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Identification and characterization of virulence-related effectors in the cucumber anthracnose fungus *Colletotrichum orbiculare*☆

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ABSTRACT

The anthracnose fungus *Colletotrichum orbiculare* invades hosts and establishes biotrophy, later switching to necrotrophy, together with the secretion of an arsenal of effectors. In this review, we describe current progress in the study of pathogen effectors. We identified three virulence-related effectors in *C. orbiculare*, and revealed part of the strategy for effector-mediated infection, including suppression of immunity triggered by particular effectors. The virulence-related effectors accumulate in a unique interfacial region between *C. orbiculare* and cucumbers around the neck of primary biotrophic invasive hyphae. We found that the secretion of effectors toward the interface involves exocytosis and SEC22-dependent ER-Golgi traffic.

1. Introduction

Infectious plant diseases cause annual losses of approximately 15% of all food production in the world. More than 70% of these diseases are caused by thousands of species of phytopathogenic fungi [1], a threat that continues to increase [2]. The way that fungal pathogens disrupt the protective shields of their host plants remains to be elucidated. One of the key factors responsible for compatible interactions between pathogens and hosts is a suite of small proteins called “effectors,” which are secreted from the pathogens and subsequently manipulate their host environment in ways that promote infection [3–6]. The ascomycete genus *Colletotrichum* comprises over 600 species that cause typical anthracnose diseases in a wide range of plant species [7,8]. Many members of this genus exhibit initial biotrophic and late necrotrophic phases in plant hosts, a lifestyle referred to as hemibiotrophy [7,9,10]. In the biotrophic stage, pathogens synthesize and secrete an arsenal of effectors that suppress plant immunity and sustain the viability of host tissues [3–6,11]. After a period of time, the fungi switch to a necrotrophic infection mode and kill host tissues, probably using toxins, lytic enzymes, and other types of effectors that destroy host cells. Nutrients from the dead tissues support fungal growth [9,10,12].

Colletotrichum fungi enter host tissues directly via a penetration peg that emerges from dome-shaped appressoria, which are darkly pigmented with melanin [7,13,14]. Subsequently, the tip of the peg develops into bulbous biotrophic primary hyphae in living host cells before the pathogen switches to necrotrophy. Although it is believed that this infection-related morphogenesis is strongly linked to regulation of effector production and secretion, until several years ago, little was known about effector-mediated infection strategies of *Colletotrichum* fungi such as expression timing, secretion sites, and virulence-related functions of their effectors. Recent genome projects involving four *Colletotrichum* species, *Colletotrichum higginsianum* (pathogen of Brassica spp), *Colletotrichum graminicola* (pathogen of maize [*Zea mays*]), *Colletotrichum orbiculare* (pathogen of cucumber [*Cucumis sativus*]), and *Colletotrichum gloeosporioides* (pathogen of strawberry [Fragaria spp]) have opened the door to genomic and transcriptomic analyses of these pathogens [9,10]. The use of genomic information about *Colletotrichum* fungi has revealed a large number of genes that are candidate effector-encoding genes. Comparative studies indicate that each *Colletotrichum* species has a large set of unique effector gene candidates, implying that effector sets individually deployed by each *Colletotrichum* species are likely to play crucial roles in their

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corresponding adaptation to specific hosts. Furthermore, comprehensive transcriptomic analyses of *C. higginsianum* and *C. orbiculare* that revealed orchestrated expression of these effector genes during the stage transition from biotrophy to necrotrophy strongly suggests a correlation between morphogenesis-related infectious stage shift and sequential expression of effectors [9,10,12]. To better understand how *Colletotrichum* fungi establish compatible interactions with their plant hosts, we need to identify and characterize important effectors of these pathogens. In this review, we describe the current status of studies of *C. orbiculare* effectors, including effector function, the effector-related pathogen–plant interface, and effector secretion machinery.

2. Virulence-related effectors in *C. orbiculare*

Recently, we identified three effectors from *C. orbiculare* that show various effects on plants [15,16]. All these effectors are small secreted proteins with no conserved functional domain or motif. The properties of each effector are introduced below.

2.1. Hypersensitive response-like cell death is induced by the biotrophy-expressed effector NIS1

Plants initially detect exposed microbial molecules called pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) through pattern-recognition receptors localized in the plasma membrane [17,18]. PAMPs/MAMPs are widely conserved among microorganisms and elicit PAMP/MAMP-triggered immunity (PTI/MTI), which constitutes the first tier of the plant immune system. However, through evolution pathogens have acquired effector proteins that are capable of suppressing PTI signaling in plants in a variety of ways [17]. If a compatible interaction is established between the pathogen and the plant by effector-triggered susceptibility, the plant is considered a “host” of the pathogen. To counter pathogen invasion via effectors, plants deploy a second line of defense that is usually reliant on a cytoplasmic resistance (R) protein that activates effector-triggered immunity (ETI) by recognizing a specific pathogen effector in a direct or indirect manner. In such cases, the effector recognized by the plant is referred to as an avirulence (AVR) protein. ETI is a defense response that is often accompanied by localized cell death known as the hypersensitive response (HR). In general, ETI has a strong effect against adapted pathogens, although PTI is thought to be effective against nonadapted pathogens, which is termed nonhost resistance [19]. For instance, *C. orbiculare* can form melanized appressoria but fail to develop biotrophic invasive hyphae on nonhost plants including *Arabidopsis thaliana*, in which the nonhost resistance strongly blocks the appressorial invasion step of this pathogen [20], while the invasion of nonadapted *Colletotrichum* fungi activates a second layer of defense [21].

At least two natural isolates of *C. orbiculare* infect the solanaceous plant *Nicotiana benthamiana*, which is distantly related to cucurbitaceous plants [22,23]. *N. benthamiana* is the most widely used experimental model plant for virus-induced gene silencing (VIGS) and transient protein expression using *Agrobacterium tumefaciens*-mediated transformation [24,25]. Effectors with critical functions are often recognized by host plants as AVR effectors, which trigger ETI often accompanied by HR cell death. Notably, there are reports that particular instances of ETI can be suppressed by other effectors [26,27]. Thus, we hypothesized that *C. orbiculare* might possess an effector that elicits HR cell death in susceptible *N. benthamiana*. Based on this concept, we performed functional screening of *C. orbiculare* cDNA to find *C. orbiculare* effectors that induce cell death in *N. benthamiana* [15]. We adopted Takken's method using a potato virusX-based expression vector [28]. Out of 6669 clones, we found that expression of four cDNAs induced chlorotic or necrotic lesion formation in *N. benthamiana* leaves. One of these cDNAs encoded a small secreted protein designated NIS1 (necrosis-inducing secreted protein 1). NIS1 is composed of 162 amino acids and has no conserved domain or motif that would allow the prediction of its function, which is a typical feature of pathogen effectors. Homology searching with BLAST revealed that *NIS1* homologues are conserved in genome sequences of other fungi belonging to Sordariomycetes (including *C. graminicola*, *C. higginsianum*, and *Magnaporthe oryzae*), Dothideomycetes, and Orbiliomycetes at the time of this homology search [15]. A growing number of sequenced fungal genomes has exponentially increased the number of fungal genomes known to carry *NIS1* homologues, with fungi belonging to Leotiomycetes, Agaricomycetes, and Tremellomycetes now added to the previous list. Thus, NIS1 is regarded as an evolutionarily ancient and conserved fungal effector. A green fluorescence protein (GFP)-based promoter assay of *NIS1* expression revealed induced expression of *NIS1* in bulbous biotrophic primary hyphae of *C. orbiculare* formed inside both *N. benthamiana* and cucumber (*Cucumis sativa*) plants, whereas the activity of the *NIS1* promoter was strongly reduced in thin necrotrophic hyphae [15]. Thus, we conclude that *C. orbiculare* secretes NIS1, which has the ability to induce cell death in *N. benthamiana* when the pathogen is establishing biotrophy with the plant. Many instances of HR cell death induced by AVR-R protein direct or indirect interactions require SGT1, RAR1, and/or HSP90, which stabilizes the R protein complex [29,30]. VIGS assays silencing these genes suggested that NIS1-induced cell death requires both SGT1 and HSP90 in *N. benthamiana*, but not RAR1 [15], suggesting that NIS1 is recognized by an unidentified R protein in *N. benthamiana*. We also found that the NIS1 orthologue of *M. oryzae* did not induce cell death in *N. benthamiana*, while the orthologue of *C. higginsianum* caused clear necrosis [15]. The NIS1 protein of *C. orbiculare* shows higher homology to NIS1 of *C. higginsianum* than to NIS1 of *M. oryzae*, suggesting that a conserved sequence of NIS1 proteins in these *Colletotrichum* species causes induction of HR-like cell death in *N. benthamiana*. The necrosis-inducing ability of *C. orbiculare* NIS1 is largely dependent on the C-terminal 30 amino-acid region of the protein. Furthermore, the corresponding C-terminal 30 amino-acid region is absent in the NIS1 orthologue of *M. oryzae*, whereas the *C. higginsianum* NIS1 orthologue retains a truncated form of the corresponding region [15].

2.2. Suppression of NIS1-induced cell death by effector DN3

AVR effectors induce a strong second layer of defense in the host plant, which confers a lack of virulence on adapted pathogens that produce them. Following this logic, *C. orbiculare* is expected to be at a disadvantage for efficient infection of its susceptible plant *N. benthamiana* caused by deploying an effector, NIS1, that induces HR-like cell death. However, we found that the loss of *NIS1* did not affect the virulence of *C. orbiculare* on *N. benthamiana* [15]. This finding raised the possibility that the putative avirulence effect of NIS1 on *N. benthamiana* is suppressed during the biotrophic infection stage of *C. orbiculare*. There are examples of plant pathogens that have likely developed ways to contend with this second layer of defense. To escape from ETI, pathogens lose or modify their effectors to avoid their recognition by the host plant, or alternatively they gain new effectors that are able to interfere with ETI signaling triggered by another effector, possibly through horizontal gene flow [26,27,31]. Thus, we next searched for an effector of *C. orbiculare* that could suppress NIS1-induced cell death in *N. benthamiana*. *CgDN3* of *C. gloeosporioides* encodes a secreted 74-