

**Studies on host specificity
of the cucurbit anthracnose pathogen *Colletotrichum orbiculare*
via comparative analyses with closely related pathogens**

(近縁菌との比較解析によるウリ類炭疽病菌の宿主特異性に関する研究)

小川 泰生

SUMMARY

CHAPTER I. Multiple *Colletotrichum* species commonly exhibit focal effector accumulation in a biotrophic interface at the primary invasion sites in their host plants

Fungal plant pathogens deploy a suite of secreted proteins, called effectors, to facilitate successful infection. Several fungal pathogens have been reported to secrete and accumulate their effector proteins in the host–pathogen interfacial spaces. *Colletotrichum orbiculare* strain 104-T was known to accumulate effectors in host–pathogen biotrophic interfacial space around the neck of the primary invasive hyphae beneath the appressoria to form ring-like signals. Here, to see whether the other strains or species in the genus *Colletotrichum* exhibit similar accumulation patterns of effectors, I generated mCherry-tagged CoDN3 effector expressing reporter lines of *C. orbiculare* MAFF306589, *C. trifolii* MAFF305078, which infects alfalfa, and *C. higginsianum* MAFF305635, which infects Brassicaceae plants. The immunoblotting analysis confirmed that these strains secrete the CoDN3:mCherry protein from hyphal cells. Microscopic observations of these strains showed that all tested reporter lines focally accumulated the CoDN3:mCherry protein around the neck region of the primary hyphae, which were detected as ring-shaped fluorescence signals, during their biotrophic interaction with their host plants. This result suggested that ring-shaped effector accumulation at the hyphal neck region is a conserved mechanism among the *Colletotrichum* genus and derived from their common ancestor. This suggested that *in planta* effector secretion machinery of the genus *Colletotrichum* is an evolutionarily conserved trait and is not related to the host specificity.

CHAPTER II. Multifaceted comparative analyses with a closely related species suggested the contribution of species-specific effectors to host specificity of *Colletotrichum orbiculare* on cucurbits

C. orbiculare and *C. trifolii* are closely related pathogens belonging to the same clade termed *C. orbiculare* species complex (COSC). Despite their close evolutionary relationship, the two species have completely different host specificities. Through inoculation assay on their host plant with the pathogens, I found that *C. trifolii* cannot invade cucumber epidermal cells despite the stable invasion of alfalfa epidermal cells. Interestingly, co-inoculation assay of cucumber cotyledons with *C. orbiculare* and *C. trifolii* revealed that *C. trifolii* also could invade cucumber epidermal cells when *C. orbiculare* develops invasion hyphae to nearby epidermal cells. This result suggested that *C. orbiculare* suppresses the non-host resistance of cucumber by non-diffusive factors. Thus, I hypothesized that, during the host invasion process, *C. orbiculare* secretes virulence effector(s) that *C. trifolii* does not have or does not express. Through comparative analysis between *C. orbiculare* and *C. trifolii*, I identified a known effector gene *EPCI* as an effector candidate involved in the host specificity of the pathogen. By comparing *EPCI* homolog sequences in COSC, I found that *EPCI* homologs of *C. sidae* and *C. spinosum* lost start codons in the genomes. Based on the topology of the phylogenetic tree, these deletion mutations are assumed to have occurred independently in COSC. Although *C. trifolii* was shown to have a functional *EPCI* homolog, this gene (*CtEPCI*) was barely expressed during the early invasion phase on the native host plant alfalfa. Furthermore, I found that the expression of *CtEPCI* might be suppressed by epigenetic mechanisms. Because *EPCI* contributes to *C. orbiculare* virulence on cucumber, these differences in *EPCI* homologs possibly reduce the potential virulence on cucumber, which might be related to differences in host specificity of pathogens in COSC. However, the constitutive expression of *CtEPCI* did not alter the virulence of *C. trifolii* on alfalfa and cucumber, suggesting that more factors are involved in the host specificity of *C. orbiculare* on cucurbits.

CHAPTER III. Specific contribution of effectors to virulence of *Colletotrichum orbiculare* on particular melon lines in cucurbits

Although *EPC1* was analyzed in Chapter II, it was suggested that there are still undiscovered factors involved in the host specificity of *C. orbiculare*. Here, I newly identified 16 effector candidate genes of *C. orbiculare* strain 104-T involved in host specificity of the pathogen using RNA-seq data of 12 strains belonging to three COSC species: *C. orbiculare*, *C. trifolii*, and *C. sidae*. The 16 genes included *EPC1*. Aside from *EPC1*, single-gene deletion of these candidates had no detectable effects on 104-T virulence on cucumber. Then, I considered the possibility that each candidate gene has functional redundancy or a relatively small contribution to virulence. I generated mutant strains lacking multiple effector candidate genes to cope with this possibility. I selected six genes that were barely expressed in *C. sidae* appressoria from the 16 candidate effector genes. Whereas two independent sextuple knockout mutants lacking the all six genes did not show a detectable reduction in the virulence on cucumber compared to the parental strain, these mutants showed reduced virulence on particular lines of melon (*Cucumis melo*). This suggested that some of the six genes contribute to the virulence specifically toward these melon lines. Melon is classified into two subspecies, *C. melo* subsp. *melo* and *C. melo* subsp. *agrestis*. The inoculation assays on melon genetic resources revealed that the sextuple KO mutant and $\Delta epc1$ strain of 104-T show reduced virulence on most of the melon accessions classified as subsp. *agrestis*, while they did not show reduced virulence on most of the accessions classified as subsp. *melo*. These findings indicate the specific contribution of the effectors to the virulence of *C. orbiculare* on particular melon lines, mostly classified as subsp. *melo*, in cucurbits.