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Characterization of the *hrp* Genes Cluster in *Erwinia pyrifoliae*

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Abstract:

Erwinia pyrifoliae, a shoot blight pathogen, was collected from different pear growing orchards of Chuncheon, Korea. Pathogenicity, physiological, and biochemical tests were performed on these collected strains. The strain WT3 was selected as a representative strain for the characterization of *hrp* genes cluster of *E. pyrifoliae* and for the genomic library preparation. Two thousand cosmids were screened by hypersensitive response (HR) on tobacco leaves and the cosmid pCEP33 was selected for its characterization. The cosmid pCEP33 contained homologous *hrp* elicitor genes and *hrp/hrc* genes cluster including *dspEF*, *hrpW*, *hrpN*, *hrpV*, *hrpT*, *hrcC*, *hrpG*, *hrpF* and partial *hrpE* genes. Additionally, two ORFs, ORFD and ORFE, were also determined at downstream of *dspEF* region. But, their nucleotide sequences have no homology to *hrp* genes of any other pathogenic bacteria.

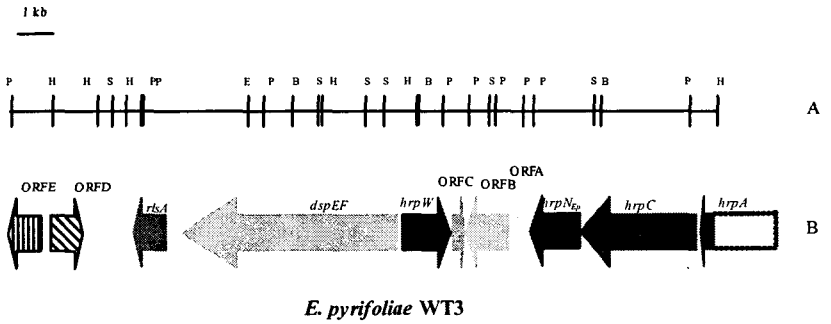
Although structure, size and orientation of transcription of most *hrp* genes were homologous to those of *E. amylovora*, the HR elicitor $hrpN_{Ep}$ gene of *E. pyrifoliae* was bigger in size than $hrpN_{Ea}$ gene due to five intergenic nucleotide fragments insertions (INFIs). The $hrpN_{Ep}$ gene produced HR elicitor protein ($HrpN_{Ep}$) with molecular mass of 44 kDa and was expressed in *Escherichia coli* under T7 promoter of vector pET-15b. Comparative HR assay on tobacco leaves showed faster and stronger HR by purified $HrpN_{Ep}$ than $HrpN_{Ea}$. These results suggested that both the $hrpN_{Ep}$ and $hrpN_{Ea}$ evolved independently at different geographical regions, although they might have originated from the same ancestral gene. Despite HR elicitation, expression of $hrpN_{Ep}$ gene could not be detected in cosmid clone pCEP33 by western blot analysis indicating its minimal expression. However, when clone pEPL2 was mobilized into the cosmid clone pCEP33, expression of $hrpN_{Ep}$ gene was observed by western blot analysis using antiserum raised against $HrpN_{Ep}$ showing the role of the *hrpL* gene in its expression. The minimal expression of $HrpN_{Ep}$ observed in the cosmid pCEP33 also supported that the *hrp* genes of *E. pyrifoliae* and *E. amylovora* might have evolved independently although their structures and functions are similar to each other.

Summary

Erwinia pyrifoliae is a phytopathogenic bacterium, which causes shoot blight disease in Asian pear tree (*Pyrus pyrifolia* cv. Singo) (1, 10). The shoot blight disease was first observed in the pear orchards of Chuncheon, Korea in 1995 (9). Disease symptoms in pear tree were similar to those of

Erwinia amylovora which causes fire blight in apple, pear, and other rosaceous plants (11). Infected shoots and leaves with necrotic disease were collected from different pear orchards from 1995 to 1998. Forty-nine strains showed their pathogenicity on immature fruit and shoot of pear and were studied for their colony morphology, physiological, and biochemical characteristics. Among these strains, detailed characterization of *E. pyrifoliae* WT3 was reported by Shrestha et al. (9). *E. pyrifoliae* WT3 was further selected for the characterization of *hrp* gene clusters since this strain was collected from the first outbreak area of Jichonri, Chuncheon in 1995.

The hypersensitive reaction and pathogenicity (*hrp*) genes, which are involved in pathogenicity and induction of hypersensitive response (HR) in non-host plants (2), are the characteristic features of most of Gram-negative phytopathogenic bacteria. The *hrp* genes have been characterized in *Erwinia amylovora*, species of *Pectobacterium*, *Pseudomonas syringae*, *Ralstonia solanacearum* and species of *Xanthomonas* (1, 3, 4, 5). These phytopathogens commonly produce HR elicitor, harpin, which was first reported in *E. amylovora* by Wei et al. (15). However, *hrp* genes and HR elicitor have not currently been reported from *E. pyrifoliae*. In order to study the genes related to pathogenicity and HR in *E. pyrifoliae*, genomic library was prepared and two thousand cosmids were screened by HR test. The cosmid pCEP33 of 19.7 kb was selected for its characterization since this cosmid showed HR when sonicated and boiled protein was infiltrated into tobacco leaves. The cosmid pCEP33 contained a cluster of the *hrp* genes including *dspEF*, *hrpW*, *hrpN*, *hrpV*, *hrpT*, *hrcC*, *hrpG*, *hrpF* and partial *hrpE* genes (Fig.1). The *hrp* genes of *E. pyrifoliae* showed highest homologies to those of *E. amylovora* and then to those of *Pantoea agglomerans*. Comparatively, low homology identities were obtained with soft rot erwinias, such as *Pectobacterium carotovorum*, and *P. chrysanthemi*. Besides *hrp* genes, two open reading frames (ORFs), ORFD and ORFE, were also identified downstream to a region containing genes homologous to *dspEF* and *rlsA*. The BLAST search analysis performed for ORFD indicated its homology to the transcriptional regulator protein (LysR) of *P. aeruginosa* PA01 and *P. syringae* pv. *tomato* strain DC3000. Another partial ORFE, which was opposite in its transcriptional orientation to ORFD, showed homology to a number of proteins, such as the putative oxidoreductase of *Streptomyces coelicolor* involved in NAD-binding, and siderophore biosynthesis including secondary metabolites biosynthesis, transport, and catabolism.



***E. pyrifoliae* WT3**

Fig.1. A 19.7 kb region of *hrp* genes cluster of *E. pyrifoliae* WT3. **A.** Physical map of cosmid clone pCEP33 showing different restriction sites. **B.** The *hrp* genes and their organization in *E. pyrifoliae*. Arrows indicate the orientation of transcription for each gene. Opened and dotted line indicates predicted structure for *hrpA* gene including partial *hrpE* gene. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; and S, *Sma*I.

All identified genes in cosmid pCEP33 had similar sizes and same transcriptional orientations as in *E. amylovora*. The major difference between *E. pyrifoliae* and *E. amylovora* was observed in their respective HR elicitor $hrpN_{Ep}$ and $hrpN_{Ea}$ genes. In *E. pyrifoliae*, the $hrpN_{Ep}$ gene was located in a 8.4 kb *Hin*dIII-*Hin*dIII fragment whereas the $hrpN_{Ea}$ gene was found in a 1.3 kb *Hin*dIII-*Hin*dIII fragment. Moreover, the $hrpN_{Ep}$ gene (1287 bp) was bigger in size than the $hrpN_{Ea}$ gene of 1212 bp due to five intergenic nucleotide fragments insertions (INFIs). These INFIs were observed when alignment was performed for $hrpN_{Ep}$ and $hrpN_{Ea}$ using the software package MegaAlign (DNASTAR, Inc., Madison, WI, USA). Because of INFIs, insertions were also observed at the amino acids level of HR elicitor $HrpN_{Ep}$. When purified HR elicitors were used for comparative HR assay at the concentrations 5 to 30 μg/ml, $HrpN_{Ep}$ showed faster and stronger HR than $HrpN_{Ea}$. These observations indicated that INFIs may have a synergic effect in the HR response on tobacco leaves and may cause the different three-dimensional (3D) structure of $HrpN_{Ep}$ to that of $HrpN_{Ea}$ by combining with the α-helical (H) and acidic (A) units, which enhanced the HR activity of $HrpN_{Ep}$. Based on these results, we concluded that the $hrpN_{Ep}$ of *E. pyrifoliae* and $hrpN_{Ea}$ of *E. amylovora* might have been evolved independently at different geographical regions, although their structures and functions are similar to each other.

Another interesting finding of this study was HR elicitation by sonicated and boiled supernatant of cosmid pCEP33. Full nucleotide sequence analysis clearly showed that pCEP33 had no regulatory genes required for regulation of *hrp* genes. In other phytopathogenic bacteria, including *E. amylovora*, mutations in *hrp* regulatory genes abolished its pathogenicity to the host plant and its ability to elicit HR in non-host plant (12, 13, 14, 16). Despite HR elicitation, expression of $HrpN_{Ep}$ was not observed by western blotting in cosmid pCEP33. In order to understand the role of the *hrpL* gene in the expression of $HrpN_{Ep}$, the *E. coli* pCEPL33 was constructed. The *E. coli* pCEPL33 was structurally the same as cosmid pCEP33, but contained an additional mobilized homologous *hrpL* gene of *E. pyrifoliae* WT3 which was cloned into expression vector pHCE IA. In contrast to the cosmid pCEP33, the clone pCEPL33 showed HR by infiltration of living cells. In addition, expression of the $HrpN_{Ep}$ was detected by western blot analysis. Therefore, we speculated that *hrpL* gene is required for enhancing regulation of the $HrpN_{Ep}$, but it could minimally express without regulatory gene at least in cosmid pCEP33.

In conclusion, the *hrp* genes of *E. pyrifoliae* and *E. amylovora* might have been evolved independently at different geographical regions, although their structures and functions are similar to each other. Besides *hrp* genes, it is believed that such independent evolution is occurring in other characters since previous study on the effects of temperature on the growth of these two pathogens showed that *E. pyrifoliae* is more cold tolerant than *E. amylovora* (8). In Korea, pears have been cultivated since the 19th century, and are important fruit crop. From the beginning of pear cultivation, neither bactericide nor any breeding program of resistant host cultivars has been used in the pear orchards for controlling shoot blight and other possible bacterial diseases. This suggests that there has been no direct selection pressure on the pathogenicity of *E. pyrifoliae*. Therefore, it is speculated

that the *hrp* genes of *E. pyrifoliae* are closer to those of the ancestral one.

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