pathogens into the plant nucleus [14,15]. These nuclear-translocated effectors are termed nuclear effectors and appear to be important for fungal pathogenesis. However, the knowledge of the molecular mechanism of the nuclear effector of phytopathogenic fungi in plant disease is limited. Below we summarize the types and *in-silico* predictions of NLSs and then provide examples of nuclear effectors that have been studied in the plant pathogenic fungi to date. These examples show that nuclear effectors affect essential processes, such as DNA replication, gene expression regulation, or epigenetic state of host chromatin in favor of the disease development. Last but not least,

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we come up with questions that should be answered in future works in order to gain deeper understanding of functions and evolution of nuclear effectors in fungal pathogens.

2. NLS diversity and their biological significance

Since the first identification of NLS in Simian Virus 40 (SV40) large T-antigen (TAq) in 1989, a large number of NLS have been identified from different organisms. They are broadly classified into two categories: classical nuclear localization signals (cNLSs) and nonclassical nuclear localization signals (ncNLSs). Based on the number of basic amino acid clusters, cNLSs are further divided into two subcategories: monopartite and bipartite cNLSs. Monopartite cNLSs contain one short stretch of the basic amino acid (e.g., KR[K/R]R and K[K/R]RK), whereas bipartite cNLSs have two stretches of basic amino acids separated by a linker 10–12 amino acids (e.g., $KRX_{10-12}K[K/R][K/R]$) [16]. cNLSs are recognized by an adapter protein, importin α, which harbors two domains: N-terminal Impβ1-binding ($\alpha 1\beta\beta$) and C-terminal domain consisting of 10 tandem armadillo (Arm) repeat and binds to the major and minor binding sites on the concave surface of the Arm repeat domain. While bipartite NLSs interact with both binding sites of importin α, monopartite NLSs preferentially bind to the major binding site [17–19]. In addition to cNLS, several additional types of NLSs (that unusual signals dissimilar from the cNLSs), which are termed as non-classical localization signals (ncNLSs), have been identified. ncNLS-containing proteins are predominantly transported into the nucleus *via* interaction with the karyopherin-β [20]. Among the ncNLSs, the "proline-tyrosine" category known as PY-NLS have been studied in detail [4,21,22]. Major characteristics of PY-NLS involve (i) structurally disorder, (ii) overall basic character, and (iii) a hydrophobic or basic region upstream of a C-terminal $R/H/KX_{2-5}PY$ motif [21]. The PY-NLS are recognized by karyopherin-β2 (kap β2/transportin-1). Other ncNLSs identified are isoleucine-lysine NLS, arginine serine repeat NLS (RS-NLS) cryptic NLS, epitope NLS, etc.

In general, nuclear-localized proteins contain a single NLS that mediates nuclear localization either through classical or non-classical nuclear localization pathway. However, nuclear proteins without an NLS may also be localized to the nucleus by complexing with a protein containing NLS. For example, Steidl et al. reported that the protein subunit HapC and HapE can be transported to the nucleus of the filamentous fungus *Aspergillus nidulans* after complexing with NLS-containing protein HapB *via* a piggyback mechanism [23]. The presence of multiple NLS has also been reported in several nuclear proteins such as *nuclear factor 1-A* [24], BRCA1 [25], BRCA2 [26], *S. cerevisiae* Mcm10p [27], *A. nidulans* HapB [28], 5-lipoxygenase [29], Dot1a [30], etc. Although the reasons behind the phenomenon have not yet been fully explained, selected studies indicate that multiple NLS may work collaboratively to promote nuclear localization resulting in significant nuclear accumulation of the target protein [29,31,32].

3. Prediction of nuclear localization and or nuclear localization sequence (NLS)

A number of bioinformatic tools have been developed to aid in predicting the nuclear localization of the protein (Table 1). Most of these tools rely on sequence similarity, machine learning approach, and the property of the amino acids and provide faster identification of the candidate protein (for example, nuclear effectors) from a pool of proteome dataset (for example, secretome) for subsequent analysis. Commonly used tools to detect NLSs includes PSORT [33], PredictNLS [34], NLStradamus [35] and cNLSmapper [36]. PSORT (or PSORT II) uses a machine learning approach based on known protein sorting signals and predicts nuclear localization in the query sequence by matching with the defining features of the NLS. Clusters of basic amino acids K and R and gaps between the clusters are the key determinants for the prediction of NLS by PSORT II. On the other hand, PredictNLS predicts NLSs based on a query database of putative NLSs that include known cNLS and M9 domain motif [34]. NLStradamus uses an experimentally validated yeast NLS dataset and predicts novel NLSs in proteins using hidden Markov models (HMMs) [35]. cNLS Mapper detects the cNLSs by calculating the NLS activities (scores) of the peptide but not by the conventional sequence similarity search or by the machine learning strategy [36]. Furthermore, the updated NLSdb may be used to screen the NLS information in the protein of interest [37]. Computational tools that were developed to predict the localizations (subcellular or nuclear) of a protein were *WoLF PSORT* [38] and NucPred [39]. WoLF PSORT is an extension of PSORT II, which converts amino acid sequences into numerical localization features based on the sorting signals, amino acid composition, and functional motifs. After conversion, a simple k-nearest neighbor classifier is used for prediction [33,38]. NucPred predicts the nuclear localization of a protein using algorithms that utilize both nuclear and non-nuclear protein as their training set [39].

Table 1. Nuclear effectors in plant pathogenic fungi.

No.	Effector	Property	Fungus	NLS	NLS Prediction tool	Target	Lifestyle	Function	References
1	MoHTR1	Zinc finger protein	*Magnaporthe oryzae*	–	WoLP-PSORT	OsMYB4	Hemibiotroph	Transcriptional reprogramming of the genes involved in plant immunity	[15]
2	MoHTR2	Zinc finger protein	*Magnaporthe oryzae*	–	NLStradamus	OsWRKY45	Hemibiotroph	Bind to the promoter region of the immunity-related genes and alter their expression	[15]
3	VdSCP41	small cysteine-rich protein	*Verticillium dahliae*	RKLKRRKLF	cNLS Mapper	CBP60	Hemibiotroph	Bind to host transcription factor and interrupt its activity	[14]
4	VdSCP7	small cysteine-rich protein	*Verticillium dahliae*	MAKRERVEMGNMAPTMKKTKG	cNLS Mapper	–	Hemibiotroph	Targets the host nucleus to modulate plant immunity	[59]
5	VdSSR1	secretory silencing repressor	*Verticillium dahliae*	–	–		Hemibiotroph	Reduce cytoplasmic miRNAs in fungal cells and increased fungal virulence	[50]
6	VdRTX1	RNase effector	*Verticillium dahliae*	RSAIEKRATTCGSTYYTTAQVNAAANAACQHV	cNLS Mapper	-	Hemibiotroph	The protein translocates into the plant nucleus and contributes to pathogenesis	[52]
7	Vd424Y	glycoside hydrolase family 11	*Verticillium dahliae*	RRTKRTSGSVN	cNLS Mapper	-	Hemibiotroph	activate PTI responses in the plant	[60]
8	$VlsPLA_2$	phospholipases	*Verticillium longisporum*	KDPKRCRWDSDGC, and DDSIVKRAETEEAEEEFEYF DANDVDPDIEG	cNLS Mapper	-	Hemibiotroph	suppressing PTI-related HR through interference in signal transduction pathways, altering gene expression	[54]
9	CgEP1	DNA binding protein	*Colletotrichum graminicola*	GAAGGKKNKAKANAANAA	NLStradamous and Wolf-PSORT	Genomic DNA	Hemibiotroph	Binds to DNA and affects host Transcription	[42]
10	BTF3a	transcription factor	*Fusarium proliferatum*	PRRKVKRAPARSGADDKKLQLALKKLNT	cNLS Mapper	-	Hemibiotroph	Unknown	[55]
11	Zuotin	transcriptional repressor	*Fusarium proliferatum*	ENRDQKRHQERKNTNARKKKKAD	cNLS Mapper	-	Hemibiotroph	Unknown	[55]
12	rAsp f 9	allergens	*Fusarium proliferatum*	WSKIALAGLFASAAAQTYSECNPMKKTCDP	cNLS Mapper	-	Hemibiotroph	Unknown	[55]
13	rco-1	Transcriptional repressor	*Fusarium proliferatum*	LDRTIKMWELSAPRQGNQPGPKGGKCVKT	cNLS Mapper	-	Hemibiotroph	Unknown	[55]
14	See1	Organ specific effector	*Ustilago maydis*	–	-	SGT1	Biotrophs	Induce tumor *via* and interaction with maize cell cycle regulator SGT1	[46]
15	*vp1*	Virulence promoting 1	*Ustilago maydis*	RKRSVPFSSGFLRRHRSK	LOCALIZER	-	Biotroph	NLS is necessary for the virulence-promoting function of Vp1	[56]
16	CSEP0064/BEC1054	RNase like effector	*Blumeria graminis*	–	–	PR10	Biotroph	Binds to the nucleic acid and prevents degradation of host ribosomal RNA, and represses plant immunity	[51]
17	Mlp124478	member of the CPG2811 family	Melampsora larici-populina	RHKNGGGSRK	NLStradamus	TGA1a	Biotroph	Remodeling the host transcription process	[41]
18	UvSec117	-	*Ustilaginoidea virens*	–	–	OsHDA701	Biotroph	Negatively regulates defense-related genes in the host	[49]
19	*Pst* GSRE1	Glycine-serine-rich effector	*Puccinia striiformis* f.sp. *tritici*	–	–	TaLOL2	Biotroph	Binds to TaLOL2 and prevent its nuclear localization to decrease host defence	[43]
20	*PNPi*	*Puccinia* NPR1 interactor	*Puccinia striiformis*	–	–	NPR1	Biotroph	Disrupt the NPR1- TGA2 complex and suppression of SA signaling	[45]
21	*Pp*EC23	small, secreted cysteine-rich proteins	*Phakopsora pachyrhizi*	–	–	GmSPL121	Biotroph	Interact with the soybean transcription factor GmSPL121 and suppress plant immunity	[44]
22	ArPEC25	PEXEL motif–containing nuclear effector	*Ascochyta rabiei*	RKRRRRR	NLStradamus	CaβLIM1a	Necrotroph	Suppression of lignin production	[53]
23	CcSp84	cysteine-rich secreted protein	*Cytospora chrysosperma*	PSKCKKRRHARD	cNLS Mapper	–	Necrotroph	Triggers cell death and immune responses in the host plant	[58]
24	CcCAP1	Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein	*Cytospora chrysosperma*	–	–	–	Necrotroph	Suppress plants immune responses	[57]

Despite the development of various bioinformatics tools, the accurate identification of NLS is often hindered by the occurrence of false-positive results that could be due to the sequence similarity between the cNLS sequence and the non-nuclear protein sequences [40]. Moreover, most of these tools are made for cNLS detection and their detection accuracy of NLS for a particular protein depends on their training datasets of known NLSs (mostly cNLSs) and therefore cannot these tools are not useful to predict other types of NLSs such as PY-NLS. It is also important to note that different tools used different strategies to predict NLS in a particular protein. Therefore, the use of multiple predictors is often advantageous and has been successfully applied to identify NLS from phytopathogenic fungal effector protein [15]. However, these bioinformatics tools only suggest probable residues that can acts as an NLS and thus require experimental validation to confirm the putative NLS as functional targeting sequence.

4. Nuclear effectors as transcript factors in host cell

Plant immunity relies on transcription factors (both activators and repressors) for precisely coordinated regulation of many defense genes. Several microbial pathogens including the fungal phytopathogens evolved multiple strategies to subvert host immunity by targeting these transcription factors or other types of protein or cellular processes in the nucleus (Table 1, Figure 1). For example, effector-mediated host transcriptional reprograming has been observed in the hemibiotrophic pathogen *Verticillium dahliae*. They secrete small cysteine-rich NLS protein VdSCP41 that is transported from the fungus to the host nucleus and targets immune regulatory factors calmodulin-binding protein 60 g (CBP60g) and SAR deficient 1 (SARD1) which, in turn bind to the promoter region of the gene involved in SA synthesis [14]. The binding of VdSCP41 with the transcriptional activation domain (TAD) in the C-terminal portion of CBP60g leads to the interruption in the transcription factor activity either by interfering with the activity of the TAD domain or in the recruitment of associated co-activators *via* this domain. This results in the modulation of both SA-dependent and SA-independent regulators and inhibition of plant immunity [14].

Some nuclear effectors can directly bind to the plant DNA and regulate the host transcription process. Kim et al. predicted sixteen effector proteins that carry one or more NLS from the rice infecting pathogen *Magnaporthe oryzae* [15]. Among them, two effectors, MoHTR1 and MoHTR2 are secreted *via* blast interfacial complex (BIC) and translocated into the plant nucleus where they function as transcriptional repressors to reprogram the transcription of host genes associated with the immunity. This study showed that MoHTR1 and MoHTR2 bind to the promoter region of the immunity-related genes and alter their expression. Interestingly, transgenic rice plants expressing these two effectors showed increased susceptibility toward hemibiotrophic pathogen *M. oryzae* and *X. oryzae*, but showed

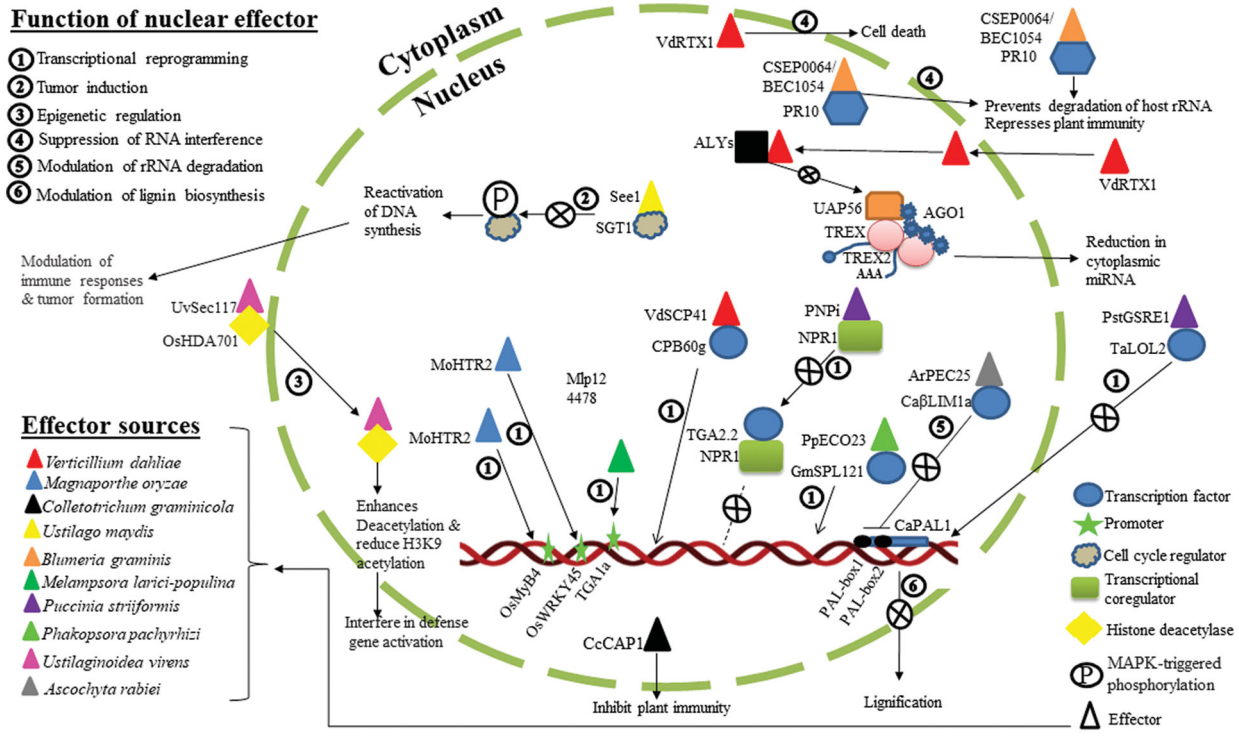

Figure 1. Mode of actions and target sites of phytopathogenic fungal nuclear effectors.

resistance to necrotrophic pathogen *Cochliobolus miyabeanus*. These findings illustrate that the effector-induced manipulation of the host cell could alter the host–pathogen interaction [15].

Similarly, the *Melampsora larici-populina* effector Mlp124478 has also been shown to reprogram host transcription by directly binding to host DNA. They belong to the member of the CPG2811 family containing a putative NLS and a putative DNA-binding domain. They have been shown to localize in both the nucleus and nucleolus of the plant cell. They bind to the TGA1a promoter of the host and remodel the transcription process to suppress genes induced in response to pathogen infection [41]. Another study by Vargas et al. characterized the DNA-binding effector protein of the hemibiotrophic pathogen *Colletotrichum graminicola* CgEP1 revealed that it binds to the promoter regions of several genes of the maize DNA and disrupts the expression of the immune-related genes, leading to successful disease development [42].

Interestingly, few fungal effectors have been shown to target the plant nuclear functions without being localized into the nucleus. These effector proteins interact with the plant transcription factor and alter their nuclear localization leading to disruption of the host nuclear process and defence system. For example, the *Puccinia striiformis* f.sp. *tritici* effector *Pst* GSRE1 has been shown to interact with ROS promoting transcription factor TaLOL2 and disrupts its nuclear localization in plant cells resulting in loss of the host defense response and increasing fungal proliferation [43]. Another cysteine-rich effector protein of *Phakopsora pachyrhizi*, PpEC23, interacts and alters the activity or stability of the soybean transcription factor GmSPL12l, thereby suppressing the expression of the host defense gene in plants. However, in contrast with CBP60g and SARD1, *Gm*SPL12l acts as a negative regulator in the plant defense system [44].

The phytopathogenic fungal effector may also indirectly target the activity of the host transcription factor by binding with the transcriptional regulator to subvert immunity. In wheat plants, for example, the non-expressor of pathogenesis-related 1 (NPR1) protein acts as a transcriptional coregulator for systemic acquired resistance, and during pathogen challenge, it translocates from the cytoplasm to the nucleus, interacts with transcription factor TGA-bZIP, and activates immune gene expression. However, during infection by *P. striiformis*, the effector PNPi interacts with NPR1 and competes with the interaction between NPR1 and TGA2.2 resulting in decreased host defence response [45].

5. Nuclear effector induce tumor in host cell

Plant tumor formation is a particular hallmark of a specific group of phytopathogenic fungal infections. It has been reported that the biotrophic fungus *Ustilago maydis* secretes the novel seedling-specific effector See1 that can localize to both the nucleus and cytoplasm of the plant cell and contributes to tumor progression in maize leaves [46]. Analysis of See1-deletion mutants indicates that protein is required for reactivation of the host DNA synthesis and mitosis, needed for tumor formation in maize leaf cells. Further analysis showed that this protein interacts with the maize cell cycle regulator SGT1 (suppressor of G2 allele of skp1) and interferes with its MAPK-triggered phosphorylation resulting in the modulation of the host immune responses and formation of severe disease [46] (Figure 1). However, isolation and functional characterization of other nuclear effectors associated with the plant cell cycle regulation is required for a deeper understanding of the process of tumor formation and disease development in the plant (Table 1).

6. Nuclear effectors as epigenetic regulators

Histone acetyltransferases protein target histones in the nucleus and epigenetically regulate the global gene transcription and emerged as a key player in plant-pathogen interactions [47,48]. It has been reported that microbial pathogens could have specific effectors to interfere with the usual epigenetic process of the host (Table 1). For example, the rice false smut fungus, *Ustilaginoidea virens* secretes an effector protein UvSec117 that interacts with the histone deacetylase OsHDA701 and promotes its translocation to the nucleus where OsHDA701 negatively regulates defense-related genes in the host. Moreover, transgenic plants expressing UvSec117-silencing RNAs showed increased resistance against phytopathogen [49]. These results demonstrate novel strategies by fungal pathogens that disrupts histone modifications of host chromatin and interfere with plant immunity (Figure 1). However, the complete mechanism behind the nuclear import of UvSec117 would require further investigation.

7. Suppression of trans-kingdom RNA interference by nuclear effectors

Growing evidence demonstrated that small RNAs (sRNA) can bidirectionally travel between interacting organisms to silence targets in the recipient organisms and these trans-kingdom RNA interference play a key role in the plant–pathogen interactions. For example, plants can export sRNAs into fungal cells to suppress virulence genes, and phytopathogen can also deliver sRNAs into the plant to silence immune genes. A recent study by Zhu et al.

indicated that fungal phytopathogen can counteract these trans-kingdom antifungal RNAi using the secretory protein VdSSR1 (secretory silencing repressor 1) [50]. They showed that VdSSR1, containing a conserved RRM_Aly_REF_like domain and a C/NLS motif, is translocated to the plant nucleus and sequesters ALY family proteins, the adaptors of the TREX (Transcription-Export) complex, inhibiting the nuclear export of the AGO1–microRNA (AGO1–miRNA) complex. This results in the reduction in cytoplasmic miRNAs in fungal cells and increased fungal virulence (Figure 1). These reports suggest the importance of nuclear effector proteins in fungal virulence through exploiting the RNA silencing-dependent plant immunity [50].

8. Modulation of host ribosomal RNA degradation by nuclear effectors

RNA processing is an important step in the plant immune pathway. It has been reported that several phytopathogenic fungi possess RNase-like effector proteins that target plant immunity by interfering with the RNA processing pathway (Figure 1). For instance, the RNase-like effector protein CSEP0064/BEC1054 of the biotrophic fungal pathogen *Blumeria graminis* can bind to the nucleic acid and prevent degradation of host ribosomal RNA and cell death by inhibiting the ribosome-inactivating proteins and promote susceptibility [51]. Similarly, a recent study by Yin et al. identified RNase like effector, VdRTX1, in *V. dahlia* that contains an NLS sequence in the mid-region of the protein and translocates into the plant nucleus to modulate the immunity [52].

9. Modulation of lignin biogenesis in host cell

The mechanism of nucleus-targeted effectors in necrotrophic fungi is poorly characterized in comparison to the biotrophic and hemibiotrophic fungal phytopathogens. Recently, one novel strategy has been discovered in necrotrophic fungi to target a key defense pathway associated with the biosynthesis of secondary metabolites and antifungal compounds to evade plant immunity (Figure 1). Singh et al. characterized the effector ArPEC25, which is secreted by the necrotroph, *Ascochyta rabiei*, the causal agent of Ascochyta blight disease in chickpea (*Cicer arietinum).* The protein was predicted to contain PEXEL motif and a nuclear localization signal (NLS) and enter the host nucleus and inhibit the transactivation of Ca*β*LIM1a by interfering with its DNA binding ability. Ca*β*LIM1a acts as a transcriptional regulator of *CaPAL1* that encodes the enzyme phenylalanine ammonia-lyase. Therefore, inhibition of Ca*β*LIM1a causes negative regulation of the phenylpropanoid pathway, resulting in suppression of lignin production and weakening of cell wall in favor of fungi for successful penetration and colonization. However, future investigations are needed to understand the detailed mechanisms of action behind this phenomenon [53].

10. Other nuclear effector involved in modulating plant immune responses

Rapid advances in molecular biology combined with the use of next generation sequencing techniques have led to the availability of genome information from a wide range of organisms, including plant pathogenic fungi. Subsequently, the identification of effector proteins also increased rapidly. However, their functional characteristics are still limited and most of their target molecules could not be detected in the host cells. In a recent study, Rafiei et al. [54] characterized the phospholipase effector VlsPLA$_2$ of *V. longisporum* and proposed that VlsPLA$_2$ disrupts the plant immunity by suppressing pattern triggered immunity (PTI)-related hypersensitive response (HR) through interference with signal transduction pathways. They predicted two functional NLS (one monopartite and one bipartite) of which the bipartite NLS2 seemed to be more critical for nuclear localization of VlsPLA$_2$. However, the precise role of *VlsPLA$_2$* in *V. longisporum* infection biology has yet to be revealed [54]. Based on the secretome analysis, Li et al. predicted nuclear-localized effector transcription factor BTF3a and transcriptional repressor rco-1, zuotin, and rAsp f 9 allergens during fungal (*F. proliferatum*) infection in banana fruit. However, their functional role in the host plant has not been studied [55]. The importance of NLS-mediated nuclear localization in host plants for inducing pathogenicity has also been reported by several studies. For example, Hoang et al. demonstrated that the NLS sequence is required for the virulence-promoting function of Vp1 (Virulence promoting 1), in *U. maydis,* and could localize the protein to the plant nucleus if the Vp1 translocates to plant cells [56]. It has also been reported that nuclear localization of the *Cytospora chrysosperma* CcCAP1 effector protein belonging to the CAP superfamily is required to inhibit the plant immunity and promote the infection of the host cell [57]. Recently Xu et al. characterized *Cytospora chrysosperma* effector protein CcSp84, which was strongly induced during infection stages and plays a major role in the fungal pathogenicity. This protein contains a predicted NLS motif in the C-terminal region and translocates to the plant nucleus and triggers host defence

responses, such as ROS accumulation, callose deposition, and induced expression of jasmonic acid and ethylene defense-related genes [58]. The secretome analysis of *V. dahliae* revealed the effector protein VdSCP7 which can alter the immunity of the host plants. This protein carries a functional bilateral NLS and its activity was highly dependent on its nuclear localization [59]. Another study by Liu et al. identified the glycoside hydrolase family protein *Vd424Y* from *V. dahliae* that can induce *BAK1*- and *SOBIR1*-dependent cell death and activated both salicylic acid and jasmonic acid signaling in the host. Further study revealed that both signal peptide and the nuclear localization signal are essential for Vd424Y-induced cell death in plant hosts [60]. However, the detailed molecular mechanism has not yet been revealed in these nuclear targeting effectors (Table 1) (Figure 1).

11. Emerging questions and future directions

Phytopathogenic fungal species have evolved with multiple strategies to survive from the host immunity and induce pathogenicity. One such key strategy is to target the host nucleus using nuclear effectors. However, until now, only a few nuclear effector proteins have been functionally characterized, and therefore, many questions in this field remain unanswered.

Does removal of the signal peptide affect the localization of nuclear effectors? Intracellular effector proteins of the plant pathogenic fungus are secreted into the extracellular space using N-terminal signal peptide (SP). It is interesting to know whether the removal of signal peptides affects the localization of fungal effectors. Study conducted by Voß et al. in the arbuscular mycorrhizal fungus *Rhizophagus irregularis* has shown that the full-length effector protein RiCRN1 localize in nuclear bodies when expressed *in planta* but removal of SP results in variable patterns of nuclear localization and only sometimes at nuclear bodies [61]. In another study showed no difference in the localization of the nuclear effector protein $VlsPLA_2$ of *V. longisporum* even after the removal of SP [54]. Kim et al. cloned and expressed the fungal effector MoHTR without SP (MoHTR1-Δsp and MoHTR2-Δsp) in rice protoplasts and observed that MoHTR1-Δsp:RFP and MoHTR2-Δsp:RFP were able to localize to the plant nucleus. However, MoHTR1-Δsp and MoHTR2-Δsp failed to accumulate in the fungal (*M. oryzae*) nucleus [15].

This leads to following questions: Is there any difference between NLS proteins localized to the fungal nucleus and NLS proteins secreted and localized to the host nucleus? Would NLSs in effector proteins be similar to the NLSs in plants but dissimilar to the NLS in fungi?

How does plant receptor interact with the fungal effectors for their nuclear localization? Higher eukaryotes have evolved with a number of receptor proteins such as importin-α isoforms that exhibit different affinities for different NLS proteins. Silencing of *Nicotiana benthamiana* importin-α1 and α2 results in the inhibition of the nuclear localization of the *Phytophthora infestans* effectors Nuk6 and Nuk7, while nuclear import of Nuk12 remains unaffected [62]. Similarly, Importin αs are reported to interact with the oomycete effector PvAVH53 and translocate into nuclei of *N. benthamiana* cells, and trigger cell death. Silencing of importin αs expression leads to increased susceptibility of the pathogens indicating the key role of importin αs in the localization process [63]. This evidence indicates that the interaction between NLS and specific importin-α plays an important role in defining the nuclear localization of NLS protein in different organisms.

What are the biochemical nature of interactions between nuclear effectors and their targets? The nuclear effector of phytopathogenic fungi has multiple targets (DNA, RNA, proteins) in the plant cell, and modulate their activity to establish fungal pathogenicity. It has been evidenced that nuclear effector may silence the targets and/or alter their activity or stability, resulting in the dysregulation of the host immunity. However, we still know relatively little about the functional role of these targets and the mechanism that regulate the interaction between effector and their targets. Therefore, a detailed understanding of the biochemical nature of the interactions between nuclear effectors and their targets, including the post-transcriptional modifications involved in these processes, may shed light on the detailed molecular mechanisms of fungal pathogenesis in the host plant.

What are the targeting signals that allows effector protein to localize both in nucleus and nucleolus? How such effector protein function in the plant cell? Growing evidence revealed that few effector proteins of phytopathogenic fungi can localize both in the nucleus as well as the nucleolus [41]. However, limited information is available about the targeting signals that carry out this process. These effector proteins may possess NLS and NoLS (Nucleolar localization sequence) regions for nuclear transport. However, they often utilize joined NoLS-NLS regions as targeting signals, and thereby their identification is hindered [64]. Furthermore, the functional properties of these specific effect proteins that localize to both the nucleus and the nucleolus are largely absent. Characterization of these effector proteins as well as identification of the joined NoLS-NLS regions is indeed a challenging task, so future research needs to focus on this to fill the knowledge gap.

12. Conclusions

Although a lot of effector proteins were identified, only a few nuclear effectors were functionally characterized and shed light on the mechanism of inducing fungal pathogenicity and suppressing plant immunity. Complex life cycles, incomplete genomic data, and the lack of appropriate effector screening methods and tractable experimental systems are major obstacles to the discovery of nuclear effectors and their functional characterization in most of the phytopathogenic fungi. Detailed molecular mechanisms involving the localization and activities of the nuclear effectors in the plant nucleus are still in the initial stages of research. Blocking the nuclear transport of these virulence effector proteins by targeting the NLS could successfully prevent plant diseases from developing, as it has been successfully used to develop nuclear transport inhibitors against microbial infections [65,66]. Therefore, identifying and analyzing the functionality of NLS and nuclear proteins, understanding their interaction with the host targets, and investigating the biochemical nature of interactions that regulate the function of nuclear effects in host cells are important in elucidating disease mechanisms and developing novel strategies for disease prevention.

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