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Abstract

Several plant pathogenic fungi including *Colletotrichum* species form specialized cells called appressoria to directly penetrate the intact cuticles of their host plants. These appressoria-forming fungi can infect a wide range of plant species including many commercially important fruits, vegetables, and cereals. The differentiation of appressorium relies on the detection and transduction of physical and biochemical signals present on the plant surface. Working with the cucumber anthracnose fungus *Colletotrichum orbiculare*, our research has revealed that long chain aliphatic molecules produced through the hydrolysis of plant surface cuticle by conidial cutinase serves as signaling molecules. Following signal detection, the morphogenesis related NDR (nuclear dbf2-related) kinase pathway, and its downstream transcription factor *Co*MTF4, play a crucial role in the transduction of plant-derived signal involved in appressorium development. In the stage of plant penetration from appressoria, we have also uncovered the recognition of plant surface signals mediated by a pair of peroxidase/copper radical oxidase (CRO) enzymes. The corresponding pair of genes conserve tandem localization in the genome of most *Colletotrichum* species and the enzyme pair is co-secreted during the penetration stage. The oxidative action of the paired enzymes on plant cuticular derived long-chain alcohols generates signaling molecules (i.e., aldehydes) that potentially functions in a biochemical cascade. In this article, we review our current understanding of fungal-plant communication in adapting to the environmental conditions required for infection-related morphogenesis.

1. Introduction

In the warfare between plants and pathogens, to penetrate the first barrier encountered when landing on plants - i.e. the plant cell wall and cuticle - some phytopathogens have evolved a specialized cell known as the appressorium [1]. In several plant pathogenic fungi, it is well known that the detection of various physical and biochemical compounds on the plant surfaces is necessary for appressorium differentiation [2,3]. *Colletotrichum orbiculare*, a causative agent of anthracnose disease in cucurbits, is one of those appressorium-forming fungi that establishes a hemibiotrophic lifestyle [4]. After appressorial penetration, the fungus initially forms biotrophic primary hyphae, which then differentiate necrotrophic secondary hyphae that kill and degrade host tissues.

In *Colletotrichum* spp., various secreted proteins are involved at each stage of infection [5]. During the early stages, germlings detect host signals and activate genes for hydrolases and small secreted proteins to manipulate host cells. The next stage is biotrophic penetration, during which intracellular hyphae secrete small secreted proteins (SSPs) and secondary metabolites that involve biotrophy. This stage is followed by activation of necrotrophy, during which rapid growth of secondary hyphae is supported by the production of plant cell wall degrading enzymes, proteases, and nutrient transporters, leading to the destruction of host cells.

In the present review, we describe recent findings regarding the molecular mechanism by which *C. orbiculare* detects and responds to plant surface signals and regulates infection-related morphogenesis in two sections: 1) Plant-derived signal sensing and infection structure development mediated by the NDR kinase pathway, and 2) Cuticle oxidation, by a tandem of metalloenzymes at appressorial stage, involved in a signaling cascade that drives plant penetration.

2. Plant-derived signal detection and appressorium development mediated by the NDR kinase pathway

In many plant pathogenic fungi including *Colletotrichum orbiculare*, the initial steps of invasion into the host plant are conidial adhesion and plant surface recognition. The detection of various host-derived signals triggers the germination of conidia and differentiation of appressoria. Previous research has shown that various physical and biochemical signals such as surface hydrophobicity, hardness, cutin monomers, and cuticular waxes stimulate the differentiation of appressoria in *Colletotrichum* spp., *Magnaporthe oryzae* (syn. *Pyricularia oryzae*), and *Ustilago maydis* [2,6-8].

However, the exact molecular basis underlying the link between surface recognition and appressorium development remain unclear. Previous studies have demonstrated that intracellular signal transduction such as the cAMP-PKA and the MAP kinase cascades, are required for morphological differentiation in response to plant-surface signals in *C. orbiculare* [9]. Our research has identified a native molecule and a related signal transduction pathway that are necessary for appressorium morphogenesis of *C. orbiculare.*

To better understand the precise relationship between fungal intracellular transduction and plant surface signals involved in appressorium morphogenesis of *C. orbiculare*, we focused on *CoPAG1*, which was identified through the screening for factors involved in plant surface recognition using mutants showing defects in appressorium development and pathogenesis [10]. The *Saccharomyces cerevisiae PAG1* (*TAO3*) is known to be a component of the RAM (Regulation of Ace2 and Morphogenesis) signal transduction pathway that regulates cell morphogenesis [11,12], where PAG1 serves as a scaffold protein for the activation of the NDR (nuclear Dbf2-related) kinase *CBK1*, which plays a

central role in this pathway [13] (Fig. 1). In filamentous fungi, this pathway is referred to as the morphogenesis-related NDR kinase network (MOR) and is important in controlling cell polarity and differentiation [14]. Targeted gene deletion analysis showed that *CoPAG1* is a key factor in appressorium development triggered by plant-derived signal molecules (cutin monomers such as *n*-octadecanal and 1, 16-hexadecanediol) and is involved in pathogenesis. We found that *Co*Pag1 physically interacts with *Co*Cbk1 and is essential for its phosphorylation. Analysis of an ATP analog-sensitive *Co*Cbk1 protein kinase showed that *Co*Cbk1 is essential for appressorium differentiation and pathogenesis. Altogether, these findings imply that the formation of a functional appressorium in *C. orbiculare* requires the activation of *Co*Pag1-*Co*Cbk1 through the recognition of plant-derived signals. Furthermore, the deletion of *CoPAG1* and the inactivation of *Co*Cbk1 suppressed the expression of a subset of the plant-signal-induced genes with potential roles in pathogenicity, such as transcription factors, SSPs, plant cell wall degrading enzymes, and membrane transporters [15-18]. As a result, MOR plays a crucial role in linking plant surface signals with infection-related morphogenesis and pathogenesis.

Although components of the MOR, including Pag1-Cbk1, are widely conserved among eukaryotes, the phenotypes of these mutants vary among species, suggesting that upstream/downstream factors and their functions are likely to be species-specific [14]. In *C. orbiculare*, we identified a gene coding for a transcription factor, namely *Co*MTF4, the expression of which is regulated downstream of the MOR [19]. The protein *Co*Mtf4 contains a $Zn(II)_2Cys_6$ zinc binuclear cluster DNA binding motif [20] and a fungal-specific transcription factor regulatory domain. The deletion of *comtf4* resulted in phenotypes similar to those of *copag1* deletion. The protein *Co*Mtf4 localized to the nucleus of the conidium during appressorium formation in response to cucumber leaf

surface compounds (more details below), and this nuclear localization was dependent on CoPag1-CoCbk1. *Co*MTF4 is also thought to contribute to the regulation of genes with potential roles in cell wall metabolism of *C. orbiculare*.

Overall, these findings indicate that *C. orbiculare* senses plant surface compounds, to use them as signals inducing appressorium formation through the regulation of genes coding for proteins involved in cell morphogenesis, such as the MOR pathway and *Co*MTF4.

A question of utmost importance remains: what are the components of cucumber leaves that are sensed by this pathway? To address this question, cucumber leaves were incubated during 1 hour with a conidial suspension of *C. orbiculare* prior to analysis of compounds released in the water solution. This experiment revealed the presence of the aliphatic long chain aldehyde *n*-octadecanal, which was released to a significantly lower extent in a control experiment without conidia cells [10]. It was previously reported that the extracellular matrix of *Colletotrichum* spp. contains high molecular weight mannose-rich glycoproteins, germination inhibitors, and a variety of enzymes, including cutinases [21]. As expected, esterase activity was detected in conidial extracellular matrix and at conidial adhesion sites, suggesting that the conidial surface esterases produce *n*-octadecanal from the cucumber cuticle layer. Furthermore, we found that the addition of *n*-octadecanal activated CoCbk1 and induced the nuclear localization of CoMtf4. These results demonstrate that *n*-octadecanal, derived from the plant cuticle, functions as a key signal molecule for appressorium development mediated by the MOR in *C. orbiculare* (**Fig. 1**).

The MOR has been extensively studied in model fungi such as *S. cerevisiae, Schizosaccharomyces pombe* and *Neurospora crassa,* and has been implicated in the

regulation of diverse cellular processes including cell separation, maintenance of cell wall integrity, cell cycle progression, and polarized growth [14,22], yet its role in the interaction of fungal pathogens with host plant has not been reported. The MOR of *C. orbiculare* plays crucial role in translating plant surface signals for infection-related morphogenesis and pathogenesis, suggesting that this molecular cascade may be utilized by fungal plant pathogens in plant-pathogen interactions and successful plant infection. Given the high conservation of MOR components, this molecular mechanism may also be utilized by other appressorium-forming phytopathogens in the sensing and response to plant signals. Thus, further explorations of the MOR function provide insight into plant-pathogen interactions critical for appressorium formation and could contribute to the development of new control strategies targeting fungal sensing and response to plant signals in plant diseases caused by fungi.

3. Cuticle oxidization by a tandem metalloenzymes at appressorial stage driving plant penetration

Copper radical oxidases (CROs) are a well-studied class of enzymes with diverse substrate specificities, but their biological function remains unknown. CROs include galactose 6-oxidases (GalOx) [23], glyoxal oxidases [24], and broad specificity primary alcohol oxidases (AlcOx) [25,26]. While some of these enzymes are of great interest for biotechnological applications [27], the biological function of most fungal CROs remains unknown.

On the basis of the observation that genes encoding secreted AlcOx orthologs are particularly widespread among phytopathogenic ascomycete fungi [25], and that AlcOx can oxidize long-chain aliphatic fatty alcohols (which are notably found in plant cuticle)

into aldehydes, we hypothesized that AlcOx could play a role in fungal pathogenesis. In most *Colletotrichum* spp. and *Magnaporthe* spp., a gene encoding a putative peroxidase (Perox) located adjacent to an AlcOx-encoding gene in a head-to-head arrangement, suggesting that there is selection pressure to retain the pairing and that it has a critical role in the biology of these pathogens. Importantly, previous *in vitro* studies have shown that the addition of commercial peroxidases (usually horseradish peroxidase) activates *via* a still unclear mechanism the AlcOx, increasing thereby the AlcOx activity [28,29]. Strikingly, we found that the Perox/AlcOx pair gene/enzyme is co-expressed and co-secreted during plant penetration from appressoria in *C. orbiculare* [30]. The single and double deletion mutants of *Perox* and/or *AlcOx* gene(s) show impaired penetration ability and pathogenicity. Integrated studies using molecular modeling, biochemical and biophysical analysis have indicated an exquisite interaction between these enzymes, which oxidize plant cuticular-derived long-chain alcohols into aldehydes. In brief, the presence of the peroxidase allows formation of the redox-active form of the AlcOx, where a tyrosine radical and a Cu(II) atom present in the active site of the AlcOx allow the latter to catalyze the two-electron oxidation of alcohols into aldehydes. In other words, the peroxidase acts a redox switch allowing tight control over the AlcOx activity. Remarkably, for all gene deletion mutants, the addition of the aliphatic long-chain aldehyde *n*-octadecanal partially restored appressorium penetration ability and lesion formation on cucumber leaves, suggesting that the role of the Perox/AlcOx pair is to increase the local concentration of long-chain aldehydes to prime the fungus for efficient plant infection. These aliphatic compounds are well-known to function as signal molecules for fungal morphogenesis [31], raising the possibility that the Perox/AlcOx pair generates signal molecules to prime the fungus for efficient plant infection, rather than simply facilitating

invasion by decomposing the plant surface. Comparative transcriptomic analyses have shown that action of the Perox/AlcOx pair triggers subset of the plant-signal-induced genes predicted to encode carbohydrate-active enzymes (CAZymes) and SSPs. The SSPs and CAZymes are well-known fungal effectors that play a key role in the molecular interactions with host plants [32,33]. Furthermore, a phylogenetic analysis of the upregulated CAZymes indicated two categories, one including genes encoding proteins directed towards the plant cell wall (pectinolytic and cellulolytic enzymes), and the other one towards the fungal cell wall (putative effector proteins with chitin-binding LysM motifs) [30]. In *C. higginsianum*, LysM proteins have been reported as effectors that suppress chitin-triggered immunity and as proteins required for appressorium function [34]. These results suggest that the reaction products of the pair enzymes are responsible for transcriptional regulation of genes involved in host infection. We speculate that localized production of the aldehyde compounds by Perox/AlcOx pair could provide the key needed to move to the next infection stage, with the expression of the genes involved in facilitating penetration and evading host immune responses (Fig. 1).

It will be interesting to determine whether Perox/AlcOx pair-mediated long-chain aldehyde production contributes to the regulation of the intracellular signal transduction, such as the MOR and cAMP–PKA /MAPK pathways, which were reported to be involved in plant surface signal sensing in other fungal pathogens [35,36].

Furthermore, we expanded our interests to the rice blast pathogen *M. oryzae* to generalize the role of Perox-AlcOx pair and found that single and double mutants of the *Mor*Perox-*Mor*AlcOx pair showed attenuated appressorium mediated penetration, thus pathogenesis, similar to *C. orbiculare* [37]. Given the specific occurrence of the Perox-AlcOx pair in most *Colletotrichum* and *Magnaporthe* species, our observation implies the

possibility that functionally equivalent, coupled oxidative enzymatic mechanisms may operate in other appressorium-forming fungal pathogens.

4. Conclusions

In this review, we introduced that there are two phases in fungal-plant communication during *C. orbiculare* infection. Phase1: adapting to environmental condition for appressorium development through the NDR kinase pathway, and phase2: appressorium penetration mediated by the Perox/AlcOx pair (Fig. 1). In the early stages of appressorium development, *C. orbiculare* utilizes long chain aliphatic compounds such as *n*-octadecanal as signal molecules for plant-microbe communication. The MOR cascade and its downstream factor *Co*MTF4 translate plant surface signals for appressorium morphogenesis and pathogenesis. In order to progress to the penetration stage by appressoria, the Perox/AlcOx pair provides the localized production of long chain aldehydes for transcriptional regulation of genes that enable host infection. Studies on the sensing and response to plant surface signals by appressorium-forming fungi demonstrate that the plant infection through these specialized infection structures is a highly complex and finely regulated process. Our study using *C. orbiculare* as a model fungus has opened new avenues for understanding the role of plant surface derived compounds and infection related morphogenesis in plant pathogenic fungi.

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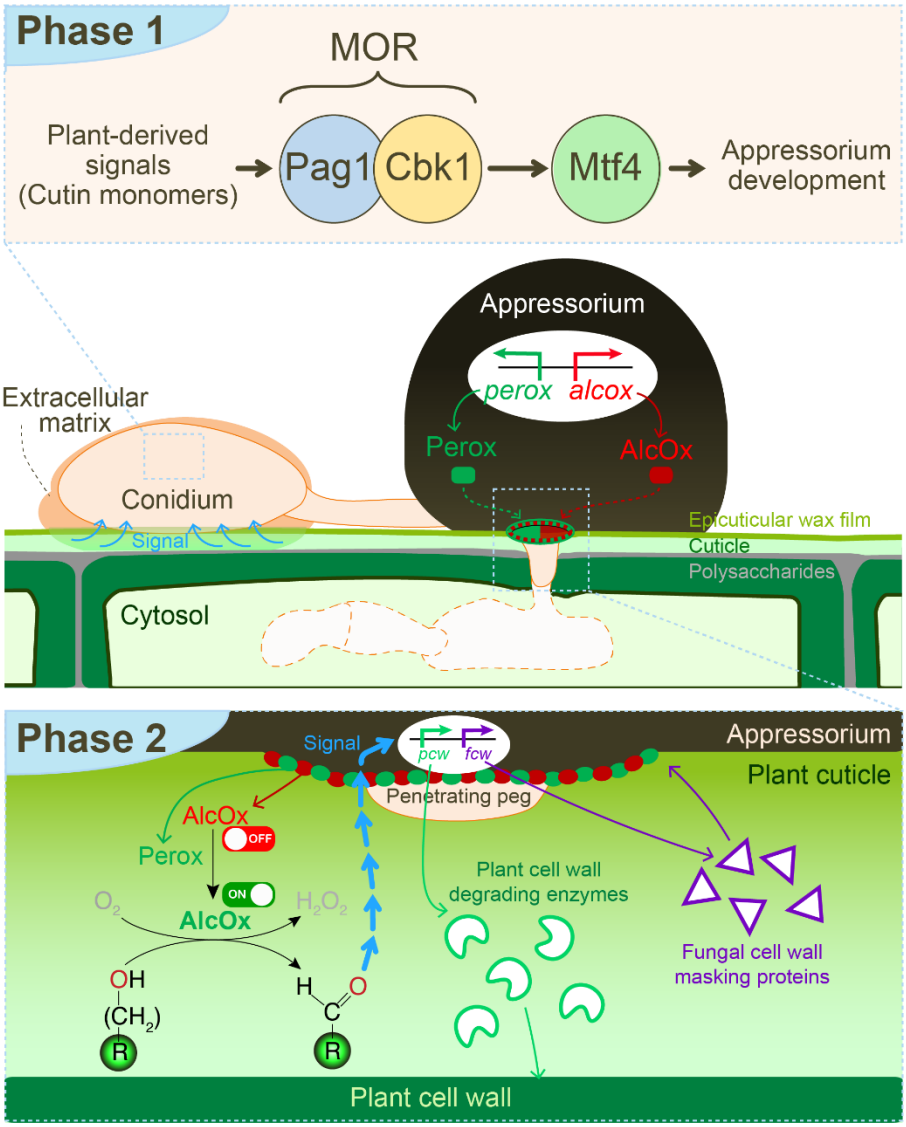

Fig. 1. Schematic summary of plant surface signal recognition and infection-related morphogenesis of *C. orbiculare.*

Phase 1: Esterase activity in the conidial extracellular matrix produces release cutin monomers from plant surface cuticle to serve as signal molecules for appressorium development *via* the MOR. Enhanced activation of CoCbk1 dependent on CoPag1 and the downstream CoMtf4 is required for appressorium morphogenesis. Phase 2: the fungal Perox-AlcOx pair is secreted during early plant penetration and its proposed role in the induction of a biochemical cascade.