HrpN_{Ea} Induces Chinese Cabbage Resistance to Bacterial Soft Rot by Inhibiting the Bacterial Attachment to Root Surfaces

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ABSTRACT

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HrpN_{Ea} is a harpin protein produced by the bacterial plant pathogen *Erwinia amylovora*. When applied to aerial parts of plants, the protein induces systemic acquired resistance in a variety of plant species. Here, we report that treating Chinese cabbage roots with HrpN_{Ea} induces resistance of the plant to *Pectobacterium carotovora* subsp. *carotovora*, the pathogen that invades roots and causes bacterial soft rot in cruciferous plants. Treating Chinese cabbage roots with HrpN_{Ea} significantly decreased severities of soft rot symptoms on the plant. The root treatment decreased the number of *P. carotovora* subsp. *carotovora* cells attached to root surfaces and inhibited the ability of *P. carotovora* subsp. *carotovora* to produce quorum-sensing signals, which regulate pathogenicity in a bacterial population-dependent manner. The inhibitory effects of HrpN_{Ea} on the root attachment and quorum-sensing signals accompanied the induced expression of several defense response genes. These results suggest that HrpN_{Ea} induces Chinese cabbage resistance to bacterial soft rot by inhibiting the bacterial attachment to root surfaces.

Bioactive natural products are widely recognized as alternative crop protection agents (27,29,55) with great potential for use in agricultural settings (10,11,18). Harpins are multifunctional type-III proteins secreted from gram-negative plant-pathogenic bacteria (27,29). External application of the proteins to plants causes several beneficial effects and, in particular, induces plant resistance to pathogens (20). A well-studied harpin is HrpN_{Ea} from *Erwinia amylovora*, the pathogen that causes fire blight in rosaceous plants (56). When HrpN_{Ea} is applied to aerial parts of a plant, systemic acquired resistance (SAR) can be induced throughout the plant (5,20,29). Development of induced resistance accompanies promoted expression of resistance-related genes such as the *NPR1* gene (45) and genes that encode pathogenesis-related (PR) proteins (20). It has been accepted that the application of harpins activates distinct signaling pathways to regu-

late plant defensive responses (10,11). To date, however, the dogma has been established almost exclusively based on effects of harpins applied to aerial parts of plants (29). There is, as yet, no study to show any effects of harpin application to plant roots.

We sought to address the question by studying whether HrpN_{Ea} application to Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) roots impacts *Pectobacterium carotovora* subsp. *carotovora*, previously *E. carotovora* subsp. *carotovora* (14,39). Chinese cabbage is a popular species of cruciferous plants, and *P. carotovora* subsp. *carotovora* is the pathogen of bacterial soft rot in cruciferous plants (46). Previously we have shown that invasion from roots of Chinese cabbage is an important path for the bacterial establishment in the plant (54). Under natural conditions, *P. carotovora* subsp. *carotovora* survives in small numbers in the soil during the fallowing season. Once Chinese cabbage is planted, *P. carotovora* subsp. *carotovora* is enriched by root exudates to increase its population (54) sufficiently for root invasion via wounding or natural openings (51). Then, *P. carotovora* subsp. *carotovora* takes a period of latent infection (51,53,54) in the vascular bundles and intercellular spaces (53). When conditions favor pathogenicity, *P. carotovora* subsp. *carotovora* produces cell-wall-degrading enzymes and causes tissue maceration. As an essential step in infection, enrichment

of *P. carotovora* subsp. *carotovora* population by root exudates facilitates contact recognition (23), which occurs via bacterial attachment to root surfaces (19,23,51). During this period, *P. carotovora* subsp. *carotovora* infection benefits from functions of surface polysaccharides in either contact recognition (19,21) or evading plant immunity (17). In the first case, biotic and abiotic interferences of contact recognition contribute to plant defense responses and have been assumed to take a part in induced resistance (21,42,46).

Virtually every species of pathogenic bacteria can use quorum-sensing (QS) signals to regulate pathogenicity and interactions with plants (48,52). *N*-acyl-homoserine lactone (AHL) and *N*-(3-oxo-hexanoyl)-L-homoserine lactone (OHHL) are diffusible QS signals that partially control the production of the main virulence factors and extracellular cell-wall-degrading enzymes in *P. carotovora* subsp. *carotovora* (2,9,12,13). By contrast, plants have a potential to disrupt QS from pathogenic bacteria by enzymatic decomposition of the QS signals or by production of compounds that mimic QS signals to confuse bacteria (7,8,52). Several species of higher plants have been shown to secrete various compounds that act like bacterial QS signals (49) and that may disrupt or manipulate QS regulation in associated bacteria (26,60). The interference may be associated with disruption in plant-bacterial recognition and arrestment of infection when plants respond to a resistance elicitor (21,41). These demonstrations stimulate a great interest to study HrpN_{Ea} effects on Chinese cabbage-*P. carotovora* subsp. *carotovora* interaction on the plant roots.

This study aims at determining whether the inhibition of *P. carotovora* subsp. *carotovora* attachment to roots is a part of HrpN_{Ea}-induced resistance in Chinese cabbage. We found that the treatment of Chinese cabbage roots with HrpN_{Ea} significantly alleviated soft rot symptoms caused by inoculation with *P. carotovora* subsp. *carotovora*. Bacterial attachment to root surfaces, production of AHL signals, and genes related to pathogenesis were investigated as correlated with changes in defensive response and soft rot severity. We show that interference of the plant-*P.*

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*The e-Xtra logo stands for "electronic extra" and indicates that Figures 1, 2, and 5 appear in color online.

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carotovora subsp. *carotovora* interaction on roots plays a role in HrpN_{Ea}-induced resistance of the plant.

MATERIALS AND METHODS

Plant treatment. Chinese cabbage (cv. Zaoshu 5) seed were sterilized for 5 min in a 30% (vol/vol) sodium hypochlorite aqueous solution, sterilized for 30 s in an ethanol (70%) aqueous solution, and washed five times with sterile water immediately and after sterilization. Sterilized seed were sown on Murashige and Skoog (MS) agar medium (40) in petri dishes. Petri dishes were vertically placed and incubated in an incubator at 22°C and 200 µE/m²/s illumination with a cycle of 12 h of light and 12 h of darkness. Two days later, germinal roots were treated by 15 min of immersion with H₂O applied as a control or with a filter-sterilized aqueous solution containing HrpN_{Ea} and the surfactant Silwet-77 (Momentive Performance Materials Inc., China Branch, Beijing). HrpN_{Ea} was produced by the prokaryotic expression system (20) based on the use of the pET30a(+) vector (10,33) and was purified by chromatography (10). HrpN_{Ea} was used at 15 µg/ml and Silwet-77 was used at 0.03% (vol/vol) in all experiments (10,11). Two days after treatment, seedlings were transferred into a new plate of MS agar medium and placed in a pit, which had been made by removing the agar medium and filling with liquid MS medium to support plant growth.

Bacterial multiplication and plant inoculation. The virulent *P. carotovora* subsp. *carotovora* strain RL4 (54,55) was used in this study. To label RL4, the gene encoding a green fluorescence protein (GFP) was cloned from the vector pEGFP-C1 (Clontech Laboratories, Inc., Palo Alto, CA) and then was cloned into the pUC19 vector (Takara Biotech. Co., Ltd., Dalian, China), which contains an ampicillin-

resistance gene. Transformation of RL4 cells with the recombinant pUC19 vector generated the GFP-expressing and ampicillin-resistant strain RL4 GFP. RL4 and RL4 GFP were multiplied by overnight incubation in liquid Luria-Bertani (LB) medium (38) in the absence and presence, respectively, of ampicillin at 100 µg/ml. Bacterial cells were collected to make a suspension of 1 × 10⁸ CFU/ml. The suspension was mixed with liquid MS medium in an agar medium plate pit to inoculate roots of Chinese cabbage seedlings growing in the pit. One hour later, seedling roots were washed thoroughly with 2 mM phosphate-buffered saline (pH 7.3) by 5 min of incubation in a shaker. This step was used to remove bacterial cells that had not tightly adhered to root surfaces (48). Samples of washed roots were observed by fluorescence microscopy using a Bio-Rad Laser Molecular Imager FX (FITC fluorophore setting). Root samples were also observed under a HITACH S-3000 scanning electron microscope equipped with digital field emission. Two hours after inoculation, seedlings were sterilized by immersing roots in a 75% ethanol solution (40 s), washed in sterile water (2 min), and then distributed on wet filter papers in petri dishes. To induce soft rot symptoms, petri dishes were placed in a jar and the jar was subjected to anaerobic treatment by pump-driven replacement of oxygen with gaseous nitrogen. Anaerobic treatment was maintained for 3 days at 25°C. Severities of soft rot symptoms were surveyed and quantified as the ratio of centuplicate value of the rotten tissue length to total tissue length.

Detection of AHL signals. Diffusible AHL signals produced by RL4 GFP were detected with an established protocol that used the bacteria *Chromobacterium violaceum* strain CV026 as a reporter of AHLs (37,49). To test AHLs from bacterial

cells grown on LS agar medium, an RL4 GFP suspension (1 × 10⁷ or 1 × 10⁸ CFU/ml) was loaded into round pits made on top of the medium, and a mixture of a CV026 suspension (1 × 10⁹ CFU/ml) and *N*-hexanoyl-L-homoserinelactone (C6-HSL) (1, 2, or 4 µM) was distributed in a restricted rectangular areas close to RL4 GFP-containing pits. A mixture of CV026 suspension and 20 µM C6-HSL was used as a positive control and a solution containing only C6-HSL (20 µM) was used as a negative control. For assays with Chinese cabbage seedlings, pits of approximately 1.5 cm in depth and 1 cm² in size were made by removing the LB agar medium in glass boxes, and the pits were filled with liquid MS medium to support plant growth. RL4 GFP suspension (1 × 10⁸ CFU/ml) was added to MS, and a mixture of CV026 (1 × 10⁹ CFU/ml) and C6-HSL (1 µM) was distributed on the left sides of pits. Roots of 40 seedlings that had been treated with H₂O or HrpN_{Ea} were placed into liquid MS medium in a pit. Reactions in both experimental types were performed at 28°C for 12 h and AHL signals were shown in purple. Quantitative analysis of AHLs in photos was conducted with the Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Inc., Nanjing Branch, China).

Gene expression analysis. Total RNA was isolated using the Tripure Isolation Reagent (Roche Diagnostics, Indianapolis, IN) and from roots of Chinese cabbage 0, 6, 12, 24, 36, 48, and 72 h after treatment with purified HrpN_{Ea} and pure water, respectively. Gene expression was analyzed by reverse-transcription polymerase chain reaction (RT-PCR) using specific primers (Table 1). The elongation translation factor gene *EF1α* expressed constitutively in eukaryotes was used as a reference (45). RT-PCR products were resolved by electrophoresis and visualized by staining with

Table 1. Information on genes tested in this study

Gene	Accession number	Primers used in real-time reverse-transcription polymerase chain reaction	Size (bp)
<i>Chi</i>	AB257452	5'-GAAACCGTCAAGTCTCAAAACTGC-3' 5'-TACTCACTAGCTCGGCTGGCCTA-3'	539
<i>EF1α</i>	AK226639	5'-AGACCACCAAGTACTACTGCAC-3' 5'-CCACCAATCTTGTACACATCC-3'	495
<i>GST</i>	AY062676	5'-TATCAATGGCAAGAGCAGACGAG-3' 5'-AAGAAGATCTCACTCTCTGCCAT-3'	336
<i>LTP</i>	AF093751	5'-GAAGTTGGCATGCTTGGTCT-3' 5'-GTCCCC TAAAGCTTCATCACAC-3'	371
<i>MAPK3</i>	AY642433	5'-CACTCGGCTAAAGTTATCCACAGG-3' 5'-TGGCTAGGTACGGGTGATTCAGAG-3'	512
<i>NADPH</i>	NM_105079	5'-TTAGCAACAAGATGGTTTAGGCG-3' 5'-TAATTGAGAATGGATGCCACTCG-3'	400
<i>NPR1</i>	U76707	5'-CAGCGAGAAGCTCTTTCTTCAAGAG-3' 5'-GTACCTCCAAACCAAGCTCTTTACG-3' (5'-GCGGTTGAATGTAATAATATCCCG-3' 5'-CAGCTTGTGCTTCCGTTGG-3')	506 (217)
<i>PR-1</i>	M90508	5'-CAAGATAGCCACAAAGATTATCTAAGGGTT-3' 5'-GGCTTCTCGTTACATAATTCCACG-3' (5'-CTATGCTCGGAGCTACGCAGAAC-3' 5'-CCAGACAAGTCACCGCTACCC-3')	408 (105)
<i>TCH4</i>	AF051338	5'-TGGGACGAGATAGATTTCGAGTT-3' 5'-ATCGGTTTGGACCAACCACCTC-3'	321

ethidium bromide. Real-time RT-PCR was conducted as described and used *EF1 α* and *ACTIN2* genes as references (55,58).

Data treatment. All experiments were repeated at least three times with similar results and, each time, 10 seedlings were tested. Variance for quantitative data was assessed using the SAS general linear model (version 6; SAS Institute, Cary, NC). Student's *t* test at $P < 0.01$ was used to compare data from plants treated with HrpN_{Ea} versus pure water.

RESULTS

Resistance induction by root treatment. After 3 days of anaerobic treatment, Chinese cabbage seedlings in the control (without inoculation) remained healthy, whereas soft rot symptoms appeared in plants whose roots had been inoculated with *P. carotovora* subsp. *carotovora* (Fig. 1A). The symptoms were expanded throughout the plants when they were treated with H₂O, which was used as a control in contrast to purified HrpN_{Ea}. The protein was highly active in eliciting Chinese cabbage resistance to soft rot because the soft rot symptoms were observed on only a few leaves of HrpN_{Ea}-treated plants. In these plants, soft rot severities were much less severe than they were in H₂O-treated plants (Fig. 1A). In contrast, the development of soft rot was more progressive in plants treated with H₂O compared with HrpN_{Ea} (Fig. 1B). At 3 days after inoculation, only a 39% proportion of HrpN_{Ea}-treated plants incurred soft rot, in contrast to greater than 95% soft rot proportion in H₂O-treated plants (Student's *t* test, $P < 0.01$). Evidently, HrpN_{Ea} applied to Chinese cabbage roots was effective at inducing plant resistance shown as the inhibition in the development of *P. carotovora* subsp. *carotovora*-caused soft rot symptoms.

Impairment in *P. carotovora* subsp. *carotovora* attachment to root surfaces. To monitor *P. carotovora* subsp. *carotovora* behaviors on Chinese cabbage roots, the recombinant strain RL4 GFP was generated and confirmed as similar as RL4 in virulence to Chinese cabbage (Fig. 1C and D). After 3 days of incubation under anaerobic conditions, plants inoculated with RL4 GFP or RL4 formed soft rot symptoms over roots and stems, in contrast to the absence of soft rot in control (not inoculated) plants (Fig. 1C). Symptom severities were similar whenever caused by RL4 or RL4 GFP (Fig. 1D). With the aid of GFP fluorescence, RL4 GFP cells attached to root surfaces were observed clearly 1 h after inoculation (Fig. 2A). Scanning electron microscopy showed different forms of bacterial attachment, including polar attachment, to root surfaces (Fig. 2B). A survey on bacterial population on roots revealed inhibitory effects of HrpN_{Ea} on bacterial attachment. *P. carotovora* subsp. *carotovora* cells attached to root surfaces

of HrpN_{Ea}-treated plants were approximately four times less than those on root surfaces of H₂O-treated plants (Fig. 3). The population of *P. carotovora* subsp. *carotovora* attached to root surfaces (Fig. 4A) was consistent with the population recovered from root tissues (Fig. 4B), suggesting a correlation between root attachment and growth of the pathogen after invasion.

Inhibition of QS. We investigated whether root treatment with HrpN_{Ea} has any impact on *P. carotovora* subsp. *caroto-*

vora in the production of AHLs, which represent an important type of diffusible QS signals (2). The established method using *C. violaceum* strain CV026 as a reporter of AHL signals (37,49) was used to determine the production of AHLs from RL4 GFP grown on the LB agar medium (Fig. 5A). Exogenous C6-HSL was appended so that total signals reached the critical mass needed to color (37). In the presence of 1, 2, and 4 μ M C6-HSL, amounts of AHLs detected were greater when RL4 GFP suspension was applied at

Fig. 1. Effects of HrpN_{Ea} on soft rot in Chinese cabbage. Plants after 3 days of growth in the medium were not treated and not inoculated in (control), and were treated by immersing roots in pure water or an HrpN_{Ea} solution, followed by root inoculation with the strain RL4 of *Pectobacterium carotovora* subsp. *carotovora*. Then, plants were subjected to anaerobic conditions to induce soft rot symptoms. **A**, Plants photographed 3 days after anaerobic treatment. **B**, Soft rot severity (%) given as centuplicate value of the ratio between soft rot length and total length of the root and stem. **C**, Parts of plants photographed 3 days after anaerobic treatment. Plants remained not inoculated in control and had been inoculated with RL4 or RL4 GFP, a recombinant RL4 strain expressing the gene coding for green fluorescence protein (GFP). **D**, Centuplicate value of the soft rot tissue length to the total tissue length ratio determined for plants from C. In B and D, data represent means $\pm$ standard deviation bars ($n = 4$ repeats).

Fig. 2. Attachment of *Pectobacterium carotovora* subsp. *carotovora* cells to Chinese cabbage root surfaces. Plants after 3 days of growth in the medium were not treated and not inoculated in (control), and were treated by immersing roots in pure water or an HrpN_{Ea} solution, followed by root inoculation with the *P. carotovora* subsp. *carotovora* strain RL4 green fluorescence protein (GFP). One hour later, root samples of inoculated plants were observed by **A**, fluorescence microscopy and **B**, scanning electron microscopy.

Fig. 3. Bacterial number (means $\pm$ standard deviation; $n = 3$ repeats) counted with fluorescence microscopy shown in Figure 2A.

10^8 than 10^7 CFU/ml in the test using pertinent controls. On the medium, neither HrpN_{Ea} nor Chinese cabbage seedlings had an evident effect on the ability of *P. carotovora* subsp. *carotovora* to produce AHLs (Fig. 5B). However, when *P. carotovora* subsp. *carotovora* cells were grown close to Chinese cabbage roots that had been treated with HrpN_{Ea}, the production of AHLs from *P. carotovora* subsp. *carotovora* cells was significantly inhibited (Fig. 5C). The level of AHLs was 3.5-fold smaller in the case of root treatment with HrpN_{Ea} compared with H₂O, suggesting the inhibition of *P. carotovora* subsp. *carotovora* QS.

Induced expression of defense response genes. Previous studies have shown that the application of HrpN_{Ea} to aerial parts of plants can induce the expression of defense response genes (10,20,24,45). Eight genes were studied with different performances (Fig. 6). The mitogen-activated protein kinase (MAPK)

Fig. 4. Comparison of RL4 green fluorescence protein (GFP) cells **A**, attached to Chinese cabbage root surfaces and **B**, present in the plant root tissues. Plants after 3 days of growth in the medium were not treated and not inoculated in (control), and were treated by immersing roots in pure water (opened histograms) or an HrpN_{Ea} solution (solid histograms), followed by root inoculation with the strain RL4 GFP. Data represent means $\pm$ standard deviation ($n = 3$ repeats).

gene is involved in *Arabidopsis thaliana* response to HrpN_{Ea} (15,16). The *GST*, *NADPH*, *TCH4*, *LTP*, and *Chit* genes function in plant response to reactive oxygen

species (3,32,36,50) or in plant defense mediated by the ethylene signal (5,28,59). These genes all have homologues in Chinese cabbage based on gene cloning and

sequencing analysis in this study, and some genes are involved in plant response to HrpN_{Ea} (5,15,16,20,45). However, none of these gene homologues change expression levels in Chinese cabbage roots following treatment with HrpN_{Ea} (Fig. 6A). Noticeably, the *PR-1* and *NPR1* genes seemed quite particular. They were induced to increase expression levels in Chinese cabbage roots following treatment with HrpN_{Ea} (Fig. 6A). The relative amount of the *NPR1* transcript was 12- and 25-fold greater and that of the *PR-1* transcript was 5- and 11-fold greater at 24 and 48 h, respectively, compared with 0 h (immediately) after treatment (Fig. 6B). Inversely, amounts of all the gene transcripts evidently did not change during the course of time after treatment with water used as a control (Fig. 6A).

DISCUSSION

Previous studies show that the application of HrpN_{Ea} to aerial parts of *Arabidopsis* can activate the SAR pathway in the plant (5,20,29). Results detailed in this article have demonstrated that Chinese cabbage resistance against *P. carotovora* subsp. *carotovora* is induced by HrpN_{Ea} applied to the plant roots, and that the induced resistance is correlated with the inhibition of *P. carotovora* subsp. *carotovora* cells in attachment to root surfaces and the production of QS signals in the environment.

The course of interaction between *P. carotovora* subsp. *carotovora* and Chinese cabbage roots involves contact recognition of both partners (23), latent infection of the pathogen in the vascular bundles, and pathogenesis in the plant tissues (54). Contact recognition relies on *P. carotovora* subsp. *carotovora* attachment to root surfaces and is essential for subsequent infection and pathogenesis to occur (21). Thus, this study was first focused on the attachment stage to elucidate the effect of HrpN_{Ea} on plant resistance to *P. carotovora* subsp. *carotovora*. The application of HrpN_{Ea} to Chinese cabbage roots impairs *P. carotovora* subsp. *carotovora* attachment to root surfaces, indicating that the bacteria–host interaction on roots has been altered by HrpN_{Ea} treatment. For the root attachment to occur, *P. carotovora* subsp. *carotovora* needs to swarm toward and congregate on host roots (22). The congregated *P. carotovora* subsp. *carotovora* population may act in a coordinated manner. An individual of the bacterial cells may benefit from cooperative group behavior to survive and invade a particular host (52). This process can be regulated by QS signals that are involved in symbiotic, pathogenic, and surface-associated relationships between microbial populations and their plant hosts (34,57). In this study, the application of HrpN_{Ea} to Chinese cabbage roots markedly reduces the levels of QS signals from *P. carotovora* subsp. *caro-*

Fig. 5. Measurement of quorum-sensing (QS) signals produced by RL4 green fluorescence protein (GFP). *N*-acyl-homoserine lactones (AHLs), an important type of diffusible QS signals, were studied. **A**, Luria-Bertani agar medium plate with the indicated reaction components, photographed after 12 h of incubation. AHLs appear in purple due to the metabolism by the *Chromobacterium violaceum* strain CV026 used as a reporter of AHL signals. *N*-hexanoyl-L-homoserinelactone (C6-HSL) was appended so that the amount of total signals reached the threshold value to color. **B**, AHLs from RL4 GFP cells grown alone in a control and in the presence of HrpN_{Ea} or 3-day-old Chinese cabbage seedlings. **C**, AHLs from RL4 GFP cells growing together with Chinese cabbage roots that had been treated with H₂O (left) or HrpN_{Ea} (right). Control was the same as in C and is not shown. In C and D, AHLs were quantified (means ± standard deviation, *n* = 5 repeats) relatively by assigning the level as 1 for the control.

tovora around roots and simultaneously inhibits adherence of the bacterial cells to root surfaces. The number of *P. carotovora* subsp. *carotovora* cells attached to roots correlates with the extents of QS signals produced by the bacterial cells in the environment, consistent with recent evidence that QS regulation governs bacterial adhesion (30,31).

Some of plant root exudates are thought to act as signals to change patterns of bacterial gene expression and influence the ability of microbial strains to colonize the roots (17,35). It has been shown that several species of higher plants, such as pea, tomato, peanut, and rice, can secrete particular compounds that affect bacterial QS regulation (7,25,49). However, our results do not support a role of any connatural exudates from Chinese cabbage roots in inhibiting the production of QS signals from the bacterial cells. In contrast, root treatment with HrpN_{Ea} inhibits the ability of *P. carotovora* subsp. *carotovora* to pro-

duce QS signals, suggesting that particular inhibitory components have been induced in the plant. The supposed inhibitory components may have a potential to disrupt the bacterial QS (7,52) that functions to regulate contact recognition (21).

Our evidence indicates that the inhibition of *P. carotovora* subsp. *carotovora* attachment and invasion activities is coincident with the activation of the SAR pathway in Chinese cabbage roots following treatment with HrpN_{Ea}. The defense response in Chinese cabbage was elucidated by investigating several pertinent genes according to the few cases where defense responses have been investigated in Chinese cabbage (1,43,44) and according to many previous studies with *Arabidopsis* (5,15,17,24,32,45,50). Genes tested in this study, including *MAPK3*, *GST*, *NADPH*, *TCH4*, *LTP*, and *Chit*, are all involved in *Arabidopsis* response to HrpN_{Ea} (5,15,16,20,45) and all have homologues in Chinese cabbage based on gene

cloning and sequencing analysis. These genes are also involved in plant response to reactive oxygen species (32,36,50) or in plant defense mediated by the ethylene signal (5,28,41), whereas reactive oxygen species and ethylene signals play important roles in defense responses induced by HrpN_{Ea} applied to aerial parts of plants (20,24). However, none of these genes was induced to change expression extents in Chinese cabbage plants following root treatment with HrpN_{Ea}. This indicates that the application of HrpN_{Ea} to aerial parts and roots of plants may have different effects on defense responses. Considered with this notion, the *NPR1* and *PR-1a* genes are exceptional. Both genes are essential components of the SAR pathway activated by HrpN_{Ea} in *Arabidopsis* (20), and both genes have increased levels of expression in Chinese cabbage following root treatment with HrpN_{Ea}, in contrast to treatment with water in the control. Therefore, the SAR pathway is apparently activated in the plant roots responding to HrpN_{Ea}.

Our results suggest that the inhibition of *P. carotovora* subsp. *carotovora* attachment to Chinese cabbage root surfaces plays a role in HrpN_{Ea}-induced resistance against the pathogen in the plant. Induced resistance accompanies inhibition of the bacterial QS and the activation of the plant defense. There is, as yet, no evidence to show physiological relationships between these responses, and they may have complicated relationships beyond findings in this study. For example, like other gram-negative plant-pathogenic bacteria, *P. carotovora* subsp. *carotovora* also produces a harpin protein, HrpN_{Ecc} (14,39), which has a high similarity to HrpN_{Ea} and other HrpN proteins (6). Like other harpin proteins, HrpN_{Ecc} may also function to induce plant defense responses (47). Studies are required in the future to characterize functional relationship between both harpin proteins during the induction of defense responses in the plant.

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Fig. 6. Expression of defense-related genes. **A**, Gene expression was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) and **B**, transcript relative levels (means $\pm$ standard deviation; $n = 3$ repeats) were determined by real-time RT-PCR. The *EF1 α* gene was used as a reference.

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