

## REVIEW

# Regulation of Pathogenicity-related Genes in Phytopathogenic Bacteria and Plant

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### Abstract

The genes required for the pathogenicity of plant pathogens can be divided into two groups: those for eliciting virulence and those for establishing host-parasite interactions. Basically, the former genes are required for efficient production of the virulence factor(s) leading to the development of symptoms, while the latter genes are needed for well-balanced production of the factors required at each step of the parasitism. To understand the overall picture of plant pathogenicity, we need to understand not only the list of both genes but also how their expressions are regulated. We chose the major virulence genes of soft rot causing Enterobacteriaceae (represented by *Pectobacterium carotovorum* subsp. *carotovorum* and *Dickeya dadantii*) as a model system for dynamic study of the former genes. For the dynamic study of the latter genes, we chose the *hrp* genes, many of which are responsible for the Type III secretion system (T3SS) and the genes for the T3SS-dependent effectors of xanthomonads. Particularly since the major effector of xanthomonads, the AvrBs3 group or TAL (transcription activator-like) effectors contain NLS (nuclear localization signal) and an acidic activation domain, their studies are also expected to lead us to dynamic analyses of plant genes too.

**Discipline:** Plant disease/Plant protection

**Additional key words:** AvrBs3 family, Dual Roles of *avr* genes, *hrp*, hyperinduction, pectate lyase, product induction mechanism, soft rot Enterobacteriaceae

### Virulence genes and their regulatory mechanisms in soft rot causing Enterobacteriaceae

Macerating enzymes such as pectinase, cellulase, and protease have been shown to be responsible for eliciting symptoms in the case of soft rot causing Enterobacteriaceae (SRE). Among these enzymes, *endo*-pectate lyase (PL), which cleaves pectate trans-eliminatively and in a random manner, is a major virulence factor. Several major and minor PL isozymes have been reported in SRE. Since PLe of *Dickeya dadantii* strain EC16 is the most abundant isozyme at the infection site, laboratories in many countries worldwide have extensively studied it. Early studies on the regulation of total PL isozymes (PLs) sparked findings of basic or common regulatory mechanisms in SRE. Subsequently, many laboratories focused on further insights to view complex regulatory mechanisms by focusing on PLe based on the sequence data of the Dd strain 3932<sup>3,7</sup>. From

these studies, we now realize that so many regulatory mechanisms are involved and in a complex manner to optimize the synthesis of even a single PL isozyme during the pathogenic life cycle.

#### 1. "Product Induction Hypothesis"

The specific activity of total PLs starts to increase considerably within 3 to 4 hours of adding pectate to the medium in the case of *Erwinia carotovora* (syn. *Pectobacterium carotovorum* subsp. *carotovorum*). Since this long lag before the onset of its induction was determined as attributable to the time required for the pectate to degrade and metabolize by the basal level of the responsible enzymes, we called this induction mechanism "Product Induction Mechanism"<sup>23</sup>. This mechanism subsequently proved to be the main regulatory mechanism of PL in SER (actually in many polymer-degrading enzymes in many different microbes) and the true inducer among metabolites was determined as 2-keto-3-deoxygluconate (KDG).

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“Product Induction Mechanism” effectively explains the mechanism whereby the SRE recognizes the environment for the necessity of PLs. Both genetic and *in-vitro* binding studies showed that the transcriptional regulator KdgR is a repressor binding at the KdgR box in the promoter regions; covering not only PL isozymes and other pectate-degrading enzymes but also their metabolizing enzymes<sup>19,20</sup>. When pectate exists in the area surrounding SRE, it must be degraded and metabolized to get energy via KDG. KDG binds at KdgR; triggering allosteric change so that the KdgR is released from the promoter. The opening at the promoter region causes the RNA polymerase to bind to it and thus initiate the transcription. The *kdgR* homologs were found and share their functions among many SRE isolates<sup>17</sup>.

## 2. “Self-catabolite repression Hypothesis”

However, the “Product induction mechanism” will result in the continuous induction of PL synthesis, even after its substrate is no longer available. This puzzle was clarified by determining the “Self-catabolite repression mechanism” of PLs<sup>24</sup>. Namely, efficiently metabolizing the breakdown pectate products leads to an accumulation of intermediate metabolites, including KDG. A high level of intermediate metabolites is known to result in the degradation of cyclic AMP (cAMP) due to the inactivation of adenyl cyclase, which catalyzes the reaction from ATP to cAMP. Since cAMP and CRP (cAMP recognition protein) act together to promote transcription by bending the promoter region to be exposed to RNA polymerase, the deficiency of cAMP due to highly efficient pectate degradation and metabolism results in the repression of PLs syntheses.

## 3. Cell-density dependent induction

During the induction study of PLs, we noted that the specific activity of PL kept increasing, even after entering the stationary phase of growth, irrespective of the presence of pectate in the medium<sup>25</sup>. This was considered attributable to the “auto-induction mechanism”, which was first found in the regulation of luciferase in *Vibrio fischeri* (syn. *Photobacterium fischeri*)<sup>15</sup>. Basically, the auto-inducer, homoserine lactone (HSL) in many bacteria, including SRE, is secreted from the bacterial cells and accumulates in the surrounding area during the early log phase of their growth. When HSL soars amid higher cell density, it starts to flow back into the bacterial cells, where it acts to induce the synthesis of the target gene(s).

## 4. *In-planta* Hyperinduction of PLs

### (1) Regulatory proteins responsible for the *in-planta* hyperinduction of PLs

The *in-planta* PLs specific activity exceeds that of PLs fully induced by the “Product Induction mechanism”. A gel-shift assay using ammonium sulfate salt fractions of

bacterial crude extracts from the culture in the medium with or without plant extract, as well as pectate and the promoter region of *pelE* (structural gene for PLe) as the target DNA, showed that at least one positive and negative regulators seem to be specifically involved in the *in-planta* hyperinduction of PLe. We isolated the former positive regulator using a gel-shift assay, called it “Pir” (plant-inducible regulator) and then cloned the *pir* gene responsible for synthesizing Pir<sup>21,22</sup>. The deletion mutant of *pir* was confirmed as losing the hyperinducing ability without affecting the Product Induction mechanism. Since the binding site for Pir overlaps with the KdgR box, hyperinduction by Pir seems to require the release of KdgR from the promoter by a product induction mechanism<sup>21,22</sup>. This is the first indicative data from an *in-vitro* study showing that competition among positive and negative regulatory molecules determines the induction rate.

### (2) Inducer(s) for the *in-planta* hyperinduction of PLs

Hyperinduction was observed in the medium containing hot-water extract of various plants as well as pectate. After extensive efforts to purify the inducers from potato extract, they were found to be simple sugars. Following a survey of sugars with or without methylation, those with or without the keto-group and rare sugars (kindly provided by Prof. Izumori at Kagawa University, Japan) were further divided into hyper-inducible and –noninducible sugars. The common configuration of inducible sugars was hexose with specific *trans*- configuration at C3 and C5 such as D-glucose, D-galactose, D-mannose, D-mannitol, D-fructose, 2-deoxy D-glucose, D-fucose with the exception of hyperinducibility by D-xylose, L-arabinose and D-ribose, even with a different configuration. Considering these exceptions and the configuration of noninducible sugars, we hypothesized that the acyclic form of these sugars is responsible for the hyperinduction of PL (Joko, Murata, Tsuyumu, unpublished data).

### (3) PhoP/PhoQ dual-component regulatory system for the *in-planta* hyperinduction of PLs

The PhoP and PhoQ two-component regulatory system (PhoP/Q TCRS) is known as one of the global regulatory systems in many different bacteria and responding to many environmental factors such as low pH and low concentrations of phosphates and magnesium ions. Most plant pathogenic bacteria inhabit intercellular spaces in plants where the above-mentioned PhoP/Q responsive environmental conditions prevail. This may be why PhoP/Q TCRS is utilized to regulate many other genes involved in pathogenicity. Using comparative microarray between wild types and the *phoQ* mutant, we found many genes, including those related to pathogenicity, e.g. to PLs, are under the control of PhoQ<sup>29</sup>. In particular, the *PhoQ* mutant could not hyperinduce PLe in a hyperinducing medium containing 10 μm (low concentration) of magnesium but

could hyperinduce PLE in one containing 10 mM (high concentration) of magnesium that would probably allow a passive intake of magnesium<sup>8</sup>. Thus, PhoQ seems to act as a sensor of low magnesium in the surroundings for bacteria to uptake it efficiently and utilize it for hyperinduction of PLs.

### 5. Other regulatory mechanisms

Besides these basic regulatory molecules, many other regulatory molecules such as PecS, H-NS, PecT, Fur, Ddl<sup>13</sup> and SlyA<sup>9</sup> have been found to be involved in regulating PLs. Readers are referred to excellent reviews on this subject<sup>4,10</sup>. In any event, we now realize that even a single structural gene responsible for pathogenicity must be regulated for optimum synthesis at each stage of the pathogenic process. This understanding of complex regulatory mechanisms for even a single virulence factor reminds us of the importance of dynamic pathogenicity analyses.

### Genes involved in pathogen-plant interactions

Transposon tagging makes it possible to isolate many different mutants deficient in pathogenicity without knowing their direct phenotypes. The genes involved in eliciting both a hypersensitive response (HR) and pathogenicity (*hrp* genes) are most frequently isolated in many plant pathogenic bacteria. Most *hrp* genes are responsible for building the Type III secretion system (T3SS) that injects many T3SS-dependent effectors into plant cells. Considering the wide distribution among plant pathogenic bacteria, this mode of trafficking the effectors seems to be the basic way that pathogenic bacteria interact with plants. It is important for us to know the regulatory mechanisms for optimum syntheses of both *hrp* genes as basic or common plant pathogenic genes and the genes for the T3SS-dependent effectors as specific genes having coevolved with plants. We isolated *hrp* mutants of *Xanthomonas axonopodis* pv. *citri* (Xac) to initiate the dynamic study of both types of genes in Xac<sup>14</sup>.

#### 1. Regulators for xanthomonad *hrp* genes

In xanthomonads, *hrp* genes are basically regulated by two major regulatory genes *hrpG* and *hrpX*<sup>30</sup> as well as the involvement of many other global regulators. In our laboratory, we found *lrp*<sup>12</sup>, XAC4131 (a putative TonB-dependent transducer)<sup>1</sup> and even KdgR<sup>16</sup> were also involved in regulating *hrp* genes in the case of *Xanthomonas oryzae* pv. *oryzae* (Xoo). Considering the fact that *hrp* genes are widely distributed among plant pathogenic bacteria, they should share not only trafficking apparatus but also the means of regulating their synthesis. However, due to space limitations in this review, I will leave this aspect for the available excellent reviews. Instead, I want to focus on the function of the T3SS effectors, particularly the AvrBs3 family (also known as the transcription activator-like family, TAL) that are

directly involved in transcribing plant genes in cascades leading to either disease-resistance or resistance responses.

#### 2. New system for elucidating the function of effectors

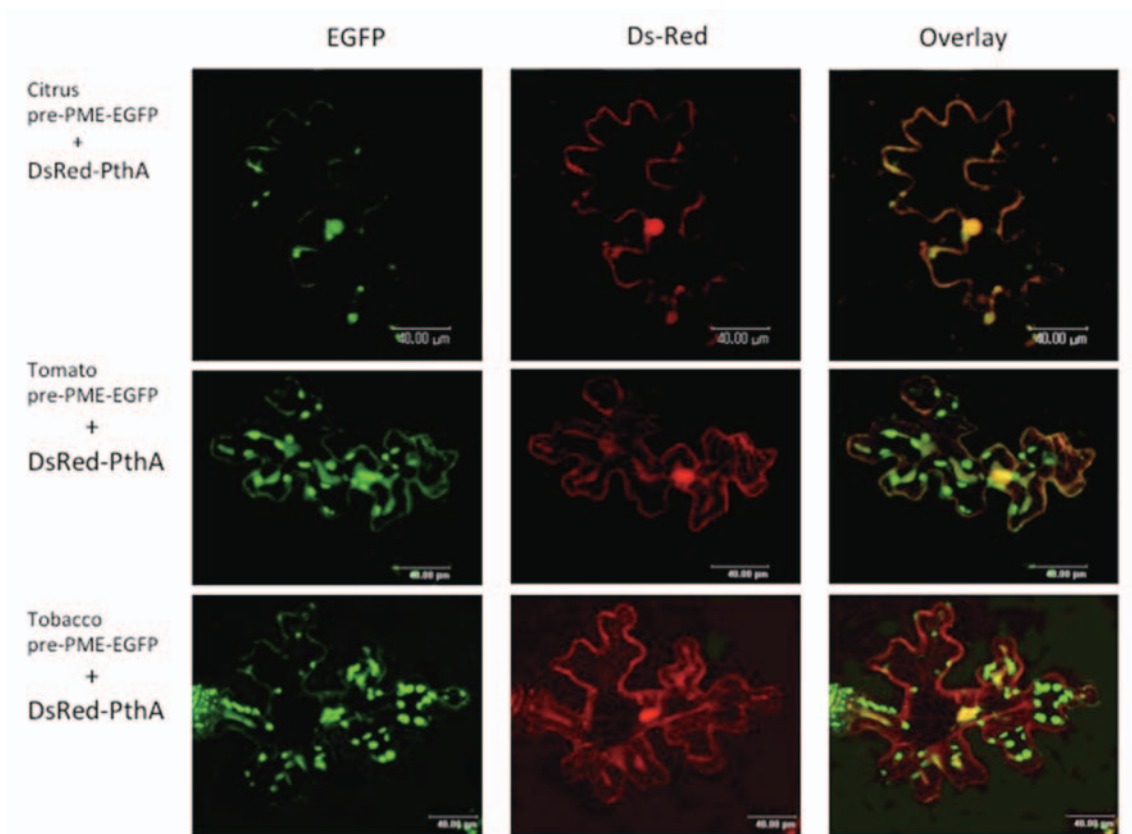
The genes of T3SS machinery are widely distributed among not only plant pathogens but also animal and opportunistic pathogens and as well as being structurally similar, are also functionally interchangeable. When *pthA*, a member of the *avrBs3* gene family but responsible for pathogenicity, was transformed into saprophyte *Pseudomonas fluorescens* (Pf) strain 55 or into *Escherichia coli* harboring the pHIR11 cosmid clone containing a full set of *hrp* genes of *P. syringae* pv. *syringae* strain 61<sup>11</sup>, both Pf (pHIR11 + *pthA*) and *E. coli* (pHIR11 + *pthA*) caused canker symptoms upon inoculation into citrus leaves<sup>26</sup>. Though Pf (pHIR11) reportedly elicited a hypersensitive response upon inoculation into tobacco leaves<sup>24</sup>, the double Pf transformant (pHIR11 + *pthA*) failed to elicit HR in tobacco leaves<sup>5</sup>. Thus, this Pf (pHIR11) system was thought to be useful to study the *in-planta* functions of various T3SS-dependent effectors.

#### 3. “Dual roles of *avr* genes hypothesis”

Using the Pf (pHIR11) system, the ability to suppress HR elicitation by major *avr* genes was examined. The Pf (pHIR11) transformants with each of the *avr* genes such as *avrXa7* and *avrXa10* of Xoo, *avrPto*, *avrB* except *avrRpt2* (kind present from B. Staskawicz at UC Berkeley) failed to show the elicitation of HR when inoculated into tobacco leaves<sup>5,6</sup>. As well as eliciting HR, no induction of an oxidative burst, of the syntheses of defense-related enzymes such as PAL and peroxidase and of the expression of the plant genes in resistance cascades such as *HIN1*, *RbohB*, *PAL* and *PR1* was observed<sup>27</sup>. Thus, the failure to elicit HR was considered attributable to the suppression of gene expression in the resistance cascade. As this suppressor function is completely opposite to the elicitation of resistance response commonly known as the major function of *avr* genes, many *avr* were hypothesized as having dual functions (elicitation and suppression of resistance response) depending on the genetic and physiological backgrounds of the plant<sup>5,6,27</sup>.

#### 4. Plant protein binds to a PthA effector

We thought that the determination of these dual functions of TAL effectors may be dependent, not only on specificity based on direct binding between the central region with a variable number of typically 34 aa repeats of TAL effector and its corresponding *UPA* (up regulating *avrBs3*) boxes<sup>2,18</sup> in the promoter responding to it but also on the binding with a third molecule in plant. In the case of PthA, we isolated pectin methyl esterase (PMC) of citrus as its binding protein. The PMC of citrus is translated into the precursor consisting of mature PMC and PMC-inhibitor



**Fig. 1. Change in localization of PthA after co-expression of PthA and precursor of PME from different plant sources in onion cells**

The genetic construct on the plant vector was made to produce PthA fused with a red fluorescent protein (Texas-Red) at its N-terminal end. Other genetic constructs were constructed to produce precursors of citrus, tomato and tobacco PME fused with green fluorescent dye, EGFP, at their C-terminal ends. The construct of PthA and that of each precursor PME (pre-PME) were co-expressed in onion cells and their localizations were examined under red-emission, green-emission, and overlay conditions under a confocal laser microscope. Note the change in the localization of PthA (red) only after it was co-expressed with citrus PME precursor but no change with tomato and tobacco PME precursors.

peptide. When the *PthA* gene fused to the Green Fluorescent Protein (GFP) at the N-terminal end was co-expressed in a plant cell with the Ds-Red-PMC precursor genes of either citrus, tobacco, or tomato, confocal laser microscopic observation indicated only the citrus PMC precursor but not these of tobacco and tomato bound to PthA, as judged from the nuclear localization<sup>28</sup> (Fig. 1). Accordingly, the specific binding of PthA with a PMC precursor was thought to change in the localization of PthA. This may be another way to determine specificity in the regulation of plant genes by a TAL effector.

## Concluding Remarks

From the dynamic analysis of PLs (especially PLe), we learnt that the optimum synthesis of virulence factor is regulated by relatively complex regulatory mechanisms. We now know that the virulence factor may not be simply involved in eliciting the symptom as the last event of para-

sitism. Determining this tight regulation mechanism indicates their optimum syntheses should vary at each stage of the infection. This understanding may be important when we want to dissect the infectious process.

Analysis of the second group of genes, particularly those of the T3SS-dependent effector, lead to dynamic analysis of plant genes in both resistance and pathogenicity cascades. Based on this understanding, we can now gain a deeper insight into the interaction between the pathogen and plants and find a new strategy for controlling bacterial diseases in plants.

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