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Development of the infection strategy of the hemibiotrophic plant pathogen, *Colletotrichum orbiculare*, and plant immunity ☆

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ABSTRACT

The hemibiotrophic fungus *Colletotrichum orbiculare* forms appressoria as infection structures and primarily establishes biotrophic infection in cucumber epidermal cells. Subsequently, it develops necrotrophic infection. In the pre-invasion stage, morphogenesis of appressoria of *C. orbiculare* is triggered by signals from the plant surface. We found that *C. orbiculare PAG1* (Perish-in-the-Absence-of-*GYP1*), a component of MOR [morphogenesis-related NDR (nuclear Dbf2-related) kinase network] plays an essential role as a key component of the plant-specific signaling pathway and that hydrolysis of cutin by a spore surface esterase creates a cutin monomer that constitutes a key plant-derived signal. Development of the infection structure of *C. orbiculare* is strictly regulated by the cell cycle and we found that proper regulation of G1/S progression *via* two-component GAP genes, consisting of *BUB2* (Budding-Uninhibited-by-Benomyl-2) and *BFA1* (Byr-Four-Alike-1) is essential for the establishment of successful infection. In the post-invasion stage, the establishment of the biotrophic phase of hemibiotrophic fungi is crucial for successful infection. We found that *C. orbiculare WHI2* (WHIsky-2), an *Saccharomyces cerevisiae* stress regulator homolog, is involved in the phase transition from biotrophy to necrotrophy through TOR (Target of Rapamycin) signaling, and is thus essential for full pathogenesis.

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1. Introduction

Colletotrichum species include a wide range of plant pathogens that cause serious diseases of various plants. They form infection structures called appressoria as host invasion structures. The penetration hyphae developed from the appressoria of *Colletotrichum* species initially form biotrophic hyphae, and the fungus later switches to a necrotrophic phase [1,2]. The biotrophic stage is a crucial step to establish infection without killing host cells. Studies on infection strategy constitute a model study of environmental signal reception and cellular development of fungal pathogens and a basic study of plant-microbe interactions. *Colletotrichum* species include not only agriculturally serious pathogens, but also models for other pathogens such as the rice blast disease pathogen *Magnaporthe oryzae*, which forms structurally similar appressoria and makes hemibiotrophic infections

similar to *Colletotrichum* [3]. Thus, *Colletotrichum* species provide excellent models for studying fungal-plant interactions and extensive studies of these species have been carried out using cytological, physiological, biochemical and genetics approaches [4]. Among them consistent and intensive research has been conducted on *C. orbiculare* strain 104-T, isolated from a cucumber plant in 1952 in Japan, which infects cucurbitaceae plants [5].

Here, we describe recent findings about *C. orbiculare*-plant interactions in three sections comprising 1) Plant-derived signal reception and infection structure development, 2) Cell cycle control and pathogenesis, and 3) Environmental signal reception and hemibiotrophic infection.

2. Plant-derived signal reception and infection structure development

Adhesion to the plant surface is the first step in initiation of the infection process in many plant pathogenic fungi [6]. Following adhesion, physical signals such as hardness and hydrophobicity or chemical signals such as cutin monomers and leaf waxes induce germination and appressorium formation in several plant pathogenic fungi. Signal transduction pathways necessary for

appressorium formation such as MAP kinase and cAMP/PKA signaling cascades have been intensively investigated in *C. orbiculare* [5,7]. However, the signal transduction pathway specifically activated by plant-derived signals has not been characterized in fungal plant pathogens.

Networks of protein kinase signaling pathways control a wide variety of key developmental processes. Conserved NDR (nuclear Dbf2-related) kinases are important for controlling cell polarity and differentiation in various organisms [8]. Studies in fungi on NDR kinases have revealed the morphogenesis-related NDR kinase network (MOR) [9], which is also called RAM (regulation of Ace2 and morphogenesis) in *Saccharomyces cerevisiae* [10]. The central component of this system is an NDR kinase, Cbk1, that associates with a regulatory subunit, Mob2 [11]. We demonstrate here that the MOR of *C. orbiculare* plays a crucial role in the signal transduction pathway for appressorium development specifically induced by plant-derived signals.

2.1. C. orbiculare Pag1 is a crucial factor for appressorium development triggered by plant-derived signals

Appressorium development is driven by the perception of several host-derived signals. It was shown that hydrophobicity and hydroxy fatty acids stimulate the differentiation of appressoria in *U. maydis* [12]. We have discovered that *KEL2* of *C. orbiculare*, a *Saccharomyces pombe tea1* homolog, is required for appressorium development on artificial surfaces, but it is dispensable for appressorium formation on the host plant surface [13]. This result suggested that there could be a bypass pathway that transduces plant-derived signals for appressorium formation independent of *KEL2*. To determine specific components of the plant-derived signaling pathway for appressorium formation, we successfully obtained insertional mutants in a *kel2Δ* background that formed abnormal appressoria not only on artificial substrate but also on the host plant surface. The mutated gene had high homology to *PAG1* of *S. cerevisiae* and *C. orbiculare PAG1* complemented an *S. cerevisiae pag1* mutant. As expected, *pag1Δkel2Δ* showed abnormal appressorium development on the host surface, but *pag1Δ* showed normal appressorium formation on artificial surfaces suggesting that Pag1 is not involved in the Kel2-dependent abiotic/physical signal transduction pathway.

2.2. Cutin monomer from the host plant cuticle functions as a signal molecule for appressorium development via the MOR

Cutin monomers have been characterized as surface signals recognized by several fungal plant pathogens [12,14]. *n*-Octadecanal was identified in the exudate from cucumber leaves and this compound induced normal appressorium formation of *kel2Δ* but not of *pag1Δkel2Δ*. This indicated that the C18 cutin monomer functions as a key signal molecule for appressorium formation through the MOR in *C. orbiculare*. The extracellular matrix that surrounds spores plays a role in attachment to the host surface and the pre-penetration development of many plant pathogens [6].

The extracellular matrix of *Colletotrichum* species contains high molecular weight mannose-rich glycoproteins, germination inhibitors, and a variety of enzymes, including cutinase [15]. Thus, we hypothesized that conidial surface esterases could be involved in the release of cutin monomers from the leaf surface. Supporting this hypothesis, esterase activity was identified on the conidial surface in *C. orbiculare*. The release of esterase was detected rapidly on contact with an aqueous environment. Importantly, *n*-octadecanal was detected in cucumber exudate containing suspended conidia, but not in exudate free of conidia, indicating that conidial surface esterases hydrolyze plant surface cuticle to cutin

monomers. These results demonstrate that, conidial surface esterases hydrolyze the cuticle to generate the signal molecule for appressorium development *via* the MOR of *C. orbiculare*.

3. Cell cycle control and pathogenesis

Takano et al. [16] reported that a precise nuclear distribution inside cells of *C. orbiculare* is required for normal appressorial formation by analysis of microtubule dynamics. In the rice blast fungus *M. oryzae*, appropriate regulation of the cell cycle is necessary to establish infection and entry into S phase is essential for the initiation of appressorium formation. Furthermore, entry into mitosis and accompanying autophagic programmed cell death in conidia are crucial for the germ tube to differentiate into a functional appressorium [17,18]. Thus, not only cell cycle regulation, but also proper cytokinesis is required for pathogenesis in *M. oryzae* [19]. However, the details of cell cycle regulation that could affect infection-related morphogenesis and pathogenesis are still to be elucidated in plant pathogenic fungi.

In *S. cerevisiae* and *Candida albicans*, exit from mitosis and entry into cytokinesis are controlled by the mitotic exit network (MEN), a GTPase-regulated kinase cascade [20,21]. The MEN consists of the central GTPase Tem1, which is regulated by the two-component GTPase-activating proteins (GAP), consisting of Bub2 (Budding-Uninhibited-by-Benomyl-2) and Bfa1 (Byr-Four-Alike-1). In *S. cerevisiae*, the Bub2/Bfa1 complex constitutes multiple cell cycle checkpoints that prevent mitotic exit [22]. Those checkpoints consist of the spindle position checkpoint (SPOC), the surveillance mechanism for proper orientation of the mitotic spindle [23], the spindle assembly checkpoint (SAC), for proper attachment of all kinetochores to the spindle [24], and the DNA damage checkpoint (DDC), for the appropriate protection of chromosomal DNA [25,26]. Although MEN and SIN components are highly conserved in yeast and filamentous fungi, the functions of each of the homologous genes are diverse. In plant pathogenic fungi, the functions of the GTPase *Tem1* and the two-component GAP consisting of *Bub2* and *Bfa1* have been reported only for the basidiomycete *Ustilago maydis*. Straube et al. [27] reported that the *TEM1* homolog *RAS3* regulates nuclear envelope breakdown rather than mitotic exit or septum formation. Other examples have been lacking in plant pathogenic fungi. Thus, the functional analysis of the two-component GAP and its GTPase in *C. orbiculare* could provide insight into the roles of MEN/SIN in fungal pathogenicity.

We revealed that in *C. orbiculare*, appropriate G1/S progression is regulated by *BUB2* and *BFA1* through *TEM1* during appressorium development and is required for infection-related morphogenesis and pathogenesis [28].

3.1. The novel function of BUB2 and BFA1 in G1/S progression in C. orbiculare

In contrast to the results obtained so far for model yeast and fungi, we found that in *C. orbiculare*, *BUB2* and *BFA1* play major roles in phase progression from G1 to S based on the following results. First, *bub2Δ* and *bfa1Δ* initiated earlier nuclear division and caused binucleation in the pre-germination conidia. Second, experiments using the LacO/LacI-GFP chromosome tagging system indicated that *bub2Δ* and *bfa1Δ* are defective in G1/S arrest. Thus, *C. orbiculare BUB2* and *BFA1* are not involved in mitotic exit or septum formation, while the primary functions of the homologous genes in *S. cerevisiae*, *S. pombe*, and *A. nidulans* are to inhibit the MEN or SIN signal cascade. Therefore, *BUB2* and *BFA1* have distinct roles in G1/S progression in *C. orbiculare*.