Proteinaceous Effector Discovery and Characterization in Plant Pathogenic Colletotrichum Fungi

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Anthracnose caused by plant pathogenic Colletotrichum fungi results in large economic losses in field crop production worldwide. To aid the establishment of plant host infection, Colletotrichum pathogens secrete numerous effector proteins either in apoplastic space or inside of host cells for effective colonization. Understanding these effector repertoires is critical for developing new strategies for resistance breeding and disease management. With the advance of genomics and bioinformatics tools, a large repertoire of putative effectors has been identified in Colletotrichum genomes, and the biological functions and molecular mechanisms of some studied effectors have been summarized. Here, we review recent advances in genomic identification, understanding of evolutional characteristics, transcriptional profiling, and functional characterization of Colletotrichum effectors. We also offer a perspective on future research.

Keywords: Colletotrichum, effector, prediction, function, pathogen-plant interaction

INTRODUCTION

Anthracnose, which is caused by the fungal genus Colletotrichum, is one of the most devastating agricultural diseases (Stephenson et al., 2000; Yoshino et al., 2012; Chen et al., 2021). Over 600 species of Colletotrichum have been identified and classified as singletons or species complexes (Irieda et al., 2014). They can infect a large variety of plants worldwide including vegetables, fruit plants, forest trees, cereals, and legumes (O’Connell et al., 2012; Gan et al., 2013; Irieda et al., 2014). Colletotrichum graminicola causes anthracnose leaf blight and stalk rot of maize and sorghum, respectively (Sanz-Martin et al., 2016). C. lentis is the causative agent of anthracnose on soybean, lentil, and pea. C. higginsianum mainly infects Brassicaceae plants (Huser et al., 2009), and C. orbiculare prefers to attack cucurbitaceous plants (Perfect et al., 1999). Moreover, C. higginsianum and C. orbiculare can also infect the model plants Arabidopsis thaliana and Nicotiana benthamiana, respectively, providing pathosystems for studies of pathogen–plant interactions (O’Connell et al., 2004). Because of its extreme destruction, widespread distribution, and scientific importance as a model pathogen–plant interaction system, the genus Colletotrichum has been ranked among the top 10 most important phytopathogenic fungi in the world (Dean et al., 2012). Presently, the two main strategies to control anthracnose are breeding resistant sources and using chemical fungicides. However, the complex genetic variation of Colletotrichum strains leads to a loss of cultivar resistance and emergence of fungicide resistance, which makes anthracnose...
difficult to control. Therefore, to devise strategies to efficiently control the spread of the disease, it is urgent to thoroughly clarify the molecular mechanism of *Colletotrichum* pathogenicity.

*Colletotrichum* fungi can infect multiple plant parts such as leaves, stems, and fruits. Upon contact with the plant, *Colletotrichum* conidium initially adheres to the host surface, and germinates to form germ tubes. Then, a specialized infection structure called the melanized appressorium forms at the tip of the germ tube and penetrates the host (Kleemann et al., 2012; Irieda et al., 2014). Post-penetration, the majority of *Colletotrichum* pathogens adapt a hemibiotrophic lifestyle. They develop penetration peg at the infection point to invade plant cells, and then produce specialized infection structures such as bulbous vesicles and primary hyphae to obtain nutrients from living plant tissues. *Colletotrichum* fungi switch to a necrotrophic stage, after which they produce secondary hyphae that invade neighboring cells and kill host tissues (Perfect et al., 1999).

To establish successful infection, phytopathogenic fungi typically secrete a large number of virulent effectors into host cells (Dou and Zhou, 2012). Effectors are proteins secreted by pathogens to manipulate plant physiology and immunity, to facilitate infection, trigger plant defense responses or both (Bozkurt et al., 2012; Rafiqi et al., 2012). According to their subcellular localization, fungal effectors can be classified into two major groups: apoplastic effectors act in extracellular spaces while intracellular ones function inside host cells (Dou and Zhou, 2012). Generally, fungal effector proteins contain an N-terminal signal peptide and are secreted via the conventional endoplasmic reticulum–Golgi apparatus secretion pathway (Giraldo et al., 2013). These fungal effectors typically lack sequence similarity to known proteins, which is thought to be the result of the evolutionary pressure that promotes the rapid diversification of effector activities, to avoid recognition by the plant immune system. Also, many effectors are small cysteine-rich proteins containing unidentified motifs and domains. Evolutional and functional studies of effectors have been important for comprehensive understanding of plant–pathogen interactions, and have facilitated the development of more effective and eco-friendly approaches to disease control.

Recent advances in high-throughput sequencing have yielded over 50 genomes of *Colletotrichum* pathogens. Computational predictions suggest that there are genes for hundreds of putative effectors in these genomes (O’Connell et al., 2012; Gan et al., 2013; Buiate et al., 2017; Lelwala et al., 2019). Comparative studies reveal that each *Colletotrichum* species contains both conserved and unique effectors, which are likely to play crucial roles in their adaptation to plant hosts. Furthermore, functional investigations of some *Colletotrichum* effectors suggest a clear contribution to the pathogenic success. Effectors have been the main focus of research on the interaction between *Colletotrichum* pathogens and plants because they directly affect the invasion, expansion, and disease occurrence of *Colletotrichum* pathogens. In this review, we summarize recent advances toward the identification and functional characterization of putative effectors from *Colletotrichum* pathogens. We focus on the genomic identification, evolutionary characteristics, and transcriptional profiling of effectors, and then on recent progress in elucidating their biological functions and effects on compatibility.

**GENOMIC IDENTIFICATION OF CANDIDATE EFFECTORS**

With the rapid development of high-throughput sequencing technologies and bioinformatics tools, analysis of entire genomes has become common practice, and can establish causal relationships between genome characteristics and the biology of plant pathogens (Raffaele and Kamoun, 2012; Plissonneau et al., 2017). So far, at least 50 genomes of *Colletotrichum* pathogens have been fully sequenced. Prediction of candidate effectors is the first step in the functional investigation of these proteins. Because fungal effectors do not possess typical motifs or other conserved sequence features, their prediction *in silico* is challenging. Note that the definition of effector proteins varies considerably among authors. Generally, computational prediction methods first predict secreted proteins, and then apply the EffectorP prediction tool, which screens high-priority effector candidates based on sequence length, molecular weight and protein net charge, as well as cysteine, serine and tryptophan content (Buiate et al., 2017; Lelwala et al., 2019). Some studies also define small secreted proteins (SSPs) as putative effectors; these are typically cysteine-rich and less than 300 amino acids in length (O’Connell et al., 2012; Gan et al., 2013). It may be important for phytopathogens to maintain effector proteins with a relatively small molecular weight for easier secretion. In recent years, several novel prediction strategies have been developed by including additional features, such as secretome analysis and *in planta* expression based on transcriptomic analysis (Ashwin et al., 2017). Due to criterion variation among studies related to effector definition and prediction, it is biased to investigate the effector size variation among *Colletotrichum* pathogens. Therefore, we predicted putative effector proteins in each *Colletotrichum* genome using a streamlined bioinformatics analysis. Secretome was predicted using a series of tools. SignalP 5.0 and WoLF-PSORT were performed to identify signal peptides and extracellular localization, respectively. TMHMM v2.0 and PredGPI were used to exclude sequences with transmembrane helices and GPI anchors, respectively. Sequences were then submitted to EffectorP 3.0 for effector prediction. Analyses of genomes have uncovered large inventories of candidate effectors (288–608 per genome) in different *Colletotrichum* species (*Figure 1* and Table 1), suggesting that putative effector numbers vary considerably among *Colletotrichum* species. Further cluster analysis in the genus *Colletotrichum* identified ~20% of core effectors which were present in each *Colletotrichum* species, while another 70% of conserved effectors had orthologs in other *Colletotrichum* species. Moreover, each *Colletotrichum* species contained 4.1–15.6% of species-specific effectors (*Figure 1*). These data suggested that the conservation patterns of candidate effectors appear to be related to the host range and virulence of *Colletotrichum* pathogens.
FIGURE 1 | Conservation patterns of putative effector proteins from Colletotrichum pathogens. A neighbor-joining species phylogeny was drawn based on the alignment of single-copy orthologs. Bootstrap values are based on 1,000 replicates. The effector candidates were predicted using a streamlined bioinformatics analysis in this study. Core: core effectors which were present in each Colletotrichum species. Conserved: conserved effectors which had orthologs in other Colletotrichum species. Species-specific: species-specific effectors.

TABLE 1 | List of genome assembly and predicted candidate effector information in Colletotrichum pathogens.

| Species              | Strain    | Host                  | Genome size (Mb) | Total gene number | Putative effector number\(^\#\) | Accession number | References                  |
|----------------------|-----------|-----------------------|------------------|-------------------|---------------------------------|------------------|----------------------------|
| C. graminicola       | M1.001    | Zea mays              | 50.9             | 12,006            | 314                             | ACOD010000001    | O’Connell et al., 2012    |
| C. higginsianum      | IMI 349063| Brassicaceae          | 49.3             | 16,172            | 417                             | CAQQ0000000000.2 | O’Connell et al., 2012    |
| C. orbiculare        | MAFF 240422| cucurbits             | 88.3             | 13,479            | 459                             | AMCV0000000000.2 | Gan et al., 2013          |
| C. fructicola        | Nara gc5  | Fruits                | 55.6             | 15,469            | 608                             | ANPB0000000001.1 | Gan et al., 2013          |
| C. fructicola (deposited as C. gloeosporioides) |                         |                     |                  |                                 |                   |                              |                            |
| C. tofieldiae        | CBS 495.85| Tofieldia calyculata  | 53.5             | 13,425            | 412                             | LFHP0000000000.1 | Hacquard et al., 2016     |
| C. incanum           | MAFF 238704| Brassicaceae, fabaceae, and solanaceae | 53.6 | 13,665 | 357 | LFW0000000000.1 | Hacquard et al., 2016 |
| C. sublineola        | CgSi1     | sorghum               | 64.8             | 13,311            | 373                             | MQVQ010000001    | Buiate et al., 2017       |
| C. truncatum         | MTCC 3414 | Capsicum annuum       | 55.4             | 13,724            | 528                             | NBAY0000000000   | Rao et al., 2018          |
| C. tanaceti          | BRI57314  | Tanacetum cinerantifolium | 57.9 | 12,172 | 288 | PJEX0000000000.1 | Leidhala et al., 2019 |
| C. lentis            | CT-30     | Legume                | 56.1             | 11,436            | 301                             | NWBT0000000000.1 | Bhaduria et al., 2019     |
| C. lindemuthianum    | 83.501    | Phaseolus vulgaris    | 97.4             | 11,673            | 370                             | MASO0000000000.2 | de Queiroz et al., 2019   |

\(^\#\)Due to criterion variation among studies related to effector definition and prediction, we predicted putative effector proteins using a streamlined bioinformatics analysis in this study.

EVOLUTIONARY CHARACTERISTICS OF EFFECTORS

Through comparative genomics analysis, the large number of Colletotrichum effectors could be divided into two classes based on their conservation patterns: lineage-specific effector candidates, which have no homology to any other protein (species-specific) or have homolog only to proteins from the same genus (genus-specific); and conserved effectors, which have homology to proteins from other fungal genera.
Cladosporium fulvum, Magnaporthe oryzae, and related secreted (BAS) protein from Bhadauria et al., 2019; Lelwala et al., 2019). C. truncatum, C. sublineola, C. fructicola, C. graminicola, C. higginsianum, C. tanaceti, C. sojae, C. musicola, and C. scovillei related to host recognition (Buiate et al., 2017). Three strains of C. scovillei, Coll-153, Coll-524, and Coll-365, exhibit variable virulence in chili pepper. Comparative genomic analysis showed that the strain Coll-524 has a remarkably greater number of candidate effectors than Coll-153 and Coll-365, and these varied effectors are mainly found in the acutatum complex (Hsieh et al., 2022). The large number of effectors may contribute to the high virulence of Col-524. In C. tanaceti, a minority of the effectors share similarity with those of other tested Colletotrichum species, which emphasizes their role in adaptation to new hosts (Lelwala et al., 2019). As well as lineage-specific effectors, conserved effectors have also been studied because they are typically important for infection of a wide range of plants. Comparison of the repertoire of candidate effectors among four Colletotrichum species pathogenic to soybean showed that 84% of C. muscicola, 85% of C. sojae, 80% of C. truncatum, and 83% of C. muscicola effectors are conserved not only within the Colletotrichum genus, but also in other microorganisms (Boufleur et al., 2021). Thus, the conservation patterns of candidate effectors appear to be related to the host range and virulence of Colletotrichum pathogens.

Of the large repertoire of predicted candidate effectors in Colletotrichum genomes, studies have found that the majority are small cysteine-rich proteins. In C. higginsianum and C. graminicola, the predicted candidate effectors are mostly small secreted proteins (SSPs), with typical lengths ranging between 110 and 175 residues, and are more cysteine-rich than the total secreted proteins (O’Connell et al., 2012). Of the predicted SSPs, 49.4% in C. fructicola and 54.6% in C. orbiculare are cysteine-rich proteins (Gan et al., 2013). Similarly, such characteristics have also been found in putative effectors derived from C. sublineola, C. truncatum, C. tanaceti, and C. lentis (Buiate et al., 2017; Bhaduria et al., 2019; Lelwala et al., 2019).

Notably, many candidate effectors are homologs of known effectors from other phytopathogens, such as biotrophy-associated secreted (BAS) protein 2 from Magnaporthe oryzae (Mosquera et al., 2009), EcP6 from Cladosporium fulvum (de Jonge et al., 2010), necrosis and ethylene-inducing protein 1 (Nep1)-like proteins (NLPs) from Phytophthora pathogens, MC69 from M. oryzae (Saitoh et al., 2012), and secreted in xylem (SIX) 5 protein from Fusarium oxysporum (Lievens et al., 2009). Furthermore, some Colletotrichum effector proteins contain functional domains. According to an analysis of the Pfam database, 46, 21, and 75 putative effector proteins in C. sublineola, C. graminicola, and C. truncatum, respectively, have functional domains (Buiate et al., 2017; Rao and Nandineni, 2017). Several domains, such as CFEM domain, chitin-binding domain, lysin motif (LysM) domain, and NPP1 domain, are related to pathogenesis. The CFEM domain is composed of eight conserved cysteine residues, and proteins containing this domain are important in pathogenesis (DeZwaan et al., 1999). C. graminicola M1.001 and S. sublineola Cgs1 share 10 and 11 SSPs, respectively, containing the CFEM domain (Buiate et al., 2017). Both of these species also contain two SSPs containing the chitin-binding domain, which is thought to bind to the chitin present in fungal cell walls to protect the pathogen from plant chitinases (van Esse et al., 2007).

Many genomes of Colletotrichum pathogens, such as C. higginsianum and C. gloeosporioides, contain both core chromosomes and minichromosomes (Plaumann et al., 2018). Supernumerary minichromosomes are common in this genus. The minichromosomes display low gene density, are highly enriched in transposable elements (TEs), and are shown to be virulence determinants on host plants. Analysis of the genomes of the strawberry-pathogenic C. fructicola, C. siamense, and C. aerengia strains identified effector gene clusters in the repeat-rich minichromosomes (Gan et al., 2021). In C. higginsianum, the minichromosomes are more enriched with putative effectors than the core genome, including seven that are strongly induced during infection (Dallery et al., 2017; Tsushima et al., 2019). Furthermore, analysis of the C. tanaceti genome show that the genomic distances between TEs and effector genes are smaller than that between TEs and random genes, suggesting that TEs are close to putative effector genes (Lelwala et al., 2019). Similarly, a significant association was detected between TEs and genes encoding putative effectors in C. higginsianum and C. truncatum (Dallery et al., 2017; Rao et al., 2018). These observations suggest that repeat-rich genomic regions tend to harbor genes that encode putative effectors and evolve at higher rates.

In the arms race model of evolution, phytopathogen pathogenicity proteins commonly evolve faster to avoid host recognition. Consistently, the analysis of selective pressure of all of the protein-coding sequences in C. graminicola showed that 224 genes undergo positive selection; such genes mainly code for putative effectors and other putative virulence factors (Rech et al., 2014). This evidence for positive selection of these putative effectors suggests that they likely evolve rapidly in response to different ecological niches.

**TRANSCRIPTIONAL PROFILING OF CANDIDATE EFFECTORS**

Many Colletotrichum pathogens employ a hemibiotrophic strategy and express effectors at different states of the infection process, including before penetration of the interface, after appressorium penetration, and during the biotrophic and necrotrophic stages. Therefore, RNA sequencing technology has been widely applied to Colletotrichum pathogens such
as *C. graminicola*, *C. higginsianum*, and *C. fructicola* at different development and infection stages. The availability of transcriptomes enables analysis of the transcriptional profiling of *Colletotrichum* candidate effectors at different stages of hemibiotrophic infection, supporting investigation of the infection phase-specific virulence roles of effectors (O’Connell et al., 2012; Bhaduria et al., 2017; Liang et al., 2018). In a microarray study of *C. orbiculare* gene expression regulation during infection of *N. benthamiana*, many SSPs were upregulated in the initial colonization stage (Gan et al., 2013). Deep transcriptome sequencing of *C. higginsianum* associated with different infection stages yielded 198 unigenes encoding candidate effectors, of which 102 are not expressed in the late necrotrophic phase. Thus, these genes are considered biotrophy-associated candidate effectors, which are relevant to appressorium penetration and the development of biotrophic hyphae (Kleemann et al., 2012). Genome-wide expression profiling of *C. graminicola* and *C. higginsianum* genes showed that most effectors are strongly induced during biotrophy. Intriguingly, one gene (*ChEC6*) encoding a candidate effector is the most strongly induced by host contact, and its transcription begins in the appressorium and continues in young biotrophic hyphae (O’Connell et al., 2012). Another set of transcriptomic data associated with *C. fructicola*-strawberry interactions revealed that 15 of the top 100 upregulated *C. fructicola* genes encode candidate effectors during plant invasion, which is the first step of infection (Zhang et al., 2018). These findings suggest that candidate effectors with transcriptional induction at an early stage of the infection process may function in host defense suppression. Also, analysis of gene expression in *C. gloeosporioides* during necrotrophy revealed that 149 SSPs are specifically expressed at that stage. Among them, a necrotrophic-stage specific SSP encoding NLP is highly upregulated, underlining the need for rapid host cell killing (Alkan et al., 2015).

In addition to analysis based on RNA sequencing date, the transcriptional patterns of some effectors with known virulence functions have also been studied deeply. For example, *CgDN3* from *C. gloeosporioides* encodes a determinant of pathogenicity associated with the biotrophic phase to regulate hyphal extension, and its homologue CoDN3 suppresses the hypersensitive reaction (HR)-like response triggered by necrosis-inducing proteins (Stephenson et al., 2000; Yoshino et al., 2012; Isozumi et al., 2019). Reverse transcriptase polymerase chain reaction analysis showed that *CgDN3* is expressed during the biotrophic stage 1–4 days after inoculation (Stephenson et al., 2000). Another two effectors, ChELP1 and ChELP2, of *C. higginsianum* prevent host chitin recognition in immune responses by associating with chitin polymer and oligomers. Expression patterns of *ChELP* genes revealed that ChELP1 and ChELP2 are the most expressed among them, and are strongly induced during the early biotrophic phase (Takahara et al., 2016). NLPs are widely distributed across many pathogenic fungi. In contrast with effectors expressed during the early phases of infection, the NLP genes of *C. orbiculare* and *C. higginsianum* are expressed specifically at the onset of necrotrophic growth and have the potential to cause necrotic lesions and accelerate host death (Kleemann et al., 2012; Azmi et al., 2018; Chen et al., 2021).

These results indicate that *Colletotrichum* effectors are host-induced and expressed in consecutive waves associated with the hemibiotrophic infection mode. Most effectors expressed during initial host penetration and biotrophic phase either act on appressorium-mediated penetration or host defense suppression, whereas others expressed precisely at the onset of necrotrophic growth can induce cell death to accelerate the switch to that stage of infection. Remarkably, some *C. higginsianum* effectors such as ChEC3 and ChEC3a, are induced at biotrophic phase, and also suppress cell death induced by ChNLP1, suggesting that *C. higginsianum* effectors interfere with ChNLP1-specific signaling components and thereby maintain host viability during initial biotrophic growth (Kleemann et al., 2012).

**EFFECTORS FOR INFECTION STRUCTURE FORMATION**

During the infection process, some *Colletotrichum* effectors may play a role in pathogenicity by regulating the development of the infection structures. For example, two LysM proteins (ChELP1 and ChELP2) in *C. higginsianum* have been shown to affect appressorium-mediated penetration. ChELP2 preferentially accumulates on the surface of biotrophic primary hyphae but is absent on necrotrophic secondary hyphae. ChELP1 RNAi mutants show considerably more abnormal spore germination than wild-type ones, and produce appressorium that fail to penetrate plant epidermal cells (Takahara et al., 2016). These data suggest that ChELP1 and ChELP2 execute their virulence functions in a penetration ability-dependent manner. Similarly, *C. gloeosporioides* genome encodes a CgDN3 effector, which was identified as a virulence effector by its induction under conditions of nitrogen deprivation. CgDN3 mutants replace the CgDN3 gene with a chimeric hygromycin resistance gene, which causes a severe reduction in the rate of appressorium formation in vitro and aids fungal infection (Stephenson et al., 2000). Taken together, the *Colletotrichum* effectors control infection structures to potentially induce pathogenicity.

**EFFECTOR DELIVERY AND SUBCELLULAR LOCALIZATION**

Effector delivery is associated with the hemibiotrophic lifestyle of *Colletotrichum* pathogens. In *C. higginsianum*, the cytological analysis shows that effectors localize to stage-specific compartments at the host-pathogen interface (Kleemann et al., 2012). Some early-expressed effectors such as ChEC36 and ChEC6 specifically localize to the penetration pore, suggesting that they are focally secreted from appressorial penetration pores before host invasion. In addition, some later-expressed effectors including ChEC89, ChEC3, ChEC13, and ChEC34, accumulate in interfacial bodies on the surface of biotrophic hyphae, implicating these hyphae in effector delivery (Kleemann et al., 2012). In another study, *C. orbiculare* effectors exhibit ring-shaped accumulations around the neck of the biotrophic hyphae (Irieda et al., 2014).
Once pathogens deliver effectors into plants, it is critical to identify host cell compartment that effectors target and how they function. Many fungal and oomycete effectors have been reported to target diverse plant compartments, such as nuclei, vacuole, tonoplast, plasma membrane, and cytosol (Petre et al., 2015). Of the 61 biotrophy-expressed effector as nuclei, vacuole, tonoplast, plasma membrane, and cytosol been reported to target diverse plant compartments, such as how they function. Many fungal and oomycete effectors have shown the ability to target and manipulate plant processes.

**EFFECTORS BLOCK FUNGAL CHITIN-INDUCED HOST SIGNALING**

Chitin is an important component in the cell walls of all pathogenic fungi, and acts as a microbe-associated molecular pattern (MAMP) that can be recognized by plant chitin receptors to activate a variety of MAMP-triggered immune responses (Miya et al., 2007; Dodds and Rathjen, 2010). Almost all of the chitin receptors have been identified as LysM-containing proteins, and three LysM domains are essential for chitin binding (Tanaka et al., 2013). To evade recognition by host chitin receptors, pathogenic fungal secrete effectors to compete with them (to bind to and protect chitin against recognition), or act as plant chitinase-degrading enzymes to specifically degrade chitinase and suppress the release of chitin fragments directly captured by plant cells. The Brassicaceae anthracnose fungus *C. higginsianum* encodes many LysM-containing proteins. Among these, ChELP1 and ChELP2 have a signal peptide and accumulate in an early biotrophic phase. The recombinant proteins of ChELP1 and ChELP2 show high affinity for chitin fragments in vitro and specifically bind fungal cell wall chitin. Analysis of mitogen-activated protein kinase (MAPK) activation showed that both ChELP1 and ChELP2 can suppress this chitin-triggered MAPK activation by sequestering chitin (Takahara et al., 2016). Another LysM-containing protein, Cgfl, which is a conserved fungalysin metalloprotease in *C. gramincola*, can bind plant chitinase for degradation. ΔCgfl mutants reduce this ability in colonized maize leaves and roots. Also, the culture filtrates of ΔCgfl show significantly reduced proteolytic activity in response to the substrate casein in vitro. Inoculation of maize leaves with ΔCgfl increases plant chitinase activity compared with inoculation with the *C. gramincola* wild-type strain (Sanz-Martin et al., 2016). These results suggest that Cgfl targets degradation of plant chitinase to protect chitin released from pathogens against cleavage and recognition. As well as chitin signaling, MAPK and plant hormone signaling including salicylic acid, jasmonic acid, and ethylene, participate in defense responses. Although the evidence shows that effectors play a role in suppressing chitin signaling and chitin-induced immunity, no *Colletotrichum* effectors have shown the ability to target and interfere with plant hormone signaling yet.

**EFFECTORS SUPPRESS HYPERSENSITIVE CELL DEATH**

Plants have evolved a sophisticated innate immune system, and the extracellular (apoplastic) and intracellular (cytoplasmic) spaces are major sites of pathogen molecule recognition and plant defense. Pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) is a substantial barrier in the apoplastic space of plants, and impedes microbial infection (Wang and Wang, 2018). Plant pattern-recognition receptors (PRRs) that recognize PAMP associate with membrane-localized receptor-like kinases (RLKs) and receptor-like cytoplasmic kinases (RLCKs) to transduce defense signaling and trigger immune responses (Monaghan and Zipfel, 2012). There is growing evidence that Brassinosteroid insensitive 1-associated kinase1 (BAK1) is an important RLK that participates in different signaling pathways to modulate various types of programmed cell death (He et al., 2007; Kemmerling et al., 2007; Jeong et al., 2010). Accordingly, pathogens have developed effectors to suppress cell death as a defense mechanism and promote pathogen infection.

The cucumber anthracnose fungus *C. orbiculare* secretes necrosis-inducing secreted protein 1 (NIS1), which is a conserved effector in filamentous fungal that targets host core immune components. The homolog CoNIS1 can suppress the cell death induced by the oomycete PAMP INF1 by interacting with BAK1 to inhibit its kinase activity. The *Arabidopsis bak1-5* mutant encodes a semidominant allele of BAK1. Inoculation of *C. higginsianum* onto the bak1-5 mutant facilitates pathogen infection compared to inoculation onto Col-0. This finding suggests that *Colletotrichum* pathogens deploy a core effector to attacks the conserved immune component (Irieda et al., 2019). Interestingly, phylogenetic analysis reveals that NIS1 is widely conserved in fungal pathogens. ChNIS1 of the crucifer anthracnose fungus *C. higginsianum* and MoNIS1 of *M. oryzae* can also suppress INF1-induced cell death in *N. benthamiana* (Irieda et al., 2019). Similarly, two virulence-related effectors of *C. orbiculare*, SIB1 and SIB2, can suppress the cell death response triggered by INF1. Overexpression of SIB1 and SIB2 increases the susceptibility of *N. benthamiana* to *C. orbiculare* (Zhang et al., 2021). Like the oomycete PAMP INF1, a mammalian proapoptotic molecule, BAX, induces programmed cell death (PCD), which is similar to the defense-related HR. Therefore, it typically acts as a reference for the HR-suppressing ability of pathogen effectors (Lacomme and Santa Cruz, 1999; Dou et al., 2008). The *C. fructicola* effector CIEC92 is an important virulence factor that infects both apple leaves and fruits. Sequence similarity analysis of CIEC92 homologs showed that this SSP is conserved across the genus *Colletotrichum*. Overexpressing CIEC92 can suppress BAX-induced cell death in *N. benthamiana* (Shang et al., 2020). These data suggest that CIEC92 and its homolog proteins possess a cell death-suppressing function for the host immune response.

Necrosis and ethylene-inducing protein 1-like proteins and NIS1 have an extremely broad distribution in filamentous fungi (Toshino et al., 2012; Seidl and Van den Ackerveken, 2019). Filamentous phytopathogens have evolved effector proteins that can suppress the host cell death triggered by NLPs and NIS1 to
promote disease. *C. higginsianum* effector candidates (ChECs) are co-expressed with the cell death-inducing protein ChNLP1 in *N. benthamiana* and exhibit virulence. Among the 102 ChECs, ChEC3, ChEC3a, ChEC5, ChEC6, and CHEC34 have been screened and exhibit significant cell death-suppressing activity. Western blot analysis indicates that these ChECs have no impact on ChNLP protein stability, and therefore reduce the necrosis of ChNLP in a suppression activity-dependent manner (Kleemann et al., 2012). As a further example, both the effector CgDN3 of *C. gloeosporioides* and its homolog in *C. orbiculare*, as pathogenicity-related proteins, suppress the HR-like response induced by NIS1 (Stephenson et al., 2000; Yoshino et al., 2012). Subsequently, sequence analysis has shown that CoDN3 suppresses the necrotic lesions caused by NLP1 homologues in a CaM-binding domain-dependent manner (Isozumi et al., 2019). Taken together, these data illustrate that a conserved strategy is employed by *Colletotrichum* species, involving the deployment of virulence effectors to suppress cell death and therefore counter plant immunity (Table 2).

### EFFECTORS INDUCE PLANT IMMUNITY

Plant pathogens adopt different virulence strategies to infect host cells and, in turn, plants evolve multi-layered immune defenses to recognize pathogen effectors and induce host immunity. During the confrontation between plants and pathogens, plant PRRs can recognize PAMPs and trigger PTI, while plant nucleotide-binding domain leucine-rich repeat containing receptors recognize effectors and induce effector-triggered immunity (Boyd et al., 2013). Plant immune responses include HR induction, ROS production, the activation of defense gene expression, extracellular alkalization, and callose deposition; together, these mechanisms provide a systemic, durable, and broad spectrum of defense (Prime et al., 2006). It is important to identify PAMPs and effectors serving as resistance inducers to promote sustainable crop protection.

Many PAMPs and effectors from the genus *Colletotrichum* have shown cell death-inducing activities (Table 3). NLPs acting as a class of well-known PAMPs, are conserved in many phytopathogens and strongly induce cell death in eudicot plants. The leucine-rich repeat receptor protein RLP23 forms a constitutive complex with the other RLKs and mediates NLP-triggered immunity by sensing a conserved 20-amino-acid fragment of NLP sequences in *Arabidopsis* (Albert et al., 2015). Six NLP homologs in *C. higginsianum* have necrosis-inducing activities, and they are expressed during the switch to necrotrophy and induce necrotic symptoms in *N. benthamiana* (Kleemann et al., 2012). Furthermore, *C. orbiculare*, which causes anthracnose disease in curcubit, secretes conserved NLP and NIS effectors. Transient expression of NLP1 or NIS1 also induces cell death in both *N. benthamiana* leaves and melon cotyledons. A mutation that deletes signal peptides and mutations in the heptapeptide motif of NLP1 blocks cell death-inducing activity in *N. benthamiana* but still causes cell death in melon. These results suggest that machinery for NLP1-triggered cell death probably differs among susceptible plants (Chen et al., 2021). Analysis of a series of deletion mutants showed that the carboxy-terminal 32 amino acids of NLP1 are recognized by Cucurbitaceae plants, which is sufficient to trigger cell death in cucumber cotyledons (Azmi et al., 2018).

As well as conserved NLPs, many potential PAMPs and proteins that induce HR have also been identified by secretome analysis. For example, *C. falcum* secretes a cerato-platanin protein called EPL1, which induces HR-like cell death 24 h after infiltration in *N. tabacum* (Ashwin et al., 2017). Another novel protein secreted by *C. falcum*, CFPDIP1, is also an HR-inducing protein. Functional characterization of distinct domain deletion variants revealed that hydroxyl-deleted variants of CFPDIP1 also rapidly trigger HR (Ashwin et al., 2018). Remarkably, some hemibiotrophic pathogens trigger cell death to signal the transition from biotrophy to necrotrophy. For example, the *Nudix* hydrolase domain-containing proteins, which are widely distributed among eukaryotes, act as important effectors in phytopathogens by manipulating host defense systems in a hydrolysis activity-dependent manner (Kong et al., 2015; Dong and Wang, 2016). The CtNUDIX effector in *C. truncatum* contains a putative 23-amino-acid *Nudix* hydrolase...
TABLE 2 | List of plant immunity-suppressing effectors in Colletotrichum pathogens.

| Effector | Colletotrichum pathogen | Expression stage | Biological functions | Sequence conservation | References |
|----------|-------------------------|------------------|----------------------|----------------------|------------|
| ChELP1   | C. higginsianum         | Biotrophic phase | Binging chitin polymer and oligomers, suppressing chitin-triggered plant immune responses, and contributing to fungal virulence and appressorium-mediated penetration | Conserved in fungi | Takahara et al., 2016 |
| ChELP2   | C. higginsianum         | Biotrophic phase | Binging chitin polymer and oligomers, suppressing chitin-triggered plant immune responses, and contributing to fungal virulence and appressorium-mediated penetration | Conserved in fungi | Takahara et al., 2016 |
| ChNIS1   | C. higginsianum         | Unknown          | Suppressing INF1-induced cell death and PAMP-triggered ROS generation | Conserved in fungi | Irieda et al., 2019 |
| ChEC3    | C. higginsianum         | Biotrophic phase | Suppressing cell death | Conserved in Colletotrichum spp. | Kleemann et al., 2012 |
| ChEC3a   | C. higginsianum         | Biotrophic phase | Suppressing cell death | Conserved in Colletotrichum spp. | Kleemann et al., 2012 |
| ChEC5    | C. higginsianum         | Saprotrophic mycelium | Suppressing cell death | Conserved in fungi | Kleemann et al., 2012 |
| ChEC6    | C. higginsianum         | Biotrophic phase | Suppressing cell death | Unknown | Kleemann et al., 2012 |
| CHEC34   | C. higginsianum         | Biotrophic phase | Suppressing cell death | Unknown | Kleemann et al., 2012 |
| CoNIS1   | C. orbiculare           | Unknown          | Suppressing INF1-induced cell death, inhibiting ROS generation triggered by flg22 and chitin, and interacting with BAK1 and BIK1 to inhibit their kinase activities | Conserved in fungi | Irieda et al., 2019 |
| SIB1     | C. orbiculare           | Early infection stage | Suppressing N. benthamiana immunity, inhibiting INF1-induced cell death, and suppressing ROS generation triggered by flg22 and chitin | Conserved in Colletotrichum spp. | Zhang et al., 2021 |
| SIB2     | C. orbiculare           | Unknown          | Suppressing N. benthamiana immunity | Conserved in Colletotrichum spp. | Zhang et al., 2021 |
| CoDN3    | C. orbiculare           | Biotrophic phase | Suppressing necrotic lesion of NIS1 and NLP1 | Conserved in Colletotrichum spp. | Yoshino et al., 2012; Isozumi et al., 2019 |
| Cgfl     | C. graminicola          | Biotrophic phase | Contributing to pathogenicity, degrading chitinases produced by plants | Conserved in fungi | Sanz-Martin et al., 2016 |
| CIEC92   | C. fructicola           | Early infection stage | Contributing to pathogenicity, suppressing BAX-triggered cell death, and inhibiting a subset of plant defense-related gene expression | Conserved in Colletotrichum spp. | Shang et al., 2020 |
| CgDN3    | C. gloeosporioides      | Biotrophic phase | Contributing to pathogenicity, maintaining appressoria formation, and suppressing cell death induced by NIS1 | Conserved in Colletotrichum spp. | Stephenson et al., 2000; Yoshino et al., 2012 |

motif in the carboxy terminus. The full-length protein of CtNUDIX can induce cell death in tobacco, but Agrobacterium tumefaciens strains carrying the CtNUDIX ASP without its signal peptide are unable to induce necrosis phenotypes. Moreover, the recombinant protein from C-terminal enhanced green fluorescent protein fusion to CtNUDIX at the plasma membrane, and precisely overlapped the area of fluorescence for the membrane-selective red fluorescent dye FM4-64. This suggests that the CtNUDIX effector causes cell death-induced activity, which is probably associated with its function in the plasma membrane and extracellular space. However, the plant targets or substrates of CtNUDIX and the mechanism by which CtNUDIX elicits cell death remains to be investigated (Bhadauria et al., 2013). Based on abundant genome resources of Colletotrichum spp., recent studies have identified some core effectors conserved within the genus. Comparative genomic analyses revealed core effector of Colletotrichum (CEC) proteins, which are conserved in seven Colletotrichum species. Taking C. higginsianum as an example, ChCEC homologs (ChCEC2-1, ChCEC2-2, ChCEC3, and ChCEC6) are highly expressed during infection, and only
TABLE 3 | List of plant immunity-inducing effectors in Colletotrichum pathogens.

| Effector | Colletotrichum pathogen | Expression stage | Biological functions | Sequence conservation | References |
|----------|-------------------------|------------------|----------------------|-----------------------|------------|
| ChNLP1   | C. higginsianum         | Biotrophy to necrotrophy switch | Inducing necrosis in N. benthamiana | Conserved in fungi and oomycetes | Kleemann et al., 2012 |
| ChCEC3   | C. higginsianum         | Pre-penetration stage and early biotrophic stage | Inducing cell death in N. benthamiana, and inducing plant nuclear expansion | Conserved in Colletotrichum spp. | Tsushima et al., 2021 |
| NLP1     | C. orbiculare           | Late infection phase | Inducing cell death in N. benthamiana | Conserved in fungi and oomycetes | Azmi et al., 2018; Chen et al., 2021 |
| CoNIS1   | C. orbiculare           | Late infection phase | Inducing necrosis in N. benthamiana and melon | Conserved in fungi and oomycetes | Yoshino et al., 2012 |
| CEC3     | C. orbiculare           | Pre-penetration stage and early biotrophic stage | Inducing cell death in N. benthamiana | Conserved in Colletotrichum spp. | Tsushima et al., 2021 |
| CiCEC3   | C. fructicola           | Pre-penetration stage and early biotrophic stage | Inducing cell death in N. benthamiana | Conserved in Colletotrichum spp. | Tsushima et al., 2021 |
| EPL1     | C. falcum              | Biotrophic phase | Eliciting systemic resistance in sugarcane and HR response in tobacco | Conserved in filamentous fungi | Ashwin et al., 2017 |
| CfPDIP1  | C. falcum              | Highly expressed between 24 and 72 hpi | Eliciting defense responses in sugarcane and HR response in tobacco | Unknown | Ashwin et al., 2018 |
| CtNUDIX  | C. truncatum           | Late biotrophic phase | Eliciting HR-like cell death in tobacco | Conserved in fungi | Bhadauria et al., 2013 |

ChCEC3 can induce cell death in N. benthamiana. Notably, homologs of ChCEC3 from C. orbiculare, C. fructicola and C. graminicola can also induce cell death. Also, analysis of subcellular localization shows that plant cells expressing ChCEC3 homologs have greater diameter nucleuses, although the mechanism of this expansion is unknown (Tsushima et al., 2021).

Besides inducing PCD, certain effectors of Colletotrichum spp. can also promote host resistance by inducing extracellular alkalization, ROS production, callose deposition, and the expression of defense-related genes. In C. falcum, the effector EPL1 and its deletion mutant induce acute extracellular alkalization and H₂O₂ accumulation in both the model plant N. tabacum and host sugarcane. Relative expression analysis showed that the deletion mutant of EPL1 significantly promotes the expression of pathogenesis-related genes (Ashwin et al., 2017). Similarly, the effector CfPDIP1 of C. falcum and its deletion mutant also induce a high level of H₂O₂ and result in rapid alkalization in the extracellular space. The expression of important candidate defense-related genes is upregulated in CfPDIP1ΔN1-21-primed canes (Ashwin et al., 2018). Accumulating evidence suggests that some effectors are perceived by plants and act as resistance inducers to activate systemic resistance.

CONCLUDING REMARKS

Colletotrichum pathogens pose a serious threat to food security worldwide. Understanding the molecular basis of Colletotrichum pathogenesis is very important for developing effective control strategies. In recent years, advances in sequencing technologies and bioinformatics tools have greatly accelerated the identification and evolutionary characterization of a large number of putative effectors in Colletotrichum pathogens, and the biological functions of some of these putative effectors have also been investigated. Our understanding of Colletotrichum effectors is improving and studies have revealed that these molecules are capable of both suppressing plant defense responses and inducing host resistance. However, our understanding of the plant targets of these effectors and the detailed molecular mechanisms are still in their infancy. Therefore, it is essential to elucidate the modes of action of these effectors and their plant targets, to identify novel modes of resistance against these pathogens. Also, little is known about the translocation routes and signals of fungal effectors. It would be interesting to investigate how Colletotrichum effectors are secreted and delivered during pathogen–plant interactions. Such studies would provide novel insights into the molecular mechanisms underlying the virulence functions of Colletotrichum effectors, and aid the development of new strategies to combat anthracnose in crops.

AUTHOR CONTRIBUTIONS

XL, JM, and DS drafted the manuscript. DS and DD revised the manuscript. All authors read and approved of the manuscript.

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