線虫、宿主マツ、媒介カミキリムシの3者関係から紐解くマツ枯れのメカニズム

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The mechanism of pine wilt disease revealed by triadic relationship among *Bursaphelenchus xylophilus*, pine tree, and vector cerambycid beetle.

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1 Research goal

Pine wilt disease (PWD) is a great threat to the local ecological environment and the global economy because it causes rapid wilting and death in the genus *Pinus*. The spread of PWD is thought to depend on two abilities of the causal pathogen *Bursaphelenchus xylophilus*: high virulence and transmissibility. Although the mechanisms of PWD have been extensively studied worldwide the last 50 years, no molecule responsible for their high virulence and transmissibility has been identified with solid evidence. It is due to the insufficient available tools for molecular functional analysis of *B. xylophilus*, host pine trees, and vector *Monochamus* cerambycid beetle. The overall aim of this thesis was to reveal the mechanism of high virulence and transmissibility of *B. xylophilus* at a molecular level. In order to address this, four sub-research were performed focusing on the relationship between *B. xylophilus* and host pine trees or beetle vectors.

2 Summary of the chapters

Thaumatin-like proteins and a cysteine protease inhibitor secreted by the pine wood nematode *Bursaphelenchus xylophilus* induce cell death in *Nicotiana benthamiana* (Chapter 2)

Physiological, histological, and molecular biological studies have shown that *B. xylophilus* induces a series of hypersensitivity responses in susceptible trees, and eventually leads to death within a short period. Recently, a highly sensitive proteome analysis identified numerous candidate pathogenic factors including molecules that induce hypersensitive responses in host pine trees. However, the actual function of these proteins during the onset of parasitism remains to be elucidated. In Chapter 2, a functional screening of 10 candidate pathogenic proteins secreted by *B. xylophilus* was performed using a leaf-disc assay based on transient overexpression in the model tobacco plant *Nicotiana benthamiana*. I found that five molecules induced significant cell death in tobacco plants compared to the GFP-only control. Three of these proteins (Bx-TH1, Bx-TH2, and Bx-CPI) may have a role in molecular mimicry and are likely to be important contributors to the induction of hypersensitive responses in host plants.

Novel functional analysis for pathogenic proteins of *Bursaphelenchus xylophilus* in pine seed embryos using a virus vector (Chapter 3)

In Chapter 3, I established a novel functional analysis method for susceptible black pine (*P. thunbergii*) seed embryos using transient overexpression by the *Apple latent spherical virus* vector. Using this method, I investigated five secreted proteins of *B. xylophilus* that cause cell death in tobacco plants to determine whether they induce hypersensitive responses in pine. I found that three of five molecules induced significantly higher expression in PR (pathogenesis-related) genes ($p<0.05$), indicating hypersensitive responses in pine seed embryos, compared with mock and GFP controls. This study is the first to analyze the function of pathogenic candidate molecules of *B. xylophilus* in the natural host pine using exogenous gene expression, which is anticipated to be a powerful tool for investigating the mechanism of PWD.

How did nematodes acquire a specific relationship with their beetle vectors? (Chapter 4)

In Chapter 4, I focused on the dauer larva (dispersal stage larva) formation of *B. okinawaensis* to elucidate how *B. xylophilus* acquired a specific relationship with *M. alternatus*. *B. okinawaensis* is a close relative species of *B. xylophilus* and has also evolved a relationship with a cerambycid beetle vector like *B. xylophilus*. It has a single dauer larval stage (DIII larva), whereas *B. xylophilus* has two distinct dauer larval stages (DIII and DIV larva). I investigated the dauer-inducing conditions of *B. okinawaensis* and compared them with those of *B. xylophilus*. The dauer percentage of *B. okinawaensis* significantly increased when the nematode population on the plate increased or when I propagated the nematodes with a crude extract of cultured nematodes that likely contained dauer-inducing pheromones. Furthermore, when I propagated the nematodes with *M. alternatus* pupae until the beetles eclosed, *B. okinawaensis* significantly developed into dauer larvae to the same level as *B. xylophilus*. The dauer larvae of these two closely related species may be induced by the same or similar compounds released by *Monochamus* beetles. Since *B. okinawaensis* is genetically tractable, genetic tools for this nematode species will provide new insights into the molecular mechanisms underlying dauer formation in *B. xylophilus*. Additionally, only 1.3% of dauer larvae were successfully transferred to *M. alternatus*. This percentage is lower than that of *B. xylophilus* (Maehara et al., 2020). DIII and DIV of *B. xylophilus* are induced by increasing the nematode population and the presence of the beetle vector, respectively. These results suggest that the specific relationship with the beetle vectors of *Bursaphelenchus* nematodes evolved first via the induction of dauer formation through vector-specific signals, followed by the acquisition of the ability to transfer to the vector.

Novel functional analysis to identify the receptor genes for dauer-inducing signals in insect-borne nematodes, using germline transformation of *Caenorhabditis elegans* (Chapter 5)

I reported a method for analyzing the function of receptors for dauer-inducing signals in even in non-model nematode species such as *B. xylophilus*, using germline transformation of *Caenorhabditis elegans*. In Chapter 5, transgenic worms developed into dauer larvae in the presence of diacetyl when I expressed odr-10, which is the receptor gene for diacetyl, on the ASJ neurons. It suggests that transgenic *C. elegans* theoretically develop into dauer larvae in the presence of vector *Monochamus* beetles when the receptor genes of *Bursaphelenchus* for recognition of its vectors are expressed on the same ASJ neuron. Although this assay system has still some limitations, it has the potential to contribute to the understanding of the dauer formation mechanism at the molecular level in the future.

General discussion(Chapter 6)

I provided insights into the evolution of *B. xylophilus* and discuss the perspective for a deeper elucidation of the mechanisms of PWD and its prevention.