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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(Summary)

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

Agriculture Program
Kirino Haru

Chapter 1: General introduction

Pine wilt disease (PWD), one of the most serious forest diseases in the world, causes rapid wilting and death in the genus *Pinus*. *Bursaphelenchus xylophilus*, native to North America, was first discovered in Japan in the early 20th century as the causal pathogen of PWD, and spread to other Asian and European countries. As *B. xylophilus* has rapidly killed many non-native pine species, PWD has become a great threat to the local ecological environment and the global economy.

Two key features of *B. xylophilus* are crucial for the rapid spread of PWD. One is its high virulence in the pines except for the native North American pine trees. *Bursaphelenchus xylophilus* is thought to induce a series of hypersensitive responses in susceptible trees, and eventually leads to death within a short period of short time, i.e., within a few weeks to months. This high virulence enables *B. xylophilus* to deprive more host resources and produce more offspring. The second feature is the high transmissibility through beetle vectors. The vector of *B. xylophilus*, a specific *Monochamus* cerambycid beetle which emerges from dead pine trees and spreads to feed on new and living pine trees, facilitates the spread of *B. xylophilus* in large numbers. This unique association with beetle vectors greatly promoted their transmissibility, resulting in the widely spread of PWD.

Accordingly, a clear understanding of the molecular interactions between *B. xylophilus* and host pine trees or beetle vectors may identify noble and more effective targets of controlling agents. Although the mechanisms of PWD have been extensively studied worldwide for the last 50 years, no molecule related to 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*, pine trees, and *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. To address this, four sub-research were performed focusing on the relationship between *B. xylophilus* and host pine trees or beetle vectors. Firstly, a functional screening of 10 candidate pathogenic proteins secreted by *B. xylophilus* was performed using a leaf-disk assay based on transient overexpression in a model plant *Nicotiana benthamiana* (Chapter 2). Secondly, a novel functional analysis method for susceptible black pine (*Pinus thunbergii*) seed embryos was established using transient overexpression by the *Apple latent spherical virus* (ALSV) vector, and five candidate pathogenic proteins causing cell death in *N. benthamiana* induced hypersensitive responses in pines (Chapter 3). The third sub-research revealed the dauer formation of *Bursaphelenchus okinawaensis*, which is genetically tractable, was promoted by *Monochamus* beetle to the same level as *B. xylophilus* (Chapter 4). It suggests that the dauer stage of these two close species is likely

to be induced by the same or similar compounds on *Monochamus* beetles. Hence, genomic and genetic tools for *B. okinawaensis* will provide new insight into the molecular mechanisms of dauer induction of *B. xylophilus*. The fourth sub-research reported a functional analytic method of receptor genes for vector beetle recognition using germline transformation of *Caenorhabditis elegans* (Chapter 5). It will apply in even not model nematodes like *B. xylophilus*. In conclusion, the contribution of this thesis to the prevention of PWD and its perspective was discussed in Chapter 6.

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

Understanding of PWD has advanced in recent years through the use of a highly sensitive proteomics procedure and whole genome sequence analysis; in combination, these approaches have enabled the identification of proteins secreted by *B. xylophilus*. However, the roles of these proteins during the onset of parasitism have not yet been elucidated. In Chapter 2, I used a leaf-disk assay based on transient overexpression in *N. benthamiana* to allow functional screening of 10 candidate pathogenic proteins secreted by *B. xylophilus*. These proteins were selected based on previous secretome and RNA-seq analyses. I found that five molecules induced significant cell death in tobacco plants relative to a GFP- only control. Three of these proteins (Bx-TH1, Bx-TH2, and Bx-CPI) may have a role in molecular mimicry and likely make important contributions to inducing hypersensitive responses in host plants.

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

PWD is thought to be initiated by sequential excessive hypersensitive responses to *B. xylophilus*. Previous studies have reported candidate pathogenic molecules inducing hypersensitive responses in pine trees susceptible to *B. xylophilus*. The functions of some of these molecules have been analyzed in model plants using transient overexpression; however, whether they can induce hypersensitive responses in natural host pines remains unclear due to the lack of a suitable functional analysis method. In Chapter 3, I established a novel functional analysis method for susceptible black pine (*P. thunbergii*) seed embryos using transient overexpression by the ALSV vector and investigated five secreted proteins of *B. xylophilus* causing cell death in tobacco to determine whether they induce hypersensitive responses in pine. I found that three of five molecules induced significantly higher expression in pathogenesis-related genes ($p<0.05$), indicating hypersensitive responses in pine seed embryos compared with mock and green

fluorescence protein controls. This result suggests that tobacco-based screening may detect false positives. This study is the first to analyze the function of pathogenic candidate molecules of *B. xylophilus* in natural host pines using exogenous gene expression, which is anticipated to be a powerful tool for investigating the PWD mechanism.

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

Bursaphelenchus xylophilus, the causal agent of PWD, is transmitted by the cerambycid beetle *Monochamus alternatus*, which is associated with pine tree host. Their specific phoretic ability to appropriate vectors depending on their life cycle is critical for efficient transfer to the correct host. In Chapter 4, I evaluated how *B. xylophilus* acquired a specific relationship with *M. alternatus* with a focus on *B. okinawaensis*, a close relative of *B. xylophilus* that has evolved a relationship with a cerambycid beetle vector. *Bursaphelenchus okinawaensis* has a single dispersal stage (dauer) larva (third-stage dispersal [DIII] larva), whereas *B. xylophilus* has two distinct dispersal stages (DIII and fourth-stage dispersal [DIV] larva). Also, the dauer formation in *B. okinawaensis* is not completely dependent on its beetle vector, whereas DIV larvae of *B. xylophilus* are induced by volatile from the beetle vector. I investigated the induction conditions of dauer larvae in *B. okinawaensis* and compared to with *B. xylophilus*. The dauer percentages 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, which likely contained dauer-inducing pheromones. In addition, dauer formation tended to be enhanced by the crude extract at high temperatures. Furthermore, when I propagated the nematodes with *M. alternatus* pupae until the beetles eclosed, *B. okinawaensis* significantly developed into dauer larvae. However, only 1.3% of dauer larvae were successfully transferred to *M. alternatus*, the rate lower than that of *B. xylophilus*. DIII and DIV of *B. xylophilus* were induced by increasing the nematode population and the presence of the beetle vector, respectively. These results suggest that not only dauer induction by the presence of beetle vectors but also two dauer stages, which only the 'xylophilus' group has acquired, are required for high transfer ability to the beetle vector.

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

For *B. xylophilus*, dauer larvae are crucial as dispersal-stage larvae. After *B. xylophilus*

have consumed old host pines, dauer larvae are developed from the propagation stage and are vectored to new hosts by the specific cerambycid beetle *M. alternatus*. Since only dauer larvae can disperse via beetle vectors and transfer to new hosts, the formation of dauer larvae is essential for the life cycle of *B. xylophilus* and the occurrence of PWD. *Bursaphelenchus xylophilus* induces dauer formation when late pupae and callow adults of beetle vectors are near them. However, any receptors receiving these dauer-inducing signals of *B. xylophilus* have not yet been identified although it is important not only to better understand the ecology of *B. xylophilus* but also as an effective target for control agents that act specifically on *B. xylophilus*. Chapter 5 reported a novel method for identifying the receptor genes for dauer-inducing signals in *B. xylophilus*, using *C. elegans*. Dauer formation of *C. elegans* is promoted when multiple ascarosides are received by the counterpart receptors on dauer-inducing neurons. Accordingly, the dauer formation of *C. elegans* is theoretically induced when other receptors for not even ascarosides are expressed on dauer-inducing neurons and activated. Here, I established transgenic worms odr-10, which is the receptor for diacetyl, on the ASJ neurons using microinjection. Then, I revealed that these transgenic worms could develop into dauer larvae in the presence of diacetyl. It suggests that transgenic *C. elegans* expressing the receptor genes of *Bursaphelenchus* for vector beetle recognition on the same ASJ neuron theoretically develop into dauer larvae in the presence of vector *Monochamus* beetles. 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.

Chapter 6: General discussion

Chapter 6 discussed the contributions of this study to PWD prevention and the perspective for a deeper elucidation of the PWD mechanisms. In this summary, I would like to talk about only the former one.

Globally, there are two general control methods for PWD: injection of nematicides into the host pine trees to kill *B. xylophilus* and aerial spraying of pesticides to kill *Monochamus* cerambycid beetle vectors. Injection of nematicides into host pine trees has been applied to protect a small number of valuable pine trees although it requires high labor and cost. However, when a larger number of vector cerambycid beetles infest host pine trees, resulting in an increasing number of nematodes in the trees, these nematicides sometimes fail to prevent the disease. Aerial spraying of pesticides is more effective in preventing the spread of PWD on a large scale and has been widely recognized as the primary method. Nevertheless, the psychological resistance of neighborhood residents to aerial chemical spraying and concerns about potentially harmful effects on chemically sensitive individuals have prevented to use of this control method in some

local governments. To solve these issues, it is essential to develop new controlling agents that are more effective and safer. In general, controlling agents that act on specific pests or pathogens are considered more effective and safer. First, they often have a more direct and rapid effect, resulting in improved pest control outcomes. Additionally, they reduce the risk of toxicity and other negative effects on non-target organisms, including humans and other beneficial insects.

The results in this thesis, which revealed the mechanism of the high virulence and transmissibility of *B. xylophilus*, contributed to the development of *B. xylophilus*-specific controlling agents. As described in Chapter 1, the spread of PWD is thought to depend on two abilities of *B. xylophilus*: high virulence and transmissibility. Accordingly, the pathogenic candidate proteins investigated in this thesis are valued as new targets of more effective and safer controlling agents that act specifically on *B. xylophilus* (Chapters 2 and 3). In addition, elucidating the molecular mechanisms underlying the beetle recognition of *Bursaphelenchus* will have significant implications for the development of noble agents which specifically prevent the spread of nematodes (Chapters 4 and 5). Furthermore, novel functional analysis methods to determine the pathogenic molecules or the receptors recognizing their beetle vectors were established (Chapters 3 and 5). These methods have the potential as powerful tools to reveal the mechanism of PWD at the molecular level and will identify more target molecules for *B. xylophilus*-specific controlling agents in the future.

Overall, this thesis significantly contributed to the understanding of PWD at the molecular level and to the establishment of functional analysis methods for important molecules in this disease. Additionally, this thesis also provided new insights into the evolution of virulence and transmissibility of *B. xylophilus*. Understanding how pathogens evolve is required to predict their ecological and economic impact and may help us develop new strategies to control or prevent the spread of disease. These studies will be important resources in the prevention and eradication of PWD.