(54) Title: NEW BIOPESTICIDE USING GENE FROM ERWINIA PYRIFOLIAEWT#3, NOVEL PATHOGEN THAT AFFECTS ASIAN PEAR TREES

(57) Abstract: The present invention relates to a biopesticide using a plant pathogen, and more particularly, to a biopesticide having an improved resistance against plant pathogens and an improved plant growth effect, which is superior to that of HrpN, a plant-sensitive protein isolated from Erwinia amylovora ATCC 15580, by using a gene isolated from WT#3 (Erwinia pyrifoliae WT#3[KCCM 10283]) thus enabling to be used as a fertilizer as well a biopesticide.
NEW BIOPESTICIDE USING GENE FROM ERWINIA PYRIFOLIAE WT#3, NOVEL PATHOGEN THAT AFFECTS ASIAN PEAR TREES

TECHNICAL FIELD

This invention relates to a novel biopesticide using a gene derived from a novel plant pathogen, Erwinia pyrifoliae WT#3 (KCCM 10283), isolated from Chunchon, Kangwon Province, Korea. This pathogen is endemic to Korea. This novel biopesticide has more effective properties, such as improved resistance to plant disease, promotion of plant growth, and insect repellency as compared to those of HrpN, a hypersensitive response inducing protein, isolated from Erwinia amylovora (ATCC15580T), which does not exist in Korea. Thus, it can be utilized as a biopesticide effective to prevent plant diseases caused by pathogens and insects and to enhance plant growth as well as a fertilizer.

BACKGROUND ART

The food shortage has been emerged as one of the most serious problems that the people are suffering around the world at present. However, the yields of crop production have been greatly decreasing due to the outbreak of harmful pests, such as pathogens and insects. Currently, chemical pesticides have been mostly used to prevent or control the spread of harmful pests. But their excessive and continued applications become responsible for inducing resistance to those chemical pesticides in pests, although rapid pesticidal effects can be demonstrated by killing the pests directly by using an easier spraying method. In addition, most effective pesticides, which are strongly toxic, have caused social problems due to serious soil and water pollution. Therefore, it is very urgent for the development of an environmentally-friendly biopesticides which do not induce any resistance to pests while having effective pesticidal activities.

The general purpose of using a biopesticide is to control pests through a direct application of antagonistic microorganism itself to plants but this is not considered very effective in controlling of the pests. Therefore, recent researches
have been progressing to control harmful pests by stimulating the self-defensive system of plants using products of antagonistic microorganisms instead of using microorganisms themselves. In other words, an essential aim of biological control is to decrease or prevent of pests by activating the self-immunological function of a plant via treatment of microorganism-derived materials to plants.


More essential plant disease resistance refers to a hypersensitive response (HR), is a rapid, localized necrosis for preventing spread of pathogens that is associated with the active defense of plants against many pathogens to stimulate their self-defensive system using some microorganism-derived materials. [Richberg, M. H., Aviv, D. H. & Dangl, J. L. 1998. Dead cells do tell tales. Curr. Poin. PlantBiol. 1:480-485]. The first method for plant disease resistance associated with HR induction is that plants mobilize their early alert system to adjacent cells infected with bacterial pathogens so that these adjacent cells can increase the resistance to pathogens. This defense system is called as ‘LAR’ (local acquired resistance).

The second method is that through the activation of defense system in non-infected parts of a plant, more potent defense system is activated against the secondary infection. Consequently, the whole plant may exert a more strongly defense system against pathogens. This defense system is called as “SAR” (systemic acquired resistance). The SAR may be sustained for several weeks or more and the plants exhibit some resistance to a variety of other unrelated pathogens [Hunt, M. D., Neuenschwander, U. H., Delaney, T. P., Weymann, K. B., Friedrich, L. B., Lawton, K. A., Steiner, H. Y. and Ryals, J. A. 1996. Recent advances in systemic acquired
resistance research a review. Gene 179:89-95].


Harpin is a common name for proteins produced from hrp gene island of plant pathogenic bacteria and one of Harpins, called HrpN, is a protein produced from hrpN gene located at hrp gene island of about 40kb of Erwinia amylovora which is not existing in Korea. When the HrpN is inoculated to host plants, such as an apple, it acts as a pathogenic factor. In contrast, when HrpN is given to non-host plants, it is recognized as a foreign compound in plants and HR is elicited.

HrpN is an acidic, heat-stable (100°C) protein with a molecular weight of 44 kDa [The molecular weight was measured in a manner such that after electrophoresis on acrylamide gel, the protein was stained with 0.025% Coomassie Blue R-250 and compared with Molecular Weight Standard of Bio-Rad Co. (Catalog# 161-0305, Bio-Rad Laboratories, 2000 Alfred Nobel Drive Hercules, CA 94547, USA)] with glycine-rich but without cysteine [Zhong-Min, W., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A. and Beer, S. V. 1992. harpin, elicitor of the

These plant defense elicitors have been sold in a variety of formulations. Since SA (Salicylic acid) has been reported as SAR elicitor, INA (2,6-dichloroisonicotinic acid) and BTH (benzothiadiazole) having a similar structure to SA were found to induce SAR and successfully registered as plant activators. Currently, Actigard™ and BION®, have been sold in US and Europe for the protection of diseases of foliage plants, tomato and tobacco. In Japan, PBZ (probenazole) under the trade name Oryzemate® has been also marketed for controlling rice blight and bacterial leaf blight [Yoshioka, K., Nakashita, H., Klessig, D. F. and Yamaguchi, I. 2001. Probenazole induces systemic acquired resistance in *Arabidopsis* with a novel type of action. Plant J. 25:149-157].

HrpN, a non-chemical protein, was first discovered in *Erwinia amylovora*, a gram-negative bacteria that cause fire blight of rosaceous plants. When it is directly sprayed to plants, it acts as the SAR elicitor which controls a variety of a plant diseases, several insects, mites and nematodes and exhibits a plant growth promoting effect by enhancing photosynthesis and nutritional absorption [Dong, H. S., Delaney, T. P., Bauer, D. W. and Beer, S. V. 1999. HrpN induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the *NIM1* gene. Plant J. 20:207-215]. Also, HrpN has little toxicity and it does not cause in any environmental pollution as it is biologically degradable and it is easy to formulate due to heat-resistance even after boiling at 100°C [Zhong-Min, W., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A. and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science 257:85-88].

Therefore, HrpN derived from *Erwinia amylovora* was successfully commercialized in 2000 by Eden Bioscience in the trade marker Messenger® in USA for controlling the diseases of crops, such as cotton, tomato, tobacco, pepper,

DISCLOSURE OF INVENTION

The inventor et al. isolated and identified some bacterial pathogens from the infected lesions of a plant showing necrosis in pear growing orchards in Chunchon, Korea and found novel species, Erwinia pyrifoliae WT#3 (KCCM 10283) which is morphologically different from recently reported Erwinia pyrifoliae by German researchers: that is, Erwinia pyrifoliae WT#3 (KCCM 10283) does not have flagella, while Erwinia pyrifoliae, which was reported by a German research team has peritrichous flagella. Also, it was different from the well-known Erwinia amylovora causing fire blight.

After isolating a gene and a protein which induce a hypersensitive response in non-host plants from novel species, Erwinia pyrifoliae WT#3 (KCCM 10283), it was noted that the protein was proven to be more effective in imparting plant disease resistance against pathogens and insects, and in enhancing plant growth than HrpN from Erwinia amylovora causing fire blight. Thus, the present invention was completed.

Therefore, an object of the present invention is to provide a novel species Erwinia pyrifoliae WT#3 (KCCM 10283) and a protein derived from the pathogen as an effective biopesticide, a plant growth activator, a seed-treating agent, an insect repellent, and a fertilizer.

A further aspect of the present invention is to provide a gene (KCCM 10282) encoding a protein or polypeptide of Erwinia pyrifoliae in a non-infectious form to plants, which induces a hypersensitive reaction to non-hosts or resistance to pathogens in plants when plant cells are in contact with or treated with the gene.

A further aspect of the present invention is to provide transformants containing a gene (KCCM 10282) encoding a protein or polypeptide in a
non-infectious form to plants from *Erwinia pyrifoliae* WT#3 (KCCM 10283).

A further aspect of the present invention is to provide a biopesticidal composition containing the protein or polypeptide and a carrier.

A further aspect of the present invention is to provide the composition which can be applied to plants as a pesticide, a plant growth activator, a seed-treating agent, insect repellent, and a fertilizer.

A further aspect of the present invention is to provide a method for producing a protein or polypeptide that induces a hypersensitive response or resistance on a mass-scale by isolating and purifying a protein or a polypeptide from cultures of *Erwinia pyrifoliae* WT#3 (KCCM 10283) and transformants containing a plant HR-inducing gene of the strain (KCCM 10282) from *Erwinia pyrifoliae* WT#3 (KCCM 10283).

The novel *Erwinia pyrifoliae* WT#3 (KCCM 10283) of the present invention was isolated from the affected stem of a plant showing necrosis of pears orchard in Chunchon (Kangwon Province, Korea). The strain was identified as the genus *Erwinia*, the same genus of the causal pathogen of fire blight, *Erwinia amylovora* and necrotic disease, *Erwinia pyrifoliae* (necrotic disease of Asian pears; *Erwinia pyrifoliae* reported by a German research team in 1999). *Erwinia amylovora* and *Erwinia pyrifoliae* are peritrichous flagella, while our pathogen *Erwinia pyrifoliae* WT#3 (KCCM 10283) is non-flagellated, showing a great morphological difference from *Erwinia pyrifoliae* which is only found in Korea. We designated the novel strain as *Erwinia pyrifoliae* WT#3 (KCCM 10283) and deposited it to the Korean Culture Center of Microorganisms on June 11, 2001 and was assigned with Accession No. KCCM 10283.

Especially, an isolated gene encoding a protein which induces a hypersensitive response in non-host plants from *Erwinia pyrifoliae* WT#3 was compared with a *hrpN* gene encoding a HrpN protein, which was discovered by Cornell University of the US and sold by Eden Bioscience co. As a result, our novel gene showed less similarity to *hrpN* gene encoding a HrpN. Notably, several
insertions of nucleotide sequences fragments were found in the gene encoding a protein which induces a hypersensitive response in non-host plants from *Erwinia pyrifoliae* WT#3 which are not present in *hrpN* gene from *E. amylovora*. More specifically, those nucleotide sequences fragments were inserted at the sites of 222-230 bp, 249-263 bp, 348-371 bp and 397-411 bp. Therefore, the protein or polypeptide produced by the gene has a different amino acid sequence and molecular weight from those of HrpN peptide.

A recombinant pKEP3 for higher expression containing the gene derived from the *Erwinia pyrifoliae* WT#3 was constructed and transformed to *Escherichia coli*. This transformant was deposited to the Korean Culture Center of Microorganisms on June 11, 2001, and was assigned with Accession No. KCCM 10282.

The high yield of a protein or a polypeptide to elicit hypersensitive response and disease resistance can be produced by mass cultivation of the *E. coli* transformant containing the expression vector having more effective properties, such as improved resistance to plant disease, promotion of a plant growth, and repellence of insect in compared to that of HrpN from *E. amylovora*.

As a result, evaluation of biological activities of the protein or polypeptide inducing hypersensitive response or plant disease resistance proved its improved effectiveness than those of HrpN in the powdery mildew of cucumber, anthracnose on pepper, blight of pepper, downy mildew of oriental melon, blight of sweet pepper, leaf blight of rice. Further, yield of harvesting showed higher cucumber, pepper, sweet pepper, and strawberry than HrpN protein. Also, the protein or polypeptide which induces a hypersensitive response in non-host plants from *Erwinia pyrifoliae* WT#3 showed an excellent in increasing the photosynthesis and the content of chlorophylls in cucumbers and peppers. Therefore, the protein or polypeptide from *Erwinia pyrifoliae* WT#3 can be applied to plants as a pesticide, a plant growth activator, and a fertilizer.

In addition, the protein or polypeptide of this invention can be applied to plants as an insect repellent (e.g., aphid) in a conventional procedure to treat stems and leaves of a plant. Further, the seeds of rice treated with a protein or a
polypeptide of this invention showed fast growth during the seeding culture period. Thus, the protein or polypeptide of this invention can be applied to plants as an insect repellent and seed-treating agent in a conventional procedure.

5 BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows TEM (Transmitted Electron Microscope) photographs of novel Erwinia pyrifoliae WT#3 (KCCM 10283) according to the present invention, Erwinia pyrifoliae Ep16\textsuperscript{T} and Erwinia amylovora ATCC 15580\textsuperscript{T}.

Fig. 2 shows the growth curves of Erwinia pyrifoliae WT#3 and Erwinia amylovora ATCC15580\textsuperscript{T} according to temperature.

Fig. 3 shows the growth curves of Erwinia pyrifoliae WT#3 and Erwinia amylovora ATCC15580 according to pH.

Fig. 4 shows the numerical analysis of Erwinia pyrifoliae WT#3 (KCCM 10283) according to the Biolag system.

Fig. 5 shows a phylogenetic analysis based on 16S rRNA gene of Erwinia pyrifoliae WT#3 (KCCM 10283) of this invention.

Fig. 6 shows the results of phylogenetic analysis of the region encoding tRNA\textsuperscript{Ala} in ITS region of Erwinia pyrifoliae WT#3 (KCCM 10283) of this invention.

Fig. 7 shows the results of phylogenetic analysis of the region encoding tRNA\textsuperscript{Glu} in ITS region of Erwinia pyrifoliae WT#3 (KCCM 10283) of this invention.

Fig. 8 shows a plasmid profile analysis of Erwinia pyrifoliae having five plasmids (WT#3, Ep1, Ep16) and Erwinia amylovora having one plasmid (ATCC15580 \textsuperscript{T}, LMG1877, LMG1946) [Lanes 1: 1kb ladder, 2: Erwinia pyrifoliae Ep1, 3: Erwinia pyrifoliae Ep16\textsuperscript{T}, 4: Erwinia pyrifoliae WT#3, 5: Erwinia amylovora ATCC15580\textsuperscript{T}, 6: Erwinia amylovora LMG1877, 7: Erwinia amylovora LMG1946].

Fig. 9 shows a hypersensitive response observed 24 hours after inoculation of the genomic library clone constructed from Erwinia pyrifoliae WT#3 (KCCM 10283) on the leaf of tobacco [B: MES buffer, C: a plant HR-inducing protein (HrpN) from Erwinia amylovora ATCC 15580\textsuperscript{T}, 1: a plant HR-inducing protein from clone1, 2: a plant HR-inducing protein from clone2, 3: a plant HR-inducing protein from pCEP33,
4: a plant HR-inducing protein from clone 4, and NC: protein from pLAFR3 vector.

Fig. 10 shows a physical map encoding the plant HR-inducing gene from the genomic library clone (pCEP33) from Erwinia pyrifoliae WT#3.

Fig. 11 shows a comparison of genes between a gene (KCCM 10282) encoding the plant HR-inducing protein from Erwinia pyrifoliae WT#3 (KCCM 10283) of the present invention and the HR-inducing gene (hrpN) from Erwinia amylovora ATCC15580 T [A: a plant HR-inducing gene from Erwinia pyrifoliae WT#3, B: a plant HR-inducing gene (hrpN) from Erwinia amylovora ATCC15580 T].

Fig. 12 shows a plant HR-inducing protein (hereinafter referred to as "Pioneer") from the gene from Erwinia pyrifoliae WT#3 which is expressed in plasmid vector pKEP3 from the gene from [M: protein size marker, 1: Pioneer with 41.1 kD, 2: HrpN with 39.7 kD, 3: pET15b vector].

Fig. 13 shows a comparison between the amino acid sequences of Pioneer and a plant HR-inducing protein (HrpN) from Erwinia amylovora ATCC15580 T [A: Pioneer, B: HrpN].

Fig. 14 shows HR on tobacco leaves treated with the Pioneer from Erwinia pyrifoliae WT#3 and HrpN from Erwinia amylovora ATCC15580 T (a control) at different concentrations.

Fig. 15 shows the disease symptoms on the surface of immature pear fruit 4 days after inoculation of the Pioneer and a buffer (control).

The following examples are intended to be illustrative of the present invention and should not be construed as limiting the scope of the invention as defined by compounds or examples herein.

EXAMPLES
Example 1: Isolation and identification of novel strain
1) Physiological/biochemical tests based on Schaad's Laboratory guide and Bergey's manual

In order to identify the pathogenic bacteria from the affected lesions by necrotic disease on pears from orchards of Chunchon (Kangwon Province, Korea),
Table 1

<table>
<thead>
<tr>
<th>Tests</th>
<th>Erwinia WT#3 10283</th>
<th>Erwinia pyrifoliae (KCCM)</th>
<th>Erwinia Ep16T</th>
<th>Erwinia pyrifoliae ATCC 15580T</th>
<th>Erwinia amylovora ATCC 15580T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decomposition of pectate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth in MS medium</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth factor</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pink pigment on YDC</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 36°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hydrogen sulfide of cysteine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indole test</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reduction of nitrate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Liquefaction of gelatin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Yellow pigment</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Motility in 3% agar</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth of mucoid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hypersensitivity to tobacco</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid production from</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salicin</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl α-d glucoside</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Melibiose</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose</td>
<td>-</td>
<td>+</td>
<td>V</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: ND, not determined; V, variable.

Overall, the analytical results showed that the physiological tests on the strain (e.g., liquefaction of gelatin, motility in 3% agar, and decomposition of pectate) were close to those of type strain Ep16T of *Erwinia pyrifoliae*. In contrast, the biochemical tests for the utilization of carbon source revealed different results, especially trehalose and L-arabinose, suggesting that the physiological and biochemical properties of the isolated strain according to the present invention were different.
from those of type strain *Erwinia pyriformiae* Ep16\(^T\) and *Erwinia amylovora* ATCC 15580\(^T\).

2) Morphological property of strain WT\#3

The morphological property of the strain WT\#3 was observed by TEM (Transmitted Electro Microscope), was different from that of recently reported *Erwinia pyriformiae* EP16\(^T\) causing necrotic disease in Asian pear trees and *Erwinia amylovora* ATCC 15580\(^T\) causing fire blight on apples and pears [Fig. 1].

As a result, *Erwinia* spp. including *Erwinia pyriformiae* EP16\(^T\) and *Erwinia amylovora* ATCC 15580\(^T\) were rod shaped with peritrichous flagella whereas the novel strain *Erwinia pyriformiae* WT\#3 was a slight oval rod shaped “without flagella”.

3) Growth of the strain WT\#3 at different temperatures

To investigate the physiological and biological properties, growth curve of *Erwinia pyriformiae* WT\#3 at different temperatures was performed and the growth was measured by turbidity using Bioscreen C.

The temperatures ranging from 12\(^\circ\)C to 39\(^\circ\)C were measured at intervals of 3\(^\circ\)C and doubling time and specific growth rate at different temperatures of *Erwinia pyriformiae* WT\#3 and *Erwinia amylovora* ATCC 15580\(^T\) were calculated [Fig. 2].

The results showed *Erwinia pyriformiae* WT\#3 has higher growth rate (27-30\(^\circ\)C) with short doubling time than that of *Erwinia amylovora*. The optimal temperature was 27\(^\circ\)C for the strain WT\#3.

At lower temperatures below 20\(^\circ\)C, *Erwinia pyriformiae* WT\#3 also showed a better growth compared to *Erwinia amylovora* ATCC15580\(^T\), suggesting that *Erwinia pyriformiae* WT\#3 is a cold tolerant because this pathogen is well adapted in the vicinity of Chunchon. Chunchon is a relatively cold area in the winter season and has different environmental conditions from that of *Erwinia amylovora* ATCC15580\(^T\).

4) Growth of the strain WT\#3 at different pH levels

To investigate the effects of pH on the growth of *Erwinia pyriformiae* WT\#3 the growth at different pH levels was measured by turbidity using Bioscreen C. The pH
ranging from pH 5.5 to 9.5 was measured at intervals of 0.5 at a constant temperature of 28°C and doubling time and specific growth rate at different pH levels of *Erwinia pyrifoliae* WT#3 and *Erwinia amylovora* ATCC15580T were calculated [Fig. 3].

The results showed that the optimal pH range for the growth of *Erwinia pyrifoliae* WT#3 was between pH 7.0 and 8.0 and its rapid growth was observed in an alkaline condition of pH 7.5 which was different from that of *Erwinia amylovora* ATCC15580T.

5) Property of strain WT#3 using Biolog system

Biolog system [BIOLOG, Hayward, CA 94545, USA] designed to monitor the utilization of 96 different carbon and nitrogen sources was applied to investigate the biochemical property of strain WT#3 in detail.

After incubation in TSA (triptic soy agar) at 28°C for 24 hours, the isolated strain was suspended to the turbidity of 63% in a solution containing 0.4% sodium chloride, 0.03% pluronic F-68, and 0.01% gellan gum and inoculated to the wells containing 96 different carbon and nitrogen sources.

Then, the strain WT#3 was further cultivated in an incubator at 35-37°C. The violet-turned as utilization of carbon and nitrogen sources were recorded using a reader and their values were numerically divided.

As shown in Fig. 4, strains ATCC15580, LMG2068, LMG1877, LMG1946 and ea246 strains (USA), which belong to *Erwinia amylovora*, were showed to be in the same group. In contrast, the strain WT#3 was in the group as *Erwinia pyrifoliae* (Ep4, Ep8, Ep16T). Therefore, it was clear that the strain WT#3 is different from the source of fire blight pathogen, *E. amylovora*.

6) Analysis of 16S rRNA gene from strain WT#3

To investigate the phylogenetic analysis of the strain WT#3, the nucleotide sequence of its 16S rRNA gene was analyzed considering the fact that it is an essential component to manage a life where its nucleotide sequence is well conserved and can be readily placed for phylogenetic analysis.
The 16S rRNA gene was amplified by PCR using fD1 primer (SEQ. ID. No. 1) and rP2 primer (SEQ. ID. No. 2) and then cloned into pGEM-T vector to analyze its nucleotide sequence.

Fig. 5 shows a phylogenetic tree prepared by a mega program [Kumar, S., Tamura, K. and Nei, M. 1993. MEGA: molecular evolutionary genetics analysis, version 1.0. The Pennsylvania State University, University Park] based on the 16S rRNA nucleotide sequence.

The strain WT#3 had similarities to Erwinia pyrifoliae Ep16\(^T\) and Erwinia amylovora 15580\(^T\) with 98.9% and 97.5% sequence identity, respectively, being more closer to Erwinia pyrifoliae than to Erwinia amylovora. Table 2 shows the similarity of 16S rRNA gene of each strain.

**Table 2**

<table>
<thead>
<tr>
<th>Category</th>
<th>Erwinia pyrifoliae WT#3</th>
<th>Erwinia pyrifoliae Ep16(^T)</th>
<th>Enterobacter pyrinus</th>
<th>Erwinia amylovora ATCC 15580(^T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erwinia pyrifoliae WT#3</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erwinia pyrifoliae Ep16(^T)</td>
<td>98.9</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacter pyrinus</td>
<td>92.7</td>
<td>92.9</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Erwinia amylovora ATCC 15580(^T)</td>
<td>97.5</td>
<td>97.7</td>
<td>91.8</td>
<td>100</td>
</tr>
</tbody>
</table>

Also, the analysis of 16s rRNA gene revealed that the strain WT#3 belongs to the group as Erwinia pyrifolia but was different from that of Erwinia amylovora ATCC 15580\(^T\), and Enterobacter pyrinus, which was previously reported to infect apples and pears in Korea several years ago.
7) Analysis for 16S-23S ITS (Intergenic Transcribed Spacer) region of strain WT#3

To analyze the ITS of the Korea-originated pathogens *Erwinia pyrifoliae* and foreign-originated *Erwinia amylovora*, 16S-23S ITS region was amplified by PCR using R16-1F primer (SEQ. ID. No. 3) and R23-1R primer (SEQ. ID. No. 4), and then cloned into pGEM-T vector to analyze the nucleotide sequence of 16S-23S ITS region.

As a result, 16S-23S ITS region was divided into two groups; *Erwinia amylovora* had three band patterns with about 1215, 970 and 720 bp in size, whereas domestic pathogen, *Erwinia pyrifoliae* had two band patterns with 970 and 720 bp in size.

Currently, all strains were showed two groups; the band pattern of 970 bp had about 70 bp region of tRNA$_{\text{Ala}}$, whereas the band pattern of 720bp had a region of tRNA$_{\text{Glu}}$, respectively.

Fig. 6 shows a phylogenic tree prepared by analyzing the nucleotide sequences of 16S-23S ITS region of both the strain *Erwinia pyrifoliae* WT#3 and *Erwinia amylovora* ATCC15580$^\text{c}$.

As a result of analyzing the nucleotide sequences of a 970 bp band encoding 70bp regions of tRNA$_{\text{Ala}}$, it was revealed that the strain WT#3 is different from *Erwinia amylovora* group and had a similarity of 47.4% to *Erwinia amylovora*. However, we could not compare with tRNA$_{\text{Ala}}$ region of *Erwinia pyrifoliae*, which was designated by German researchers because it was not registered in GenBank.

The analysis of the nucleotide sequences of a 720 bp band encoding tRNA$_{\text{Glu}}$ region showed that the strain *Erwinia pyrifoliae* WT#3 has 85.2%- 92.7% of sequence identity to that of *Erwinia pyrifoliae* reported by a German researchers, thus being suggested as the same group [Fig. 7].

8) Analysis of *Erwinia pyrifoliae* WT#3 according to plasmid profiles

The Korea-originated *Erwinia pyrifoliae* and foreign-originated *Erwinia amylovora* were divided into the following two groups [Fig. 8].

Group I: *Erwinia amylovora* (ATCC15580, LMG1877, 10296) (a plasmid > 29 kb)
Group II: *Erwinia pyrifoliae* (WT#3, Ep1, Ep16) (a plasmid > 29 kb, a plasmid 5 kb, and three plasmids 2-4 kb in size)

That is, the necrotic disease pathogens, *Erwinia pyrifoliae*, on Asian pear trees including the strain WT#3 have five plasmids, whereas fire blight pathogens, *Erwinia amylovora*, have only one plasmid, respectively.

9) Analysis for DNA relatedness according to DNA-DNA hybridization

The relatedness in whole genome between the Korea-originated *Erwinia pyrifoliae* and foreign-originated *Erwinia amylovora* were was investigated.

The purely isolated total DNA was dissolved in 100μl TE buffer to a concentration 1ng/μl, added with 10N NaOH, and then denatured by boiling at 80℃ for 10 min. Denatured DNA was applied to Hybond-N+ nylon membrane using a slot-blot apparatus. Native DNA to be used as a probe was labeled with DIG 11-dUTP by Dig-High Prime [Roche Molecular Biochemicals, Sandhofer Strasse 116, Germany], prehybridized at 49℃ for 3 hours in the presence of DNA already fixed to a nylon membrane, and then hybridized for 16 hours at the same temperature. Development of the membranes was conducted by DIG Luminescent Detection Kit [Roche Molecular Biochemicals, Sandhofer Strasse 116, Germany].

The result showed that the foreign-originated *Erwinia amylovora* (ATCC15580^T, LMG1877, LMG1946, LMG2068) belong to group I while Korea-originated *Erwinia pyrifoliae* (WT#3, Ep16^T) belong to group II.

More specifically, the foreign-originated pathogens, *Erwinia amylovora* belonged to group I but the Korea-originated pathogens, *Erwinia pyrifoliae* belonged to group II. This result clearly indicates that the Korean pathogens, *Erwinia pyrifoliae* including strain WT#3 and the foreign pathogens, *Erwinia amylovora* were distinct, suggesting that *Erwinia pyrifoliae* is native to Korea. The following table 3 shows the similarities of related strains.
Table 3

<table>
<thead>
<tr>
<th>Source of unlabeled DNA from:</th>
<th>Erwinia amylovora ATCC 15580&lt;sup&gt;T&lt;/sup&gt;</th>
<th>Erwinia pyrifoliae WT#3</th>
<th>Erwinia pyrifoliae Ep16&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erwinia amylovora</td>
<td>100</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>ATCC 15580&lt;sup&gt;T&lt;/sup&gt;</td>
<td>101</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>LMG1877</td>
<td>102</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>LMG1946</td>
<td>97</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Erwinia pyrifoliae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ep16&lt;sup&gt;T&lt;/sup&gt;</td>
<td>61</td>
<td>88</td>
<td>100</td>
</tr>
<tr>
<td>WT#3</td>
<td>45</td>
<td>100</td>
<td>102</td>
</tr>
</tbody>
</table>

From the above physiological, biochemical and genetic properties of the strain WT#3, it was noted that, in terms of physiological and biochemical properties based on Schaad's laboratory guide and Bergey's manual, temperature and pH level-related properties, Biolog system, 16S rRNA gene, 16S-23S ITS, plasmid profiles, and relatedness by DNA-DNA hybridization, the strain WT#3 was in the group of *Erwinia pyrifoliae*, which was present only in Korea in 1999, although there were a few other different properties between *Erwinia pyrifoliae* WT#3 and Ep16<sup>T</sup>.

However, since the strain WT#3 does not have flagella in morphology which is first reported in genus *Erwinia*, it appears to be different from those of pathogen, *Erwinia pyrifoliae*, which was reported by German researchers and *Erwinia amylovora*.

We designated the strain WT#3 as *Erwinia pyrifoliae* WT#3 (KCCM 10283) and deposited to the Korean Culture Center of Microorganisms on June 11, 2001. The accession number is KCCM 10283. Also, a recombinant pKEP3 for higher expression containing the gene derived from the *Erwinia pyrifoliae* WT#3 was constructed and transformed to *Escherichia coli*. This transformant was deposited to the Korean Culture Center of Microorganisms on June 11, 2001, and was assigned with Accession No. KCCM 10282.
Example 2: Properties of specific protein triggering a plant hypersensitive reaction from *Erwinia pyrifoliae* WT#3 and a gene encoding it

1) Analysis of a plant HR-inducing gene from *Erwinia pyrifoliae* WT#3

To analyze a gene encoding specific protein eliciting a plant hypersensitive response, total DNA of *Erwinia pyrifoliae* WT#3 was incubated at 37°C to ensure that it was partially digested with Sau3AI. The DNA (1 μl) was then examined for appropriate digestion on an electrophoresis at the intervals of 1 hr, and the result revealed that about 6 hours of digestion was most appropriate. Total DNA, so manipulated, was prepared as an insertion DNA and ligated into the BamHI polylinker site of cloning vector pLAFLR3 using DNA ligase by incubating at 14°C for 12 hours. A complete plasmid DNA ligated with the insertion DNA and a vector was transformed to *E. coli* (HB101) via CaCl₂ transformation. Then, *E. coli* strains were cultivated on Luria agar medium containing tetracycline (30 μg/ml) at 37°C for 24 hours and 2,000 genomic library clones were generated.

To select clones encoding the specific protein eliciting a plant hypersensitive reaction from the 2,000 genomic DNA library clones of *Erwinia pyrifoliae* WT#3, total protein was extracted from each clone as follows. The solution, so cultured in LB medium for 12 hours, was centrifuged to obtain a pellet and after suspending the pellet in the mixture containing 5 mM MES buffer and 0.1 mM PMSF, the mixture was lysed by sonication and boiled at 100°C for 10 min. Then, the supernatant only, so centrifuged, was collected and injected to the reverse sides of tobacco leaves (*Nicotiana tabacum* L. Samsun) using a syringe, which were grown with more than four true leaves with each leaf having a diameter of more than 15 cm. The necrosis of tobacco leaves 24 hours after injection was regarded as the HR and then genomic DNA library clones showing HR were selected [Fig. 9].

From the selected clone pCEP33, an 8.5 kb DNA fragment containing the HR-inducing gene was cloned to pUC19 vector and a physical map was constructed using a restriction endonuclease [Fig. 10].
Fig. 11 compares a gene (KCCM 10282) encoding the plant HR-inducing protein from *Erwinia pyrifoliae* WT#3 (KCCM 10283) of the present invention with the HR-inducing gene (*hrpN*) from *Erwinia amylovora* ATCC15580T by analyzing the nucleotide sequences from the selected gene.

From *Erwinia pyrifoliae* WT#3 of this invention, a 1287 bp gene encoding a protein of plant hypersensitive response can be obtained. The 1287 bp gene was designated as "a plant HR-inducing gene from WT#3" and its similarity to *hrpN* gene of *Erwinia amylovora* ATCC15580T (1212 bp) was investigated.

As a result, it was shown that five novel nucleotide sequences fragments were inserted to the plant HR-inducing gene of WT#3 at the sites of 222-230 bp (TTTAACCGG), 249-263 bp (TGGCGCGGTCCTGCT), 327-333 bp (TCTGGGT), 348-371 bp (CGGCATTGGCGGCGGCGGATGGTGG), and 397-411 bp (ACCGTGCGGACCTCT), thus resulting in the increase in the molecular weight of the gene. The plant HR-inducing gene from WT#3 had a low sequences similarity of 83.2% homology to *hrpN* gene of *Erwinia amylovora*.

Therefore, it demonstrated that the gene encoding the plant HR-inducing protein of *Erwinia pyrifoliae* WT#3 has a different nucleotide sequences from *hrpN* gene of *Erwinia amylovora* and also has the insertions of novel nucleotide sequences fragments, thus showing that the plant HR-inducing gene from WT#3 has a novel gene structure which is not found in *hrpN* gene of *Erwinia amylovora*.

The nucleotide sequence of the gene encoding the plant HR-inducing protein of *Erwinia pyrifoliae* WT#3 was denoted as SEQ. ID. No. 5.

2) Construction of expression vector of a plant HR-inducing protein from *Erwinia pyrifoliae* WT#3

To extract and purify a plant hypersensitive response eliciting protein on a large scale from the plant HR inducing gene of *Erwinia pyrifoliae* WT#3, an expression vector pKEP3 containing the gene was constructed as follows:

Recombinant protein expression system in *E.coli* (Novagen, Inc. Madison, WI53711 USA) was employed so as to construct the expression vector pKEP3. This
system was derived from pBR322 plasmid, where T7 promoter and operator can bind to lac repressor before the insertion site of a foreign gene. This structure can easily facilitate the expression of an inserted gene in a larger volume by T7 RNA polymerase produced in a host E.coli genome. Notably, when a substrate IPTG 3 hours after incubation is added, the combination of both lac repressor produced from lacI gene and IPTG does not repress the expression of T7 RNA polymerase and thus, a larger amount of protein is synthesized. For the selection of complete transformants, pKEP3 was constructed to contain an ampicillin-resistance gene and ampicillin can be used to the medium as a selective marker.

The gene encoding a plant HR-inducing protein from Erwinia pyrifoliae WT#3 and the plasmid of recombinant protein expression system were digested at 37°C for 12 hrs in the presence of the restriction enzymes NdeI and BamHI to generate the same 5' and 3'-end. They were then ligated into the restriction sites using DNA ligase at 14°C for 16 hrs and transferred into E.coli via CaCl₂ transformation.

pKEP3 has many advantages that (1) ampicillin-resistant gene can be used as a selected marker, (2) its possession with His tag may make an easier purification, and (3) its possession of a strong T7 lac promoter may ensures a larger volume of protein production from ligated insertion DNA.

The E. coli transformant containing the expression vector pKEP3 was deposited to the Korean Culture Center of Microorganisms on June 11, 2001 and was assigned with the Accession No. KCCM 10282.

In the present invention, hrpN gene of Erwinia amylovora ATCC15580T was cloned by using the same recombinant protein expression system (Novagen, Inc., Madison, WI 53711, USA) and used as a control for the biological test of pKEP3, which contains novel plant HR-inducing gene from Erwinia pyrifoliae WT#3.

3) Expression of a plant HR-inducing protein

To produce a large-scale protein from E. coli transformants containing pKEP3 (KCCM 10282) bacterial cells were inoculated to a LB broth by adding ampicillin (50 μg/ml) as a selective marker and chloramphenicol (33 μg/ml) for inhibiting the synthesis of other proteins produced from E.coli genome, and subcultured at 37°C
for 12 hours. Then, the bacterial transformant (KCCM 10282) of the present invention was cultured at 30°C for 7 hours using the same medium. When the O.D. of the transformant (KCCM 10282) reached 0.6 about 3 hours after the cultivation, the culture was added with 0.4 mM IPTG and cooled down to 30°C and then cultured again for 4 hours. After a total of 7 hours cultivation, the mixture was centrifuged at 6,000 rpm for 15 min. Then, the supernatant was discarded and a pellet was suspended in a solution containing 5 mM MES buffer and 0.1 mM PMSF. The suspension of transformant (KCCM 10282) was lysed by sonication until the suspension became transparent and then boiled for 10 min at 100°C. Then, the mixture was centrifuged at 15,000 rpm for 10 min and the supernatant was discarded. After adding a protein inhibitory cocktail at the ratio of 1/1,000, the mixture was filtrated by using a 0.45 μm filter and weighed the extract amount of protein.

Through the above-mentioned process, the plant HR-inducing protein producing from transformant encoding the plant HR-inducing gene (KCCM 10282) of *Erwinia pyrifoliae* WT#3 was named as ‘Pioneer’.

According to the present invention, *hrpN* gene of *Erwinia amylovora* ATCC15580T was cloned by the same recombinant protein expression system (Novagen, Inc. Madison, WI53711 USA) and used as a control for the biological test of Pioneer.

As shown in Fig. 12, it was demonstrated that both genes of *Erwinia pyrifoliae* WT#3 and *Erwinia amylovora* ATCC15580T encoding plant HR-inducing protein were successfully expressed by the recombinant protein expression system and were synthesized a large volume of a plant HR-inducing proteins.

4) Analysis for the similarity of plant HR-inducing protein (Pioneer)

The similarity of Pioneer was compared with the HR-inducing protein of *Erwinia amylovora* ATCC15580T by analyzing the amino acid sequence of purified Pioneer [Fig. 13].

As a result, it was noted that novel protein domains of Pioneer were produced from the N-terminal at the sites of 76-79 (Thr-Gly-Leu-Leu),
88-92 (Leu-Gly-Gly-Gly-Ser), 102-113 (Gly-Leu-Gly-Gly-Leu-Gly-Gly-Asp-Leu-Gly-Ser-Thr), and 131-137 (Gly-Ala-Thr-Val-Gly-Thr-Ser).

The Pioneer had a low amino acid sequence identity of 85.9% homology to that of HrpN. The molecular weight of Pioneer was 41.1 kDa as compared with 39.7 kDa of HrpN. The molecular weight of both Pioneer and HrpN was not compared with the molecular weight standards on an acrylamide gel but a molecular weight of each amino acid deduced from the nucleotide sequence of gene using Winstar program.

Therefore, Pioneer has a novel type of a protein by the insertion of novel peptide domains and such difference is expected to bring about much improved biological activity, which cannot be found in that of HrpN.

5) Hypersensitive response of a plant HR-inducing protein (Pioneer)

Until recently, the plant HR-inducing protein is known as a pathogenic factor in host plants but induces the HR in non-host plants.

Pioneer from Erwinia pyrifoliae WT#3 and HrpN from Erwinia amylovora ATCC15580F were inoculated onto tobacco (non-host plants) leaves using a syringe, together with MES buffer (protein lysis buffer) as a control [Fig. 14].

As shown in Fig. 14, it was noted that Pioneer and HrpN exhibited a clear HR in the front tobacco leaf at the dose of 10 µg/ml and 20 µg/ml, respectively; in the same reverse tobacco leaf, Pioneer and HrpN exhibited a clear HR at the dose of 5 µg/ml and 10 µg/ml, respectively. This reflected that Pioneer induces HR at a relatively lower concentration than that of HrpN.

As shown in the following table 4, it revealed that when Pioneer was inoculated at the dose of 5 µg/ml and 10 µg/ml, a clear HR was observed at 24 hours and 14 hours after inoculation, respectively, whereas HrpN induced a clear HR at 48 hours and 18 hours after inoculation at same concentration, respectively. This demonstrates that Pioneer exhibited more rapid HR than HrpN by 24 hours at of 5 µg/ml while Pioneer exhibited more rapid HR than HrpN by 4 hours at 10 µg/ml.
respectively.

This test was repeatedly performed three times and the result suggests that Pioneer induces the immune system of a plant at a lower dose and faster than HrpN.

### Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>After 14 hrs</th>
<th>After 18 hrs</th>
<th>After 24 hrs</th>
<th>After 36 hrs</th>
<th>After 48 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pioneer</td>
<td>HrpN</td>
<td>Pioneer</td>
<td>HrpN</td>
<td>Pioneer</td>
</tr>
<tr>
<td>30 µg/ml</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>20 µg/ml</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++/2</td>
<td>++</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>+/2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>5 µg/ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/2</td>
<td>-</td>
</tr>
<tr>
<td>MES</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

6) Pathogenicity test on pear of plant HR-induced protein (Pioneer)

Purified Pioneer at a dose of 500 µg/ml was inoculated to the surface of an immature fruit by punching it a hole (0.5 mm in diameter and 10 mm in depth).

As shown in Fig. 15, the surface of the immature fruit turned black as a progressive symptom 4 days after treatment, compared with that of a control. Therefore, Pioneer might have a potent pathogenicity on pears although it needs a further investigation.

As mentioned above, the properties of Pioneer and a gene encoding it can be summarized as follows.

(1) The gene encoding Pioneer showed that several novel nucleotide sequences fragments are inserted to the several sites of Pioneer gene, which are not found in hrpN gene; the size of Pioneer gene was 1,287 bp as compared with that of the gene of hrpN (1,212 bp).

(2) In the protein structure, the molecular weight of Pioneer (41.1 kD) with novel peptide domains was larger than HrpN (39.7 kD) [The molecular weight of both Pioneer and HrpN were not compared with molecular weight standards on an acrylamide gel but their molecular weight for each amino acid were deduced from the nucleotide sequences of a gene using Winstar program].
(3) In the HR observed in tobacco leaves, it was noted that Pioneer induces the immune system of plants at a lower dose and faster than HrpN.

Therefore, it is clear that Pioneer is more suitable than HrpN from Erwinia amylovora for the development of a better biopesticide eliciting the excellent HR.

Example 3: The study of biological activity using plant HR-inducing protein (Pioneer)

1) Control effect against powdery mildew (Sphaerotheca fuliginea) of cucumbers

To evaluate the control effect of Pioneer against powdery mildew (Sphaerotheca fuliginea), a gene encoding the plant HR-inducing protein was cloned into pKEP3 vector to purify the protein.

Cucumbers were cultivated by the conventional "rain-protecting" method prevailing in agricultural farms. The test materials were applied based on the 3-time repeated randomized complete block design. The stems and leaves of cucumbers were treated with the HR-inducing protein, Pioneer, at a dose of 20 µg/ml according to the recommended dose by EDEN Bioscience corporation.

EDEN Bioscience's Messenger® was used as a control at the same dose above, together with locally produced Fenarimol (chemical pesticide) based on the instruction in use. The treatment methods are as follows:

① Treatment by three times: Pioneer, Messenger® and Fenarimol were sprayed to the stems and leaves of cucumber three times each at intervals of 10 days after early stage of powdery mildew (Sphaerotheca fuliginea).

② Treatment by four times: Pioneer, Messenger® and Fenarimol were sprayed to the stems and leaves of cucumber three times each at the implantation of day 7, 21, 35, and 49.

The disease severity of powdery mildew (Sphaerotheca fuliginea) from the upper 8 leaves to the bottom 3 leaves of cucumbers was measured 7 days after treatment based on the following criteria (0: no disease, 1: 1-5%, 2: 5.1-20%, 3: 20.1-40%, 4: more than 40%).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease Severity (%)</th>
<th>Control effect (%)</th>
<th>Increase of control effect; Pioneer vs. Messenger® (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 time</td>
<td>2 times</td>
<td>3 times</td>
</tr>
<tr>
<td>Pioneer (4 times)</td>
<td>32.5</td>
<td>25.7</td>
<td>27.5</td>
</tr>
<tr>
<td>Messenger® (4 times)</td>
<td>44.0</td>
<td>42.3</td>
<td>40.3</td>
</tr>
<tr>
<td>Fenarimol (4 times)</td>
<td>13.8</td>
<td>12.8</td>
<td>15.6</td>
</tr>
<tr>
<td>Pioneer (3 times)</td>
<td>32.9</td>
<td>26.7</td>
<td>26.4</td>
</tr>
<tr>
<td>Messenger® (3 times)</td>
<td>43.9</td>
<td>44.5</td>
<td>45.8</td>
</tr>
<tr>
<td>Fenarimol (3 times)</td>
<td>9.7</td>
<td>5.4</td>
<td>10.6</td>
</tr>
<tr>
<td>Non treatment</td>
<td>52.9</td>
<td>56.0</td>
<td>51.0</td>
</tr>
</tbody>
</table>

As shown in the table 5, Pioneer is expected to be used as an excellent pesticide, since both three-time and four-time treatments showed that its control effects were increased by 122.6% and 187.0% as compared with Messenger®.

2) Enhanced production of cucumbers

To ascertain the enhanced production of cucumbers with the treatment of Pioneer, the stems and leaves of cucumbers were treated with Pioneer at a dose of 20 μg/ml 5 times each at intervals of 14 days from day 7 before implantation. HrpN, a
control, was also given to the stems and leaves of cucumbers at the same dose as aforementioned.

The cucumbers for this experiment were harvested at the implantation of day 8, 10, 12, 14, 16, 18, 21, 23, 25, and 30. Under the judgment that the cucumbers with 20 cm in length can be commercialized, the results are indicated as a marketable rate as follows.

**Table 6**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 8</th>
<th>Day 10</th>
<th>Day 12</th>
<th>Day 14</th>
<th>Day 16</th>
<th>Day 18</th>
<th>Day 21</th>
<th>Day 23</th>
<th>Day 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pioneer</td>
<td>*27/31</td>
<td>19/27</td>
<td>13/15</td>
<td>14/25</td>
<td>12/17</td>
<td>11/14</td>
<td>26/27</td>
<td>16/22</td>
<td>21/22</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 30</th>
<th>Marketable rate (%)</th>
<th>Increase of yield; Pioneer vs. non-treatment (%)</th>
<th>Increase of yield; Pioneer vs. HrpN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pioneer</td>
<td>16/25</td>
<td>77.8</td>
<td>8.1</td>
<td>4.6</td>
</tr>
<tr>
<td>HrpN</td>
<td>18/24</td>
<td>74.4</td>
<td>3.3</td>
<td>-</td>
</tr>
<tr>
<td>Non-treatment</td>
<td>13/20</td>
<td>72.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Note: marketable fruit/total fruit*

From the above tables, it was noted that the marketable rate treated with Pioneer was increased by 8.1% as compared to that of non-treatment, and 4.6% as compared to that of HrpN.

Therefore, Pioneer can be more effectively used as a plant growth activator and a fertilizer than HrpN.

3) Increase of contents in photosynthesis and chlorophyll of cucumbers

To ascertain the physiological reaction of cucumbers, namely, the increase of
contents in photosynthesis and chlorophyll of cucumbers, when treated with Pioneer, the stems and leaves of cucumbers were treated with Pioneer at a dose of 20 μg/ml 5 times each at intervals of 14 days from day 7 before implantation. HrpN, a control, was also given to the stems and leaves of cucumbers at the same dose as aforementioned.

The cucumbers for this experiment were harvested at the implantation of day 34, 42 and 56 and investigated using a portable photosynthesis device (LCA-4 system, ADC BioScientific Ltd., UNK; light source: 1,500 μmole) and a chlorophyll device (Chlorophyll meter SPAD-502, Minolta, Japan).

**Table 7**

<table>
<thead>
<tr>
<th>Category</th>
<th>Date of measurement</th>
<th>1 time</th>
<th>2 times</th>
<th>3 times</th>
<th>Average</th>
<th>Increase of contents; Pioneer vs. non-treatment (%)</th>
<th>Increase of contents; Pioneer vs. HrpN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>*P/L</td>
<td>P/L</td>
<td>P/L</td>
<td>P/L</td>
<td>P/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contents of photosynthesis</td>
<td>Non-treatment</td>
<td>17.2/</td>
<td>15.7/</td>
<td>14.1/</td>
<td>15.67/</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(CO2 μmole m² s⁻¹)/chlorophyll</td>
<td>Pioneer</td>
<td>20.7/</td>
<td>17.4/</td>
<td>16.5/</td>
<td>18.20/</td>
<td>16.2/</td>
<td>9.8/</td>
</tr>
<tr>
<td>SPAD-502 unit</td>
<td>HrpN</td>
<td>18.7/</td>
<td>16.4/</td>
<td>14.6/</td>
<td>16.57/</td>
<td>5.7/</td>
<td>-</td>
</tr>
</tbody>
</table>

*Note: P, contents of photosynthesis; C, contents of chlorophyll

From the above table, it was noted that when cucumbers was treated with Pioneer, the contents of photosynthesis and chlorophyll were increased by 16.2% and 5.4%, respectively, vs. non-treatment and 9.8% and 2.0%, respectively vs. HrpN.

Therefore, Pioneer may be effectively used as a plant growth activator and a fertilizer than HrpN.
4) Control effect against blight of pepper (*Phytophthora capsici*)

To evaluate the control effect of Pioneer against blight of pepper (*Phytophthora capsici*), a gene encoding the plant HR-inducing protein was cloned into pKEP3 vector to purify the protein. Peppers were cultivated by the conventional "open field culture" method prevailing in agricultural farms. The stems and leaves of pepper were treated with Pioneer at the doses of 10, 20 and 40 µg/ml. HrpN, a control, was also given to the stems and leaves of pepper at the same dose as aforementioned. The treatment was performed as follows:

① At the intervals of day 8 and 12 after implantation, the stems and leaves of peppers were treated with each concentration of both Pioneer and HrpN. *Phytophthora capsici* (2×10⁶ cells/ml) was inoculated to peppers, so treated, and their disease severity was measured 63 days after treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease Severity (%)</th>
<th>Control effect (%)</th>
<th>Increase of control effect; Pioneer vs. HrpN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 time</td>
<td>2 times</td>
<td>3 times</td>
</tr>
<tr>
<td>Pioneer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10 µg/ml)</td>
<td>25.0</td>
<td>27.2</td>
<td>24.8</td>
</tr>
<tr>
<td>HrpN</td>
<td>56.3</td>
<td>49.2</td>
<td>52.8</td>
</tr>
<tr>
<td>(40 µg/ml)</td>
<td>82.1</td>
<td>79.8</td>
<td>85.0</td>
</tr>
<tr>
<td>Non-treatment</td>
<td>82.1</td>
<td>79.8</td>
<td>85.0</td>
</tr>
</tbody>
</table>
As shown in table 8, it was noted that Pioneer (10 \( \mu g/\text{ml} \)) exhibited better control effect than HrpN (40 \( \mu g/\text{ml} \)).

Therefore, Pioneer can be effectively used as a pesticide at a lower dose than HrpN protein.

5) Control effect against anthracnose of pepper (*Colletotrichum orbiculare*)

To evaluate the control effect of Pioneer against anthracnose of pepper (*Colletotrichum orbiculare*), a gene encoding the plant HR-inducing protein was cloned into pKEP3 vector to purify the protein. Peppers were cultivated by the conventional "open field culture" method prevailing in agricultural farms. The stems and leaves of peppers were treated with Pioneer at a dose of 10 \( \mu g/\text{ml} \) 5 times at the implantation of day 14 from day 7 before treatment. HrpN, a control, was also given to the stems and leaves of peppers at the same dose. The treatment was performed as follows:

1. At the implementation of day 8 and 12, the stems and leaves of peppers were treated with each concentration of both Pioneer and HrpN. At the implementation of day 20, 27, 34 and 40, implanted peppers, so treated, were indicated as health and unhealthy fruits.

### Table 9

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 20 healthy/ unhealthy</th>
<th>Day 27 healthy/ unhealthy</th>
<th>Day 34 healthy/ unhealthy</th>
<th>Day 40 healthy/ unhealthy</th>
<th>Rate of fruit infection (%)</th>
<th>Control effect (%)</th>
<th>Increase of control effect; Pioneer vs. HrpN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pioneer</td>
<td>8/4</td>
<td>9/3</td>
<td>10/6</td>
<td>5/2</td>
<td>31.9</td>
<td>52.8</td>
<td>14.3</td>
</tr>
<tr>
<td>HrpN</td>
<td>5/3</td>
<td>7/4</td>
<td>8/5</td>
<td>8/4</td>
<td>36.4</td>
<td>46.2</td>
<td></td>
</tr>
<tr>
<td>Non-treatment</td>
<td>3/5</td>
<td>2/6</td>
<td>4/6</td>
<td>2/6</td>
<td>67.6</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

As shown in table 9, Pioneer exhibited better control effect against anthracnose of pepper (*Colletotrichum orbiculare*) than HrpN by 14.3%. Therefore, Pioneer can be effectively used as a pesticide than HrpN.
6) Enhanced production of peppers

To ascertain the enhanced production of peppers with the treatment of Pioneer, Pioneer was sprayed 5 times to the stems and leaves of peppers at a dose of 10 μg/ml at implantation of day 8 and 12. HrpN, a control, was also inoculated to the stems and leaves of peppers at the same dose. The treatment was performed as follows:

① Immersion plus spray method: Peppers were immersed in both Pioneer and HrpN at a dose of 10 μg/ml and immersed at 28°C for 24 hours. Then, these peppers were sowed in a pot with soil, grown for 46 days and planted in an open field. At the implementation of day 8 and 12, the stems and leaves of peppers were sprayed with both Pioneer and HrpN protein. The peppers for this experiment were harvested at the implantation of day 18, 25, 32, 39 and 46.

### Table 10

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 18</th>
<th>Day 25</th>
<th>Day 32</th>
<th>Day 39</th>
<th>Day 46</th>
<th>Total</th>
<th>Increase of yield; Pioneer vs. non-treatment (%)</th>
<th>Increase of yield; Pioneer vs. HrpN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pioneer's immersion and spray</td>
<td>19</td>
<td>12</td>
<td>21</td>
<td>16</td>
<td>24</td>
<td>92</td>
<td>84.0</td>
<td>22.7</td>
</tr>
<tr>
<td>HrpN's immersion and spray</td>
<td>16</td>
<td>12</td>
<td>16</td>
<td>10</td>
<td>21</td>
<td>75</td>
<td>50.0</td>
<td></td>
</tr>
<tr>
<td>Non-treatment</td>
<td>10</td>
<td>9</td>
<td>13</td>
<td>9</td>
<td>9</td>
<td>50</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
As shown in table 10, it was noted that Pioneer exhibited better production by 22.7% than HrpN.

Therefore, Pioneer can be more effectively used as a plant growth activator and a fertilizer than HrpN.

7) Increase of contents in photosynthesis and chlorophyll of peppers

To ascertain the physiological reaction of cucumbers, namely, the increase of contents in photosynthesis and chlorophyll of peppers, when treated with Pioneer, the stems and leaves of peppers were treated with Pioneer at a dose of 20 \( \mu \text{g/ml} \) 5 times at the intervals of 14 days from day 7 before treatment. HrpN, a control, was also given to the stems and leaves of peppers at the same dose as aforementioned.

The peppers for this experiment were harvested at the implantation of day 34, 42 and 56 and investigated using a portable photosynthesis device (LCA-4 system, ADC BioScientific Ltd., UNK; light source: 1,500 \( \mu \text{mole} \)) and a chlorophyll device (Chlorophyll meter SPAD-502, Minolta, Japan).

**Table 11**

<table>
<thead>
<tr>
<th>Category</th>
<th>Date of measurement</th>
<th>1 time</th>
<th>2 times</th>
<th>3 times</th>
<th>Average</th>
<th>Increase of contents; Pioneer vs. non-treatment (%)</th>
<th>Increase of contents; Pioneer vs. HrpN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>*P/L</td>
<td>P/L</td>
<td>P/L</td>
<td>P/L</td>
<td>P/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contents of photosynthesis</td>
<td>Non-treatment</td>
<td>14.3/57.2</td>
<td>15.3/60.9</td>
<td>12.3/57.3</td>
<td>13.97/58.47</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(CO2 ( \mu \text{mole m}^{-2} \text{s}^{-1} ))/chlorophyll</td>
<td>Pioneer</td>
<td>16.1/60.5</td>
<td>16.8/63.8</td>
<td>15.7/62.8</td>
<td>16.20/62.37</td>
<td>16.0/6.7</td>
<td>7.5/4.9</td>
</tr>
<tr>
<td>SPAD-502 unit</td>
<td>HrpN</td>
<td>15.8/58.4</td>
<td>16.4/62.6</td>
<td>13.0/57.4</td>
<td>15.07/59.47</td>
<td>7.9/1.7</td>
<td>-</td>
</tr>
</tbody>
</table>

*Note: P, contents of photosynthesis; C, contents of chlorophyll
From the above table, it was noted that when peppers was treated with Pioneer, the contents of photosynthesis and chlorophyll were increased by 16.0% and 6.7%, respectively, vs. non-treatment and 7.5% and 4.9%, respectively vs. HrpN.

Therefore, Pioneer can be more effectively used as a plant growth activator and a fertilizer than HrpN.

8) Control effect against downy mildew of oriental melon (*Pseudoperonospora cubensis*)

To evaluate the control effect of Pioneer against downy mildew of oriental melon (*Pseudoperonospora cubensis*), a gene encoding the plant HR-inducing protein was cloned into pKEP3 vector to purify the protein. The stems and leaves of oriental melons were treated with Pioneer at a dose of 40 μg/ml 5 times. HrpN, a control, was also given to the stems and leaves of oriental melons at the same dose. The treatment was performed as follows:

① Immersion plus spray method: Oriental melon seeds were immersed in both Pioneer and HrpN protein at a dose of 10 μg/ml for 24 hours. Then, these seeds were sowed in a pot. At the implementation of day 17 and 28, the stems and leaves of melons were treated with both Pioneer and HrpN.

② Spray method: At the implementation of day 17 and 28, the stems and leaves of oriental melons were treated with both Pioneer and HrpN.

The disease severity of downy mildew was measured 55 days after treatment.

**Table 12**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>Minor</th>
<th>Moderate</th>
<th>Severe</th>
<th>Most severe</th>
<th>Disease Severity (%)</th>
<th>Control effect (%)</th>
<th>Increase of control effect; Pioneer vs. HrpN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spray with Pioneer</td>
<td>17</td>
<td>13</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>16.1</td>
<td>68.6</td>
<td>96.0</td>
</tr>
<tr>
<td>Immersion with</td>
<td>18</td>
<td>14</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>12.5</td>
<td>75.6</td>
<td>44.6</td>
</tr>
</tbody>
</table>
As shown in Table 12, it was noted that both the spray and its immersion plus spray of Pioneer exhibited better control effect than those of HrpN by 96.0% and 44.6%, respectively. Therefore, Pioneer can be more effectively used as a pesticide than HrpN.

9) Control effect against blight of sweet pepper (*Phytophthora capsici*)

To evaluate the control effect of Pioneer against blight of sweet pepper (*Phytophthora capsici*), a gene encoding the plant HR-inducing protein was cloned into pKEP3 vector to purify the protein.

Sweet peppers were implanted to a 25 cm port and cultivated at a glass house. The stems and leaves of sweet peppers were treated with Pioneer at a dose of 20 µg/ml, according to the recommended dose by EDEN Bioscience corporation. HrpN, a control, was also given to the stems and leaves of sweet peppers at the same dose. The treatment was performed as follows:

1. 8 days after implantation, the stems and leaves of sweet peppers were treated with both Pioneer and HrpN. *Phytophthora capsici* (2 × 10^6 cells/ml) was inoculated to sweet peppers, so treated, and disease severity was measured 45 days after treatment.

**Table 13**
As shown in table 13, it was noted that Pioneer exhibited better control effect than HrpN by 87.1%.

Therefore, Pioneer can be more effectively used as a pesticide than HrpN.

5 10) Enhanced production of sweet peppers

To ascertain the enhanced production of sweet peppers treated with Pioneer, the stems and leaves of sweet peppers were treated with Pioneer at a dose of 40 μg/ml 8 days after implantation. HrpN, a control, was also given to the stems and leaves of sweet peppers at the same dose.

Sweet peppers were implanted to a 25 cm port and cultivated at a glass house. Sweet peppers, so treated, were investigated at the implantation of day 41 and 45.

Table 14

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 41 (ea)</th>
<th>Day 45 (ea)</th>
<th>Total (ea)</th>
<th>Increase of yield; Pioneer vs. non treatment (%)</th>
<th>Increase of yield; Pioneer vs. HrpN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pioneer</td>
<td>42</td>
<td>48</td>
<td>90</td>
<td>69.8</td>
<td>21.6</td>
</tr>
<tr>
<td>HrpN</td>
<td>30</td>
<td>44</td>
<td>74</td>
<td>39.6</td>
<td>-</td>
</tr>
<tr>
<td>Non-treatment</td>
<td>27</td>
<td>26</td>
<td>53</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

As shown in table 14, it was noted that Pioneer exhibits better enhanced production effect of sweet peppers than HrpN by 21.6%.

Therefore, Pioneer can be more effectively used as a plant growth activator
and a fertilizer than HrpN.

11) Enhanced production of strawberries

To ascertain the enhanced production of strawberries treated with Pioneer, Pioneer, the stems and leaves of strawberries cultivated at a green house was treated with Pioneer at a dose of 20 \( \mu g/ml \). HrpN, a control, was also given to the stems and leaves of green peppers at the same dose.

At the implantation of day 30, 33, 38, 41, 48 and 55, strawberries were harvested and indicated as total weight value (g).

Table 15

| Treatment         | Day 30 (g) | Day 33 (g) | Day 38 (g) | Day 41 (g) | Day 48 (g) | Day 56 (g) | Average | Increase of yield; Pioneer vs. non-treatment (%) | Increase of yield; HrpN (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pioneer</td>
<td>348.0</td>
<td>139.9</td>
<td>316.0</td>
<td>195.9</td>
<td>279.1</td>
<td>0</td>
<td>213.2</td>
<td>21.1</td>
<td>13.8</td>
</tr>
<tr>
<td>HrpN</td>
<td>418.7</td>
<td>224.0</td>
<td>242.1</td>
<td>83.2</td>
<td>150.6</td>
<td>5.32</td>
<td>187.3</td>
<td>6.4</td>
<td>-</td>
</tr>
<tr>
<td>Non-treatment</td>
<td>408.9</td>
<td>220.7</td>
<td>251.8</td>
<td>82.1</td>
<td>92.8</td>
<td>0</td>
<td>176.1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

As shown in table 14, it was noted that Pioneer exhibited better facilitated production effect of strawberries than HrpN by 13.8%.

Therefore, Pioneer can be more effectively used as a plant growth activator and a fertilizer than HrpN.

12) Control effect against blight of rice (*Magnaporthe grisea*)

To evaluate the control effect of Pioneer against blight of rice (*Magnaporthe grisea*), a gene encoding the plant HR-inducing protein was cloned into pKEP3 vector
to purify the protein.

Rice was cultivated by the conventional "open field culture" method prevailing in agricultural farms. The stems and leaves of rice were treated with Pioneer at the doses of 10 μg/ml, and immersed at 28°C for 24 hours. Rice seeds were sowed to a seedbed, grown for 16 days and implanted to a test field.

Then, the stems and leaves of rice were sprayed with Pioneer at the intervals of day 45 and 52. HrpN, a control, was also given to the stems and leaves of rice at the same dose and method as aforementioned. The disease severity was measured 85 days after treatment.

**Table 16**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease Severity (%)</th>
<th>Control rate (%)</th>
<th>Increase of control effect; Pioneer vs. HrpN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 time</td>
<td>2 times</td>
<td>3 times</td>
</tr>
<tr>
<td>Pioneer (10 μg/ml)</td>
<td>10.5</td>
<td>13.0</td>
<td>12.0</td>
</tr>
<tr>
<td>HrpN (20 μg/ml)</td>
<td>37.5</td>
<td>11.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Non-treatment</td>
<td>38.0</td>
<td>26.0</td>
<td>37.0</td>
</tr>
</tbody>
</table>

As shown in table 16, it was noted that Pioneer (10 μg/ml) exhibited better control effect than HrpN (20 μg/ml).

Therefore, Pioneer can be more effectively used as a pesticide at a lower dose than HrpN.

13) Repellent effect against aphids
To evaluate the repellent effect of Pioneer against aphis, a gene encoding the plant HR-inducing protein was cloned into pKEP3 vector to purify the protein.

Cucumbers were selected as host plants of aphis.

Cucumbers were cultivated by the conventional "rain-protecting" method prevailing in agricultural farms. The stems and leaves of cucumbers were treated with Pioneer at a dose of 20 μg/ml. HrpN, a control, was also given to the stems and leaves of cucumbers at the same dose.

When the height of cucumbers was 1m, the stem and lead of cucumbers were treated with both Pioneer and HrpN.

The number of naturally occurring aphis at cucumbers was counted 7 days after treatment.

**Table 17**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of aphis</th>
<th>Control rate (%)</th>
<th>Increase of control effect; Pioneer vs. HrpN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 time</td>
<td>2 times</td>
<td>3 times</td>
<td>Average</td>
</tr>
<tr>
<td>Pioneer 10 μg/ml</td>
<td>9</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>HrpN 20 μg/ml</td>
<td>16</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>Non-treatment</td>
<td>42</td>
<td>35</td>
<td>52</td>
</tr>
</tbody>
</table>

As shown in table 17, it was noted that Pioneer had better repellent effect against aphis at a lower dose than HrpN by 29.4%.

Therefore, Pioneer can be more effectively used as a repellent than HrpN.
14) Enhancement effect of growth in seeding culture of rice

To ascertain the enhancement effect of growth in seeding culture of rice treated with Pioneer, a gene encoding the plant HR-inducing protein was cloned into pKEP3 vector to purify the protein.

The stems and leaves of rice were treated with Pioneer at the doses of 10, 20 and 40 μg/ml, diluted at MES buffer and immersed at 28°C for 24 hrs. Rice seeds were sowed to a seedbed, grown for 16 days and implanted to a test field. HrpN, a control, was also given to the stems and leaves of rice at the same dose and method as aforementioned.

As a result, better growth in seeding culture of rice treated with Pioneer was observed by about 3~4 cm vs. non-treatment and 1 cm higher than HrpN.

A higher dose (40 μg/ml) of Pioneer was required for better enhancement of rice's growth.

Therefore, Pioneer can be more effectively used as a seed-treating agent which can promote the growth of rice seeds than HrpN.

INDUSTRIAL APPLICABILITY

As mentioned above, a novel Erwinia pyrifoliae WT#3 (KCCM 10283) according to the present invention is isolated and identified, and a novel protein (Pioneer) or a polypeptide translated from a plant HR-inducing gene (KCCM 10282) of this strain shows that Pioneer has improved properties in inducing plant resistance, plant growth promotion, insect repellent effect, increase of photosynthesis and chlorophyll, and seed treatment effect than a HrpN from Erwinia amylovora ATCC15580®. The former readily elicits the plant hypersensitive response at a lower dose and faster than HrpN, thus, the protein from Erwinia pyrifoliae WT#3 (KCCM 10283) is quite suitable for the development of a novel and better plant hypersensitive reaction eliciting biopesticide.

Therefore, this invention is advantageous in developing a novel and improved biopesticide and a fertilizer.
BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To: ChunKeum Lim
Anshol 1 Seomyun, Chuncheon,
Kangwon-Do 200 832, Korea

RECEIPT IN THE CASE OF AN ORIGINAL issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITORIAL AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

| Identification reference given by the DEPOSITOR: Escherichia coli (pKEP3) | Accession number given by the INTERNATIONAL DEPOSITORIAL AUTHORITY: KCCM-10282 |

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

- [ ] a scientific description
- [ ] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I above, which was received by it on Jun. 11, 2001. (date of the original deposit)¹

IV. INTERNATIONAL DEPOSITORIAL AUTHORITY

| Name: Korean Culture Center of Microorganisms | Signature(s) of person(s) having the power to represent the International Depository Authority |
| Address: 361-221, Yurim B/D Hongje-dong, Seodaemun-gu SEOUL 120-091 Republic of Korea | Authority of authorized official(s): |
| Date: Jun. 18, 2001 |

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired: where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

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Sole page

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INTERNATIONAL FORM

To: ChunKyun Lim

Author: I Seomyun, Chuncheon, Kangwon-Do 200-832, Korea

RECEIPT IN THE CASE OF AN ORIGINAL
Issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
Identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the
DEPOSITOR:

Pseudomonas putidae WTK#3

Accession number given by the
INTERNATIONAL DEPOSITARY AUTHORITY:

KCCM-10283

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

☐ a scientific description
☐ a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Jun. 11, 2001. (date of the original deposit)

IV. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Korean Culture Center of Microorganisms

Address: 361 221, Yurim B/D
Hongje 1 dong,
Seodaemun gu
SEOUL 120-091
Republic of Korea

Signature(s) of person(s) having the power to represent the International Depositary Authority of the deposit:

[Signature]

Date: Jun. 18, 2001

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired: where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.
CLAMS

What is claimed is:

1. Erwinia pyrifoliae WT#3 (KCCM 10283), an Erwinia pathogen effective in controlling plant diseases and activating plant growth.

2. A gene (KCCM 10282) encoding a protein or a polypeptide in a non-infectious form originated from Erwinia pyrifoliae which induces a hypersensitive reaction or resistance to pests in plants when plant cells are in contact with or treated by said gene.

3. A gene (KCCM 10282) according to claim 2, wherein said DNA molecule has a nucleotide sequences of No. 5.

4. A gene according to claim 2, wherein said protein or polypeptide has amino acid sequences of No. 6.

5. A gene according to claim 2, wherein said Erwinia pyrifoliae is Erwinia pyrifoliae WT#3 (KCCM 10283).

6. An expression system containing the gene according to claim 2.

7. An expression system according to claim 6, wherein the gene has a nucleotide sequences of No. 5.

8. Transformants containing the gene according to claim 2.

9. Transformants according to 8, wherein said transformants are selected from the group consisting of bacteria and plants.

10. A protein or polypeptide in a non-infectious form derived from Erwinia pyrifoliae which induces a hypersensitive reaction or resistance to pathogens in plants when in contact with plant cells.

11. The protein or polypeptide according to claim 10, wherein said Erwinia pyrifoliae is Erwinia pyrifoliae WT#3 (KCCM 10283).

12. The protein or polypeptide according to claim 10, wherein said protein or polypeptide has a nucleotide sequences of No. 6.

13. The protein or polypeptide according to claim 10, wherein said protein or polypeptide is a recombinant.
14. A biopesticide composition containing said protein or polypeptide and a carrier according to claim 11.

15. A pesticide using said protein or polypeptide and a carrier according to claim 11.

16. A plant growth activator using the protein or polypeptide and carriers according to claim 11.

17. A seed-treating agent using said protein or polypeptide and a carrier according to claim 11.

18. An insect repellent using said protein or polypeptide and a carrier according to claim 11.

19. A fertilizer using said protein or polypeptide and a carrier according to claim 11.

20. A method for producing a protein or polypeptide that induces a hypersensitive response or resistance on a mass-scale by isolating and purifying said protein or polypeptide from cultures of *Erwinia pyrifoliae* WT#3 (KCCM 10283) and transformants containing a plant HR-inducing gene of the strain (KCCM 10282) from *Erwinia pyrifoliae* WT#3 (KCCM 10283).
**DRAWING**

*Fig. 1*

*Erwinia pyrifoliae WT#3  Erwinia pyrifoliae Ep16T*

*Erwinia amylovora ATCC15580T*

*Fig. 2*

[Graph showing specific growth rate (μ) vs. temperature (°C)]
Fig. 5

- **Erwinia pyrifoliae** W1W3
  - **Erwinia pyrifoliae** B1
  - **Erwinia amylovora** B1
  - **Erwinia amylovora** ATCC15680\(^T\)
  - **Erwinia persicina** LMG2691
  - **Erwinia rhapsontici** LMG2688
  - **Klebsiella pneumoniae** DSM30104

- **Erwinia tracheiphila** LMG1906
  - **Erwinia psidii** LMG7034
  - **Erwinia malloilvora** LMG2708
  - **Escherichia coli** K-12

- **Enterobacter pyrinus** KCTC2520\(^T\)
  - **Pantoea agglomerans** ATCC33243\(^T\)
  - **Pantoea ananatis** pv. ananatis ATCC33244
  - **Pantoea ananatis** pv. uredovora ATCC1932

- **Brenneria salicis** ATCC15721\(^T\)
- **Erwinia chrysanthemi** ATCC11663\(^T\)
- **P. carotovorum** subsp. **carotovorum**
- **P. carotovorum** subsp. **betavescularum**
Fig. 6

100 --- *P. carotovorum* subsp. *carotovorum*

52 --- *P. carotovorum* subsp. *betavesculorum*

79 --- *Pantoea ananatis*

61 --- *Pectobacterium chrysanthemi*

100 --- *Erwinia rhapontici*

--- *Erwinia pyrifoliae* WT3

--- AF290418 (*Erwinia amylovora*)

--- Erwinia amylovora ATCC15580^T
Fig. 7

- Erwinia amylovora Eal
  - Erwinia amylovora ATCC15580
  - Pantoea ananatis
  - Enterobacter pyrus
  - P. chrysanthmi
  100+ P. carotovorum subsp. carotovorum
  100+ P. carotovorum subsp. betavesculorum

- Erwinia rhapontici
  - Erwinia pyrifoliae WTW3
  98+ Erwinia pyrifoliae Ep1
  91+ Erwinia pyrifoliae Ep16

Fig. 10

HindIII BamHI PstI PstI PstI PstI BamHI PstI HindIII

The inducible gene of plant hypersensitive response from WT#3 strain

Fig. 11

DNA Sequence Analysis


7/10
Fig. 13

Protein Analysis

| 10| 20| 30|    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
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| G | O | O | G | S | A | A | S | G | V | O | S | A | L | D | Q | A | L | G | I | N | S | T | S | O | N | D | S | S | T | S | G | T | D | S |
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SEQUENCE LISTING

Pioneer Co., Ltd.

New biopesticide using a gene from Erwinia pyrifoliae WT#3, a novel pathogen that affects Asian pear trees

KR2001-0049047
2001-08-14

Number of SEQ ID NOS: 6

Kopatent#n 1.71

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Organism: Artificial Sequence

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Length: 21
Type: DNA
Organism: Artificial Sequence

Feature:
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Lys Gly Val Ser Asp Ala Leu Ser Ala Leu Met Gly Asn Gly Leu Ser
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420 425
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 1/20
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 1/20, 5/00, 15/00 ; A01N 37/18 ; A61K 38/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean Patents and Applications for Inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubMed, Delphion, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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☐ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

"A" document defining the general state of the art which is not considered to be of particular relevance
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"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
AHN, Mi Jung
Telephone No. 82-42-481-5593

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