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Activation of Defense Responses in Chinese Cabbage by a Nonhost Pathogen, *Pseudomonas syringae* pv. *tomato*

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***Pseudomonas syringae* pv. *tomato* (*Pst*) causes a bacterial speck disease in tomato and *Arabidopsis*. In Chinese cabbage, in which host-pathogen interactions are not well understood, *Pst* does not cause disease but rather elicits a hypersensitive response. *Pst* induces localized cell death and H_2O_2 accumulation, a typical hypersensitive response, in infiltrated cabbage leaves. Pre-inoculation with *Pst* was found to induce resistance to *Erwinia carotovora* subsp. *carotovora*, a pathogen that causes soft rot disease in Chinese cabbage. An examination of the expression profiles of 12 previously identified *Pst*-inducible genes revealed that the majority of these genes were activated by salicylic acid or BTH; however, expressions of the genes encoding PR4 and a class IV chitinase were induced by ethephon, an ethylene-releasing compound, but not by salicylic acid, BTH, or methyl jasmonate. This implies that *Pst* activates both salicylate-dependent and salicylate-independent defense responses in Chinese cabbage.**

Keywords: Chinese cabbage, Nonhost resistance, Plant defense, *Pseudomonas syringae*

Introduction

The genus *Brassica* includes many important vegetable crops, such as broccoli, cabbage, Chinese cabbage, cauliflower, mustard, rape, kale, and turnip. Although these *Brassica* species have served as good model plants for studying self-incompatibility (Takasaki *et al*., 2000), remarkably little is known about pathogen defense mechanisms in *Brassica*. And, in particular, progress on identifying defense mechanisms in Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), an important vegetable crop in Asia, has been extremely slow.

Defense responses are triggered when plants perceive invading pathogens. This recognition in turn activates a complex array of defense signaling pathways in plant cells (McDowell and Dangl, 2000; Nuernberger and Scheel, 2001). The interaction between a resistant plant and an avirulent pathogen, known as incompatible interaction, provides a good system for the study of defense responses in plants (Hammond-Kosack and Jones, 1996). Incompatible interactions are, in general, controlled by a disease resistance (*R*) gene that enables the plant to recognize and respond to pathogens carrying a specific avirulence (*Avr*) gene. This gene-for-gene resistance response also accounts for race or cultivar-specific resistance (Bent *et al*., 1996; Dangl and Jones, 2001).

Unfortunately, no host-pathogen system displaying the characteristics of an incompatible interaction has been identified in Chinese cabbage. When we attempted to identify such a system in Chinese cabbage, instead we found that *Pseudomonas syringae* pv. *tomato* (*Pst*) induces a hypersensitive response (HR). HR, defined as localized cell death at the site of attempted pathogen invasion (Hammond-Kosack and Jones, 1996), is a prevalent and effective mechanism deployed by plants to protect themselves against various pathogens (Hammond-Kosack and Jones, 1996; Lam *et al*., 2001), and is frequently observed as a component of incompatible interactions. Although *Pst* causes disease in *Arabidopsis* and tomato (Bashan *et al*., 1981; Whalen *et al*., 1991), it has not been reported to cause disease in Chinese cabbage. We observed that two *Pst* strains, *Pst* 259 and *Pst* 263, elicited HR in all eight Chinese cabbage cultivars tested (Charming Yellow, CR-Ansim, Hwangsimbong, Jangwon, Matna, Norang, Olympic, and Yeoreumhwang). Thus, it appears that *Pst* represents a nonhost pathogen of Chinese cabbage.

In this study, we characterized the defense responses of Chinese cabbage to *Pst* 259. In addition to the HR, we examined the effect of pre-inoculating cabbage plants with *Pst*

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on its resistance to another pathogen, *Erwinia carotovora* subsp. *carotovora*, which causes soft rot in Chinese cabbage. Although soft rot is devastating to Chinese cabbage, genetically defined resistance to this disease has not been described. In addition, we also examined the effect of salicylic acid (SA) and other signaling molecules on the expression of 12 *Pst*-inducible cabbage genes that we had isolated previously (Ryang *et al.*, 2002). Our results suggest that *Pst* activates both SA-dependent and SA-independent defense responses and induces resistance to soft rot disease in Chinese cabbage.

Materials and Methods

Plant materials Chinese cabbage seedlings (*Brassica rapa* subsp. *pekinensis* cultivar Norang) were grown in potting compost after germination. Unless otherwise stated, experiments were performed with cabbage seedlings at the seven- or eight-leaf stage. *Pseudomonas syringae* pv. *tomato* (*Pst*) strain 259 was prepared as described by Lee and Cho (2003). Cabbage leaves were inoculated with a bacterial suspension by syringe infiltration. *Pst*-treated cabbage leaves were then transferred to a growth chamber and incubated at 25°C under continuous light. Control plants were similarly treated with sterile water. After 6, 18, 24, or 48 h, leaf samples were harvested, weighed, and frozen immediately in liquid nitrogen.

For SA treatment, fully developed and healthy leaves from plants were cut into 1 × 1 cm pieces and floated on 20 mM MOPS buffer (pH 7.5) containing either 5 mM or no SA (Sigma Chem. Co., St. Louis) in a 10-cm or 15-cm Petri dish. The leaf samples were treated at 25°C under continuous fluorescent light. Methyl jasmonate (1 mM in 0.1% [v/v] ethanol), ethephon (1 mM), and the control of 0.1% ethanol were applied by spraying them on the leaves. The cabbage plants were then transferred to a growth chamber and incubated at 25°C under continuous fluorescent light. Treatment with benzothiadiazole (BTH) was carried out either by floating 1 × 1 cm leaf pieces on MOPS buffer containing 0.3 mM BTH, as described for SA treatment, or by spraying BTH solution onto intact leaves. Twenty-four hours after treatment, the leaf samples were harvested, weighed, and frozen immediately in liquid nitrogen. BTH (5% active ingredient in wettable powder) was donated by Novartis, Korea. Methyl jasmonate (MeJA) and ethephon were purchased from Aldrich Chemical Co. (Milwaukee) or the Sigma Chemical Co.

Detection of H_2O_2 by DAB staining Chinese cabbage leaves were excised 24 h after infiltration with sterile water or *Pst*, and were placed in DAB solution (3,3-diaminobenzidine-HCl, pH 3.8, 1 mg/ml) for 8 h at 25°C. DAB polymerizes to produce a brown precipitate on contact with H_2O_2 in the presence of peroxidase, and thus provides a useful marker of peroxide accumulation (Rusterucci *et al*., 2001). Subsequently, the leaves were cleared for 10 min in boiling 96% ethanol solution. The samples were then mounted on a slide in 60% glycerol and examined using a light microscope (Olympus AHBT-514).

Detection of cell death by trypan blue staining Trypan blue staining was performed as described by Rate *et al*. (1999). Leaf squares (1 cm × 1 cm) were removed from mock- or *Pst*-inoculated leaves. The leaf pieces were then boiled in a lactophenol solution (lactic acid : glycerol : phenol : water/1 : 1 : 1 : 1, v/v) containing trypan blue (0.05%, w/v) for 1 min, cleared by boiling in a lactophenol and ethanol mixture (2 : 1, v/v) for 2 min, and then washed in 50% ethanol for 5 min.

Induction of disease resistance To induce disease resistance in Chinese cabbage, a bacterial suspension of *Pst* 259 (OD_{600} = 0.1) was infiltrated into three fully expanded upper leaves at three places per leaf using a 1 ml syringe without a needle. For BTH treatment, cabbage plants were drenched by pouring 50 ml of 0.3 mM BTH solution into each pot. Control cabbage plants were drenched with distilled water. Three or five days after this pre-treatment, the cabbage plants were inoculated with the soft rot pathogen *Erwinia carotovora* subsp. *carotovora* (*Ecc*), as described by Lee and Cha (2001). Each cabbage plant was drenched by pouring 10 ml of a 4 : 1 mixture of *Ecc* 394 (10^4 cfu/ml) and sterile mineral oil (heavy white oil; Sigma) over the center of the plant. The inoculated cabbage plants were examined for soft rot daily after *Ecc* inoculation. The experiment was performed on at least 12 plants per treatment in triplicate.

Northern analysis Total RNA was prepared from frozen plant materials using the "hot phenol" method described by De Vries *et al*. (1988). For Northern analysis, 10 mg of total RNA from each sample was separated on 1.0% formaldehyde-agarose gel and blotted onto a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) using the standard capillary transfer method. After UV-crosslinking at 125 mJ, blots were hybridized with a DNA probe labeled with digoxigenin (DIG). Chemiluminescent detection of the hybridized probe was carried out as described by Oh *et al*. (2004). The probe DNA was prepared by PCR (polymerase chain reaction) amplification of the insert DNA in cDNA clones isolated in our laboratory (Ryang *et al*., 2002). The putative functions of the clones and their GenBank accession numbers (in parentheses) are as follows: CPE23, unknown protein (AF528169); CPE24-2, 33-kDa secretory-like protein (AF528170); CPE25-1, unknown protein (AF528171); CPE25-2, chitinase (AF528172); CPE32, CYP79B1 (AF528173); CPE34, apospory-associated-like protein (AF528174); CPE-T9, CYP83B1 (AF528175); CPE-T15, RPW8 homolog (AF528176); CPL1, PR1a (AF528177); CPL24-1, chitinase (AF528178); CPL24-2, thaumatin-like protein (AF528179); CPL29, defensin (AF528180); CPL30, PR4 protein (AF528181). A DNA probe for glyceraldehyde 3-phosphate dehydrogenase (GAPD) gene was obtained by PCR amplifying the insert in a Chinese cabbage GAPD cDNA clone isolated in our laboratory (GenBank accession no. AF536826). DIG-labeling of probe DNA, hybridization, and chemiluminescent immunodetection were performed using kits from Roche Molecular Biochemicals (Mannheim, Germany). In cases with multiple inserts, gene-specific primers were used to amplify specific sequences.

Results

Hypersensitive response elicited by *Pst* in Chinese cabbage To examine the host defense response to *Pst*, we inoculated the leaves of Chinese cabbage seedlings at the seven- to eight-leaf stage with *Pst* 259. Although the gene-for-gene model does not apply to the relationship between Chinese cabbage and *Pst*, tissue collapse resembling that following HR in incompatible interactions was observed at sites of *Pst* infiltration (Fig. 1A). Trypan blue staining of infected leaves confirmed that cell death was induced in the *Pst*-infiltrated area (Fig. 1B); visible necrotic lesions usually appeared 24 to 36h after inoculation.

In typical incompatible interactions, one of the early events of HR is an oxidative burst with the generation of superoxide (O_2^-) and the subsequent accumulation of hydrogen peroxide (H_2O_2) (Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997). Superoxide anions are thought to be produced outside the plant cell by a plasma membrane-associated NAD(P)H oxidase, and are usually rapidly converted to H_2O_2 by superoxide dismutase. To examine whether H_2O_2 also accumulated at the site of *Pst*-elicited HR, cabbage leaves were excised 24 h after *Pst* inoculation and dipped in a solution of DAB. Fig. 1C clearly shows that H_2O_2 accumulated during the HR caused by *Pst*. This indicates that the early events elicited by a nonhost pathogen are similar to those observed in typical incompatible interactions.

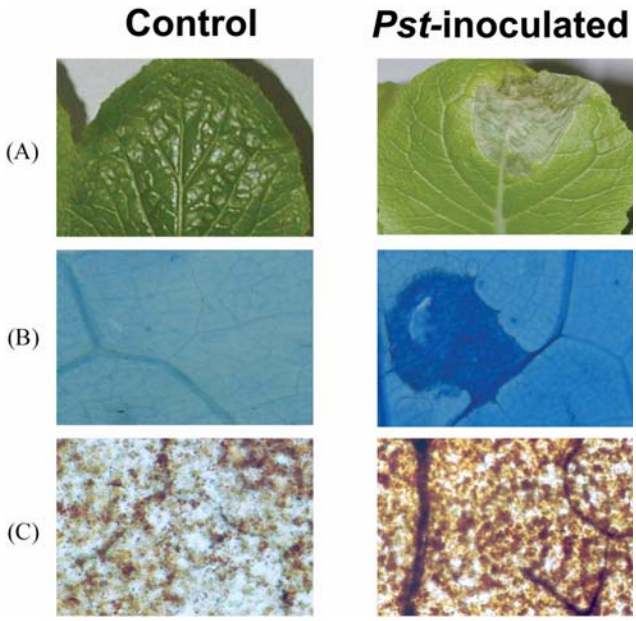

Fig. 1. Histochemical examination of HR elicited by *Pst*. (A) Photographs of a control leaf and of a lesion caused by infiltration of *Pst*. (B) Hypersensitive cell death was visualized by trypan blue staining. (C) Accumulation of H_2O_2 at the region of inoculation was examined using DAB staining and is shown at 200X magnification. The cabbage leaves were incubated in a growth chamber (25°C) for 24 h after mock treatment (left panels) or *Pst* inoculation (1×10^6 cells; right panels).

Induction of disease resistance to soft rot by *Pst* To examine whether *Pst* can induce resistance to soft rot, cabbage leaves were challenged with *Ecc* three or five days after *Pst* inoculation, and the incidence of soft rot was noted. We also examined the effect of chemical treatment with BTH, which is a functional analog of SA and activates plant defense responses (Goerlach *et al.*, 1996). In cabbage plants treated with 0.3 mM BTH, the incidence of soft rot reduced by *ca.* 50% (Fig. 2). This suggests that SA-dependent defense responses are involved in the disease resistance of Chinese cabbage to soft rot.

Cabbage plants pre-inoculated with *Pst* also showed enhanced resistance to *Ecc*, although this resistance was weaker and appeared later than the resistance induced by BTH. The reduction in soft rot was greater following pre-inoculation with *Pst* when the cabbage plants were challenged with *Ecc* five days after *Pst* treatment. Moreover, the disease resistance induced by *Pst* was systemic, since *Pst* infiltrated into the upper three leaves, whereas challenge inoculation with *Ecc* was carried out in the lower parts of the cabbage plants.

Activation of defense-related gene expression by *Pst* In plants, SA activates a disease resistance response known as systemic acquired resistance (SAR). SAR is induced locally by a pathogen or pest and then spreads to provide protection to the whole plant (Ryals *et al.*, 1996). We therefore examined whether *Pst*-inducible cabbage genes identified in our previous study (Ryang *et al.*, 2002; also see the Materials and Methods and Fig. 3) could also be induced by SA. In addition to the 12 *Pst*-inducible genes, we also examined the expression pattern of a cabbage defensin gene (CPL29). Cabbage defensin is most homologous to radish defensin RsAFP4 (95% identity), and shows strong similarity with *Arabidopsis* PDF1.2 (86% identity). Although the cabbage defensin gene did not show induction by *Pst*, we were interested in its expression profile, since it has been reported that plant defensin has strong antifungal activity (Terras *et al.*, 1995), and because the defensin gene is induced *via* an SA-independent pathway (Pennincks *et al.*, 1996; Terras *et al.*, 1998).

To examine the effect of SA on gene expression, cabbage leaves were cut into 1×1 cm pieces and floated on MOPS buffer containing 5 mM SA. Northern analysis showed that the majority of the *Pst*-inducible genes were also induced by SA (Fig. 3, column S). However, several genes were not induced by SA, and curiously, the expression of defensin (CPL29) and PR4-type protein (CPL30) genes seemed to be repressed by SA treatment. The expressions of CPE-T9, CPL1, CPL24-1, CPL29, and CPL30 appeared to be greater in leaf squares mock-treated with MOPS buffer (Fig. 3, M lanes) than in intact leaves treated with sterile water (Fig. 3, W lanes). Since the mock treatment involves physical injury to leaf tissues, these findings suggest that these genes may be induced by wounding.

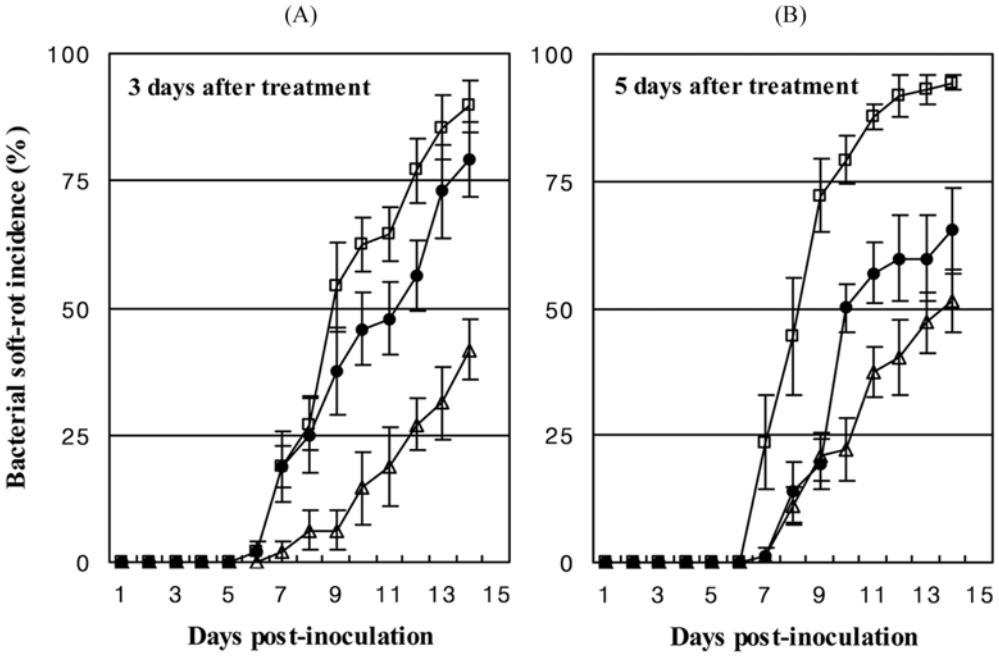

Fig. 2. Soft rot in Chinese cabbage plants pre-treated with resistance-inducing agents. An *Ecc* suspension (10^4 cells/ml) was poured onto the center of Chinese cabbage plants three (A) or five days (B) after *Pst* (filled circles) or BTH (triangles) pretreatment. Control cabbage plants (squares) were drenched with distilled water. The results shown are representative of three independent replicate experiments utilizing 12 plants per treatment. The values shown are averages ± standard error.

These results suggest that some *Pst*-induced genes are activated by signals other than SA, and that two major signaling pathways, one SA-dependent and the other SA-independent, feature in the Chinese cabbage defense system. Recently, it was shown that systemic resistance can also be mediated by jasmonate or ethylene (Piterse and van Loon, 1999). To examine how defense-related cabbage genes respond to jasmonate or ethylene, we treated cabbage leaves with methyl jasmonate (MeJA) or ethephon (an ethylene-releasing compound); we also treated them with BTH. In these experiments, the chemicals were sprayed onto cabbage leaves so as not to cause mechanical damage. The results obtained are shown in Fig. 4.

Of the 12 *Pst*-inducible genes, all except CPL24-1 and CPL30 were induced by BTH. This result is generally in agreement with the result obtained with SA. However, discrepancies were observed for CPE32 and CPL24-2, which were not induced by SA, but were induced by BTH.

The genes encoding the two cytochrome P450 proteins, CYP79B1 (CPE32) and CYP83B1 (CPE-T9), were induced by BTH and MeJA but not by ethephon. It is not surprising that these genes are induced by MeJA, a wounding signal, because the enzymes they encode catalyze essential steps in the biosynthesis of glucosinolates (Bak *et al*., 1998; Hansen *et al*., 2001), which serve as a feeding deterrent to herbivores. Moreover, the genes encoding class IV chitinase (CPL24-1) and PR4 protein (CPL30) were not induced by either BTH or MeJA, but they were induced by ethephon. Since ethylene also serves as a wounding signal, this result is also consistent with the observation that the expressions of the two genes were elevated when cabbage leaves were cut into 1 × 1 cm squares (Fig. 3). The gene expression pattern of the cabbage defensin gene (CPL29) was variable and did not show any evidence of induction by MeJA; unlike defensin genes in *Arabidopsis* and radish (Penninckx *et al*., 1996; Terras *et al*., 1998).

The 12 *Pst*-inducible cabbage genes can be divided into four groups based on the expression profiles shown in Fig. 4. One group of six genes is induced only by BTH: CPE23, CPE24-2, CPE25-1, CPE25-2, CPE34, and CPE-T15. The second group is composed of CPE32 and CPE-T9 - genes inducible by both BTH and MeJA, and the third group comprises thaumatin-like protein (CPL24-2) and PR1a (CPL1), which are induced by both BTH and ethephon. The fourth group consists of CPL24-1 and CPL30, which are induced by ethephon, but not by BTH, SA, or MeJA. That the CPL24-1 and CPL30 genes are not induced by BTH or SA suggests that *Pst* activates both SA-dependent and SA-independent pathways.

Discussion

Chinese cabbage is frequently damaged by various pathogens, which include *Peronospora brassicae*, *Plasmodiophora brassicae*, *Erwinia carotovora* subsp. *carotovora*, *Xanthomonas campestris* pv. *campestris*, and turnip mosaic virus. However, few studies on host-pathogen interactions have been performed

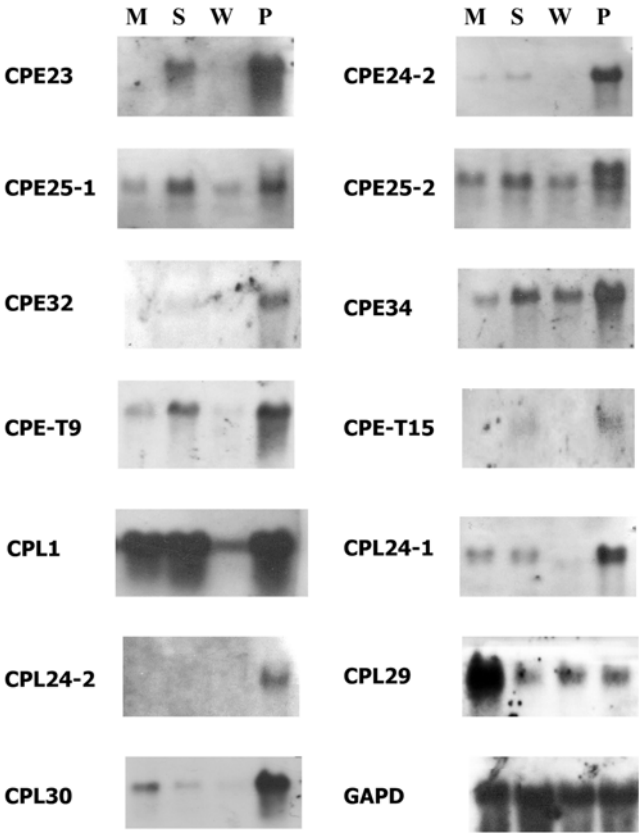

Fig. 3. Northern blot analysis of Chinese cabbage genes. 1x1cm leaf squares were collected 24 h after treating 20 mM MOPS buffer (M) or 5 mM SA (S). Cabbage leaves were also harvested 24 h after infiltration with either *Pseudomonas syringae* pv. *tomato* (P) or sterile water (W). Ten μg of total RNA from each sample was size-fractionated on 1% formaldehyde agarose gel, blotted onto a Hybond N^+ nylon membrane, and hybridized with a DIG-labeled DNA probe. The putative identifications of the clones used in this study are as follows: CPE23, unknown protein; CPE24-2, 33-kDa secretory-like protein; CPE25-1, unknown protein; CPE25-2, chitinase; CPE32, CYP79B1; CPE34, apospory-associated like protein; CPE-T9, CYP83B1; CPE-T15, RPW8 homolog; CPL1, PR1a; CPL24-1, chitinase; CPL24-2, thaumatin-like protein; CPL29, defensin; and CPL30, PR4 protein. GAPD represents the glyceraldehyde-3-phosphate dehydrogenase gene, which was used as a control.

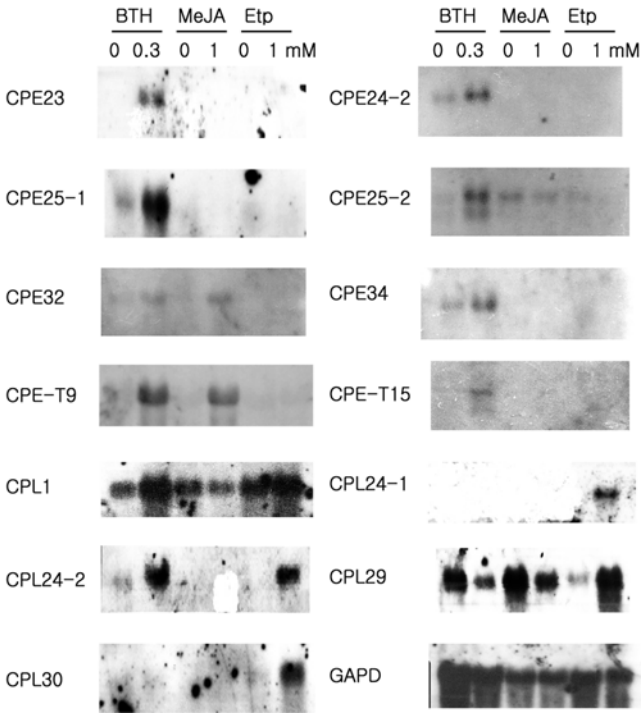

Fig. 4. Induction of gene expression by BTH, MeJA, and ethylene. 0.3 mM BTH, 1 mM MeJA in 0.1% ethanol, 1 mM ethephon (Etp) solution, or 0.1% ethanol and sterile water (control) were sprayed onto cabbage leaves. After 24 h leaf samples were collected and analyzed by Northern blot hybridization using DIG-labeled probes, as described in Fig. 3. As a loading control, samples were also hybridized with cDNA specific for cabbage glyceraldehyde-3-phosphate dehydrogenase (GAPD).

in Chinese cabbage, partly because the incompatible interactions that induce defense responses have not been identified in this plant.

Here, we studied the response of Chinese cabbage to infection by *Pseudomonas syringae* pv. *tomato (Pst)*, a nonhost pathogen of Chinese cabbage. Although it is not an interaction that fits the gene-for-gene model, the Chinese cabbage-*Pst* interaction provides a good model system for the study of defense responses in Chinese cabbage. Nonhost resistance is the most common form of disease resistance in plants. Moreover, this resistance type is weaker but more durable than the resistance induced by *R-Avr* interactions. Despite its importance, information on the mechanisms of nonhost resistance is limited. Although preformed defenses are a major component of nonhost resistance, induced defense responses that are activated by nonspecific elicitors, such as harpins and flagellins, or by *Avr* gene products, also constitute mechanisms of nonhost resistance (Heath, 2000). It has also been suggested that many cases of induced nonhost resistance involve the same signaling pathways as host resistance. These include HR-like cell death and the generation of reactive oxygen species (Mysore and Ryu, 2004). An example of key evidence supporting this idea was provided by the finding that in *Arabidopsis nho1* mutant, both host- and nonhost-resistance against *Pseudomonas* bacteria are compromised (Lu *et al.*, 2001). Another example was provided by Peart *et al.* (2002), who found that a ubiquitin ligase-associated protein, SGT1, is required for both host and nonhost disease resistance in *Nicotiana bethamiana*.

Fig. 2 shows that pre-inoculation with *Pst* enhanced resistance to a soft rot pathogen, *Erwinia carotovora* subsp. *carotovora*. However, the induced resistance was weaker than that induced by BTH. The rather weak and slow induction of disease resistance by *Pst*, as compared with that by BTH, may be because the site of challenge inoculation with *Ecc* differed

from that of *Pst*. It is also possible that the slow kinetics of defense response induced by *Pst* was responsible. Visible necrosis was only seen in Chinese cabbage leaves 24 to 30 h after *Pst* inoculation, although HR was induced within 16 h by the incompatible *Arabidopsis-Pst* interaction (Whalen *et al*., 1991). Compared to the induction of disease resistance by BTH, induction by *Pst* was similarly delayed (Fig. 2). A recent microarray analysis of expression profiles also demonstrated the slow induction kinetics of nonhost resistance; e.g., *Pseudomonas syringae* pv. *phaseolicola*, a nonhost pathogen of *Arabidopsis*, induces defense mechanisms similar to those induced by RPS2-mediated resistance, but at a slower rate (Tao *et al*., 2003).

A similar kinetic effect may explain the discrepancy between the expression profiles of CPE32 and CPL24-2 genes, whose expressions were induced by BTH but not by SA. These result probably reflects the fact that SA and BTH induce the two transcripts with different kinetics. Goerlach *et al*. (1996) reported that the time courses of gene induction by SA and BTH show distinct characteristics; SA caused a rapid and short transient induction, whereas BTH caused a slower but prolonged induction. Alternatively, this observation indicates that the two chemicals, SA and BTH, act through different mechanisms.

Genetic studies with *Arabidopsis* signaling mutants have shown that an SA-dependent response is deployed against biotrophic pathogens that obtain nutrients from living cells, whereas ethylene- or jasmonate-dependent responses are important for induced resistance to necrotrophic pathogens that kill plant tissue (Piterse and van Loon, 1999; McDowell and Dangl, 2000). In *Arabidopsis*, resistance to *Pst* depends on SA-dependent signaling, for example, the inoculation of NahG *Arabidopsis* plants, which lack SA, with an avirulent *Pst* strains leads to the development of severe disease symptoms (Delaney *et al*., 1994). Consistent with this observation, our study shows that *Pst* activates defense-related genes mainly *via* an SA-dependent pathway in Chinese cabbage.

Despite the substantial involvement of SA-dependent pathways, SA-independent pathways, and in particular the pathway mediated by ethylene, also seem to be activated by *Pst*. This activation of multiple signaling pathways is not unprecedented. A recent microarray study of the fungal pathogen *Alternaria brassicola*, resistance to which relies on the jasmonate signaling pathway (Thomma *et al*., 1998), showed that the pathogen activates SA- and ethylene-inducible genes in addition to jasmonate-inducible genes (Schenk *et al*., 2000). An expression profiling study of *Arabidopsis* responses to *Pseudomonas syringae* pv. *maculicola* showed that the jasmonate- and ethylene-signaling pathways are also activated, although activation by SA-dependent signaling is stronger (Glazebrook *et al*., 2003). Moreover, a comparison of responses to *A. brassicola* and *P. syringae* infections showed that approximately 50% of the induced genes were induced by both pathogens, despite the fact that these two pathogens *elicit* different defense responses (van Wees *et al*., 2003). Thus, it is evident that a complex array of defense signaling networks, rather than a single signaling pathway, is activated to combat individual pathogens.

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