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Approaches to the Study of Plant-Phytopathogen Interactions: *In Vivo* and *In Vitro* Assay Systems of Phytobacterial Pathogenesis

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Abstract

Plant-phytopathogen interactions, such as rice-*Xanthomonas oryzae pv. oryzae* (*Xoo*) interactions, are important for the fate of both the host plant and invading phytopathogen, particularly in the early stage of infection. Thus far, many *in vivo* and *in vitro* systems have been developed to study the plant-phytopathogen interactions to cause disease or resistance in plant and each system has its own merits and limits. *In vivo* system is easy to monitor the effector translocation from phytopathogen to plant and has been used to study the resistance mechanism of plant like Hypersensitivity Response (HR). *In vitro* system is useful to study the pathogenic mechanism of phytopathogen such as pathogenic gene expression. Recently, new *in vitro* system was developed, which enables us to monitor the time-dependent gene expression of phytopathogen upon the interaction with host plant. The *in vivo* and *in vitro* assay systems will be useful to study the mechanism of phytobacterial pathogenesis and plant resistance.

Keywords

Plant-phytopathogen interactions, Rice, *Xanthomonas oryzae Pv. oryzae* (*Xoo*), Phytobacterial pathogenesis, Plant resistance

Introduction

Hosts and pathogens have competed throughout the whole evolutionary history of life. In many cases, pathogenic mechanism between plant and phytopathogen is well conserved with that between animal and animal pathogen [1-3]. At the early stage of infection, plant-phytopathogen interactions are important for the fate of interaction to cause diseases on susceptible plants or to elicit Hypersensitive Reactions (HR) on resistant plants [4]. Rice is the most widely consumed staple food worldwide, especially in Asian countries. The gram-negative plant pathogen *Xanthomonas oryzae pv. oryzae* (*Xoo*) is the causal agent of bacterial blight on rice [5], of which outbreak easily reduces rice yields by as much as 50% [6].

In vivo and *in vitro* systems have been successfully developed to study plant-phytopathogen (rice-*Xoo*) interactions and each system has its own merits and limits (Figure 1). The resistance mechanisms of host rice could be studied better with the *in vivo* systems [7], whereas for the pathogenic mechanism of phytopathogen the *in vitro* systems are generally more suitable. Recently, a new *in vitro* system, mimicking both the *in vivo* and *in vitro* systems, was developed, which initiates and activates the *Xoo* pathogenicity by adding fresh rice leaf extracts into *Xoo* culture medium [8]. The *in vitro* system was also combined with RNA-Seq to study the genome-wide gene expression of the phytopathogen *Xoo*.

The Type III Secretion System (T3SS) is a well conserved protein translocation system in Gram-negative

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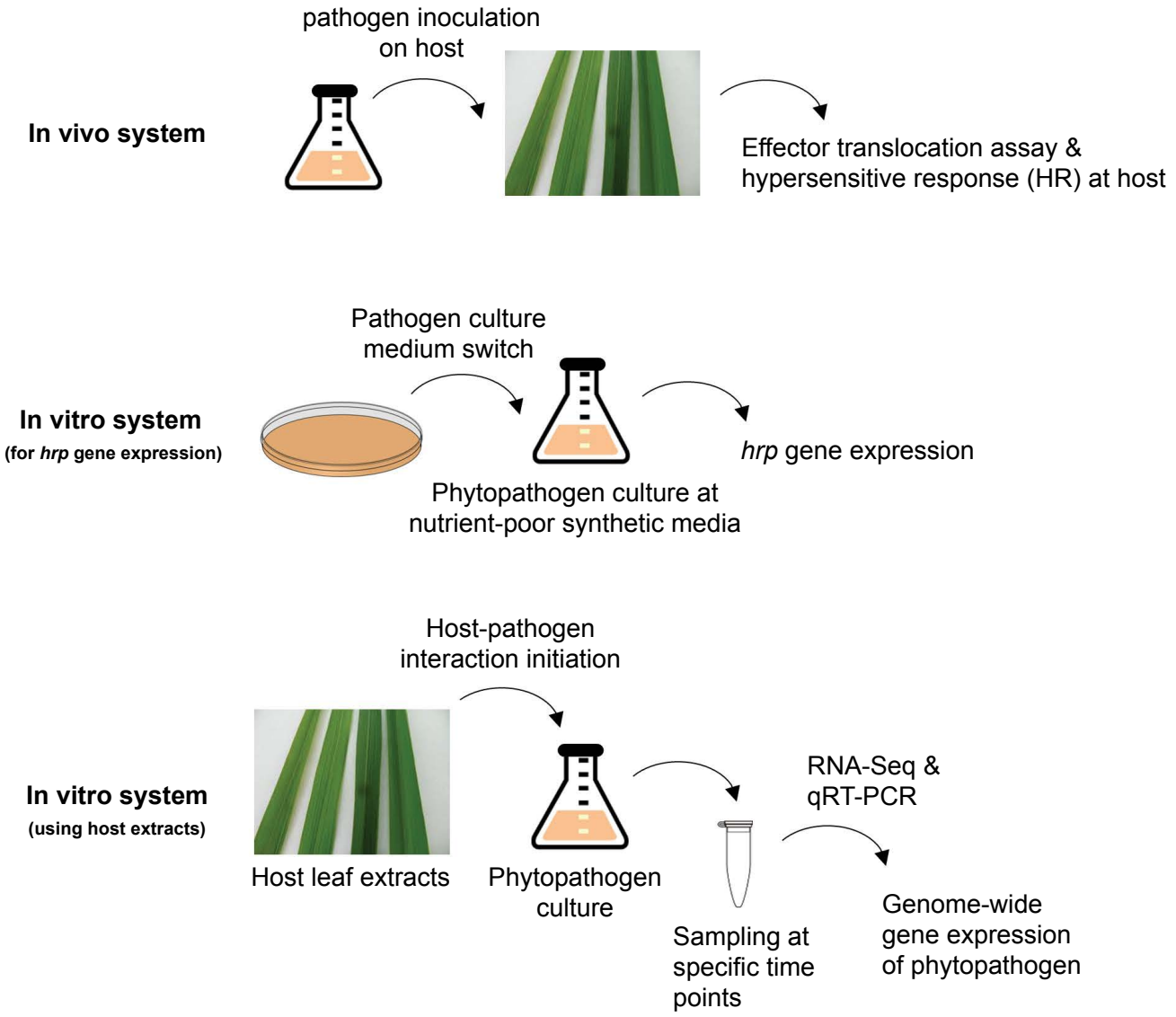

Figure 1: Three representative *in vivo* and *in vitro* assay systems for the study of plant-phytopathogen interactions. *In vivo* system consists of phytopathogen inoculation on plant and is used to study the plant resistance mechanism by monitoring the effector translocation from pathogen to host and HR in plant. *In vitro* assay system using minimal medium is used to upregulate *hrp* genes-like pathogenic gene expression in phytopathogens. *In vitro* assay system using host leaf extracts activates the pathogenicity of phytopathogen by adding host leaf extracts into phytopathogen culture media, which provides genome-wide time-dependent gene expression in phytopathogen upon the interaction with plant.

phytopathogenic bacteria which infect plants and animals [9-11], of which components are encoded by Hypersensitive Response and Pathogenicity (*hrp*) genes including *hrpG* and *hrpX* genes [12]. The T3SS delivers bacterial effector proteins into the host cells to modulate host defense signaling pathways and cause diseases. Currently, the T3SS, *hrp* genes, and effectors are crucial molecules to study the pathogenic mechanism of phytopathogen and resistance of host plant.

In vivo assay system for effector translocation and HR

Basically, *in vivo* assay system consists of phytopathogen inoculation on plant and disease or resistance mechanism study in plant [13]. The translocation of effector proteins from phytopathogenic bacteria to plants via T3SS has been successfully monitored in the *in vivo* system with the activity of reporter protein attached to the effectors [14]. Calmodulin-Dependent Adenylate Cyclase (cya) domain from *Bordetella pertussis* has been used as a reporter protein, which produces cAMP depending on the existence of eukaryotic plant calmodulin [14-17]. Accordingly, it is active only when it translocates from the prokaryotic cell into the eukaryotic host cell. For example, in *Xanthomonas campestris pv. vesicatoria*, as early as 3 h after inoculation, the translocation of effector protein AvrBs2 (avrBs2) to host pepper plants was confirmed with the *in vivo* assay system [14]. Because the *in vivo* system uses intact plant, we

could monitor HR-like plant responses resulting from the phytopathogen infection.

In vitro assay system for *hrp* gene expression

In the initial interaction with plant, the quick response of pathogenic signal activation in phytopathogen is important for successful infection. The pathogenic signaling pathway is a good target to develop pesticides against the plant disease. However, heterogenous phytopathogen population at *in vivo* infection site makes it hard to study the pathogenicity signals such as pathogenicity-related gene expressions. Simplified *in vitro* assay system could be more useful to monitor the pathogenic gene expressions in phytopathogen. The *hrp* genes in Gram-negative phytopathogenic bacteria including *Xoo* play important roles for pathogen's pathogenicity on host plants [12]. The expression of *hrp* genes is highly controlled and usually up-regulated in certain nutrient-poor synthetic media compared to nutrient-rich complex media [18-21]. The nutrient-poor synthetic media has been used to activate the *hrp* gene expression in plant pathogens xanthomonas such as the synthetic minimal medium of XOM2 and XVM2, which is known to mimic the apoplast plant environment to activate the pathogenic signal of phytopathogen [22-25]. Among the components of the synthetic minimal medium, specific carbohydrate sources are known to be important. *Xoo* propagates in rice xylem vessels, of which 60% are xylan (xylose) [26] and the xylose concentration in the synthetic minimal medium is critical to regulate the expression of *hrp* genes in the *in vitro* assay system [22]. The *in vitro* system lacks any rice-derived factors and could be the minimal condition that could activate the hrp gene expression.

In vitro assay system using host extracts

Recently, a new *in vitro* system for rice-*Xoo* interactions was developed, which activates *Xoo* pathogenicity by adding Rice Leaf Extract (RLX) into *Xoo* culture [8]. The *in vitro* system showed the upregulation of effector gene expression and T3SS-dependent effector protein secretion after RLX treatment on *Xoo* [8,27,28]. The *in vitro* system was successfully combined with RNA-Seq to analyze the time-resolved genome-wide gene expressions of *Xoo* upon the interactions with RLX. The new *in vitro* system could synchronize the pathogenicity activation signal in the RLX-treated *Xoo* cells, which enables to monitor the pathogenic signal of *Xoo* in a time-dependent way and the signal to noise ratio of RNA-Seq data was high. Because it is possible to turn on the pathogenic signal of *Xoo* at any specific time point, we could study the pathogenic signal pathways in the same genetic background of wild-type *Xoo* without making single gene-knockout mutants for comparison. The RNA-Seq results provided the expression of many pathogenicity-related genes of *Xoo* was initiated within 5 min upon the contact with RLX. The *hrpG* gene was transcribed at the maximum level within 10 min and *hrpX* gene expression reached the maximum level in 15 min.

Conclusion

Xanthomonas genus includes many pathogenic organisms like *Xoo*, *Xanthomonas oryzae* pv. *oryzicola, Xanthomonas albilineans, Xanthomonas axonopodis pv. phaseoli, Xanthomonas axonopodis pv. manihotis, Xanthomonas campestris pv. campestris, Xanthomonas campestris pv. armoraciae, Xanthomonas campestris pv. musacearum, Xanthomonas campestris pv. vasculorum, Xanthomonas citri pv. citri, Xanthomonas euvesicatoria, and Xanthomonas fuscans subsp. aurantifolii*, which infect diverse crops like rice, sugarcane, beens, cassava, crucifers, banana, citrus, tomato, and pepper [29]. Many pathogenicity-related genes in *Xanthomonas* are well conserved in other plant pathogens and even human pathogens [3].

We reviewed most commonly used and newly developed *in vivo* and *in vitro* assay systems to study the plant-phytopathogen interactions. The *in vivo* system uses intact plant and phytopathogen for assay and is useful to study the plant responses to the phytopathogen infection. However, it is hard to study the pathogenesis mechanism on the side of phytopathogen due to the heterogeneity of phytopathogen populations in the infection site. The *in vitro* system using minimal medium is the simplest system to upregulate the expression of pathogenicity-related genes, such as hrp genes, in phytopathogens. The upregulating mechanism is still unclear and needs to be further studied. Newly developed *in vitro* system activates phytopathogen pathogenicity by using host leaf extracts instead of minimum medium and enables us to study the time-dependent pathogenic responses of phytopathogen upon the interaction with plant in the same genetic background. The assay systems will help us to understand the mechanism of pathogenesis in phytopathogens and resistance in plants and crops.

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