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Priming of Defense-Related Genes Confers Root-Colonizing Bacilli-Elicited Induced Systemic Resistance in Pepper

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A group of beneficial plant bacteria has been shown to increase crop growth referring to as plant growth-promoting rhizobacteria (PGPR). PGPR can decrease plant disease directly, through the production of antagonistic compounds, and indirectly, through the elicitation of a plant defense response termed induced systemic resistance (ISR). While the mechanism of PGPR-elicited ISR has been studied extensively in the model plant *Arabidopsis*, it is less well characterized in crop plants such as pepper. In an effort to better understand the mechanism of ISR in crop plants, we investigated the induction of ISR by *Bacillus cereus* strain BS107 against *Xanthomonas axonopodis* pv. vesicatoria in pepper leaves. We focused on the priming effect of *B. cereus* strain BS107 on plant defense genes as an ISR mechanism. Of ten known pepper defense genes that were previously reported to be involved in pathogen defense signaling, the expression of *Capsicum annum* pathogenesis-protein 4 and *CaPR1* was systemically primed by the application of strain BS107 onto pepper roots confirming by quantitative-reverse transcriptase PCR. Our results provide novel genetic evidence of the priming effect of a rhizobacterium on the expression of pepper defense genes involved in ISR.

Keywords : induced systemic resistance, pepper, PGPR, priming

The rhizosphere is a zone around plant roots where microbes interact and inter- and intra-species interactions of microbes, such as bacteria, fungi and protozoa, occur due to the presence of a rich and diverse microbial food source (Bais et al., 2006). Among the interactions between plants and microbes, the role of rhizosphere bacteria (rhizobacteria) has been of great interest in efforts to stimulate plant growth, as some rhizobacteria, referred to as plant growth-promoting rhizobacteria (PGPR), have been shown to significantly increase crop yield in the greenhouses and fields (Kloepper et al., 2004). Fluorescent pseudomonads, in particular, are the focus of considerable attention by many research groups because this bacterial group has a short generation time and strong mobility, which allows it to rapidly colonize roots and elicit protection against soil-borne pathogens (Bakker et al., 2007). By comparison, *Bacillus* spp. and *Paneibacillus* spp. are considered less potent PGPR strains than Gram-negative bacteria, because bacilli typically have longer generation times and are isolated at lower population densities from plant roots than *Pseudomonas* spp. (Weller, 1988).

However, interest in endospore-forming bacilli has been revived recently in light of commercialization efforts with fluorescent pseudomonads, which revealed in early trials that biocontrol and biofertilizer products based on *Pseudomonas* spp. fail due to an insufficient shelf life (Kloepper et al., 2004). Moreover, the development of convenient molecular and biochemical tools to study bacterial determinants and plant responses involved in bacilli-elicited biological effects and plant growth have provided new insight into bacilli-plant interactions (Emmert and Handelsman, 1999).

In the early 1990s, three independent research groups reported a PGPR-elicited plant defense response in cucumber, carnation, and bean when PGPR was inoculated into plants at a separate site than the site of pathogen challenge to avoid direct contact between the two microorganisms. This response was termed induced systemic resistance (ISR) (Alström, 1991; van Peer et al., 1991; Wei et al., 1991). ISR represents an attractive means to manage plant disease because it can potentially protect a plant against a broad spectrum of pathogens, including fungi, bacteria, viruses, nematodes and even insects, and has a relatively long-lasting effect compared to conventional agrochemical application. Since its discovery, PGPR-elicited ISR has been employed extensively in intensively managed agricultural systems, such as greenhouses and fields, and the signal transduction mechanisms involved in ISR have been dissected, particularly in comparison to the response to necrotizing pathogens- or chemical-elicited systemic acquired resistance (SAR). *A. thaliana* has been used extensively as a model plant to investigate the underlying signal-

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ing pathways involved in ISR because it has a short life cycle, requires small space for growth, and is available specific gene knock-out mutants (van Loon, 2007). However, the results obtained from studying *A. thaliana* are not always comparable or relevant to crop plants. For example, root-associated *Pseudomonas fluorescens* WCS417r induces resistance, whereas *P. putida* WCS358 does not in carnation and raddish. However, neither strain WCS417r nor strain WCS358 elicits ISR in rice, but *P. fluorescens* strain WCS374 does, which indicates that the inability to acquire ISR in certain plants in response to certain bacterial strains is due to a lack of ISR determinants in the bacteria or in the plant roots, or alternatively, the inability of specific plant species to perceive certain microbial determinants (De Vleesschauwer et al., 2008; van Loon, 2007). To fully understand the mechanism of ISR in crop species, sophisticated approaches need to be used with targeted PGPR strains acting on specific plant species to elicit ISR. Unfortunately, mechanistic studies of ISR in crop plants, with the exception of rice, are not available due to limited genetic and molecular information about crop species.

Many varieties of hot and chilli pepper are raised as crop plants in many countries, including Korea (D'Arcy, 1986). Recently, in addition to expressed sequence tag (EST) data, studies of the mechanisms of defense signaling and virus-induced gene silencing in pepper have been reported (Chung et al., 2006; Kim et al., 2008; Ryu et al., 2004). Three case studies of ISR in pepper (*Capsicum annuum*) elicited by rhizobacteria and bacterial endophytes against *X. axonopodis* pv. vesicatoria and *Colletotrichum gloeosporioides* under greenhouse and field conditions have been reported (Jetiyanon et al., 2002, 2003; Kang et al., 2007; Kloepper et al., 2007). However, efforts to understand the mechanism of ISR in pepper have yet to be reported.

Early in the study of ISR, the concept of the priming of defense responses involved in rhizobacterium-mediated ISR was developed. Colonization of carnation roots with the rhizobacterium *P. fluorescens* WCS417 induces resistance against *Fusarium oxysporum* f.sp. *dianthi* (van Peer et al., 1991). In response to *P. fluorescens* WCS417, phytoalexin content in treated plants increases at a significantly faster rate at the site of inoculation following challenge with *F. oxysporum* f.sp. *dianthi* as compared to non-challenged plants. In bean (*Phaseolus vulgaris*), the rhizobacterium *B. pumilus* SE34 has been shown to induce ISR against *Fusarium oxysporum* f.sp. *pisi* (Benhamou et al., 1996). *B. pumilus* SE34 does not induce systemic resistance in bean plant root tissue before challenge with pathogen. Rather, upon inoculation with *F. oxysporum*, the root cell walls of bean plants with ISR are rapidly strengthened at sites of attempted fungal penetration by the apposition of large amounts of callose and phenolic compounds, thereby effectively preventing fungal ingress (Benhamou et al., 1996). In cucumber plants that were treated with the rhizobacterium *P. chlororaphis* O6 to elicit ISR against *Corynespora cassiicola*, the transcription of *CsGolS1*, which is involved in the synthesis of a common plant sugar, galactinol, was increased compared to water-treated control plants (Kim et al., 2008). While priming is believed to be a key aspect of ISR elicited by PGPR, details about the signaling cascades involved and the specific priming genes have yet to be uncovered. The evaluation of priming of ISR elicited by PGPR and the identification of priming gene(s) in crop plants such as pepper is a critical hurdle in the application of ISR technology to the field.

In the current study, our objective was to identify and characterize ISR priming gene(s) in pepper. Using a PGPR strain that elicited ISR in pepper in the greenhouse, we identified candidate priming genes as those genes whose expression was induced more strongly or faster in response to pathogen challenge in plants subjected to drench application of PGPR, as compared to control water-treated plants. The expression of two genes, *CaPR1* and *CaPR4*, was primed by PGPR. Further analysis of candidate genes that had previously been reported to be involved in pepper defense responses revealed that ethylene-dependent signaling is involved in ISR elicited in pepper by PGPR.

Materials and Methods

Plants and pathogen inoculation. Pepper plants (*C. annuum* L. cv. Bukang) were cultivated in a growth chamber at 25 °C under a 16 h/8 h light/dark photocycle. Isolation and screening of PGPR strains that elicited ISR in pepper were carried out as previously described (Kang et al., 2007). For pathogen challenge, a culture of the compatible bacterial pathogen *X. axonopodis* pv. vesicatoria (OD_{600}= 0.04 in 10 mM $MgCl_2$) was pressure-infiltrated into pepper leaves using a needleless syringe one week after drench-application of PGPR to the pepper roots, as described previously (Kang et al., 2007; Oh et al., 2005, 2006). The severity of symptoms was scored from 0 to 5 in the inoculates site as follows: 0, no symptoms; 1, yellowish color; 2, chlorosis only; 3, necrosis and chlorosis; 4, partial necrosis of the inoculated area; and 5, complete necrosis of the inoculated area (Fig. 1C inset). Bacterial pathogens were cultured overnight at 28 °C in LB medium supplemented with the appropriate antibiotics. Chemical treatment of pepper roots was performed as described previously (Kang et al., 2007). As a positive control, roots were treated with 0.5 mM benzothiadiazole (BTH) that was kindly provided by Syngenta co. Leaves were harvested at the indicated times and then frozen immediately in liquid nitrogen for total RNA extraction. Intact pepper leaves

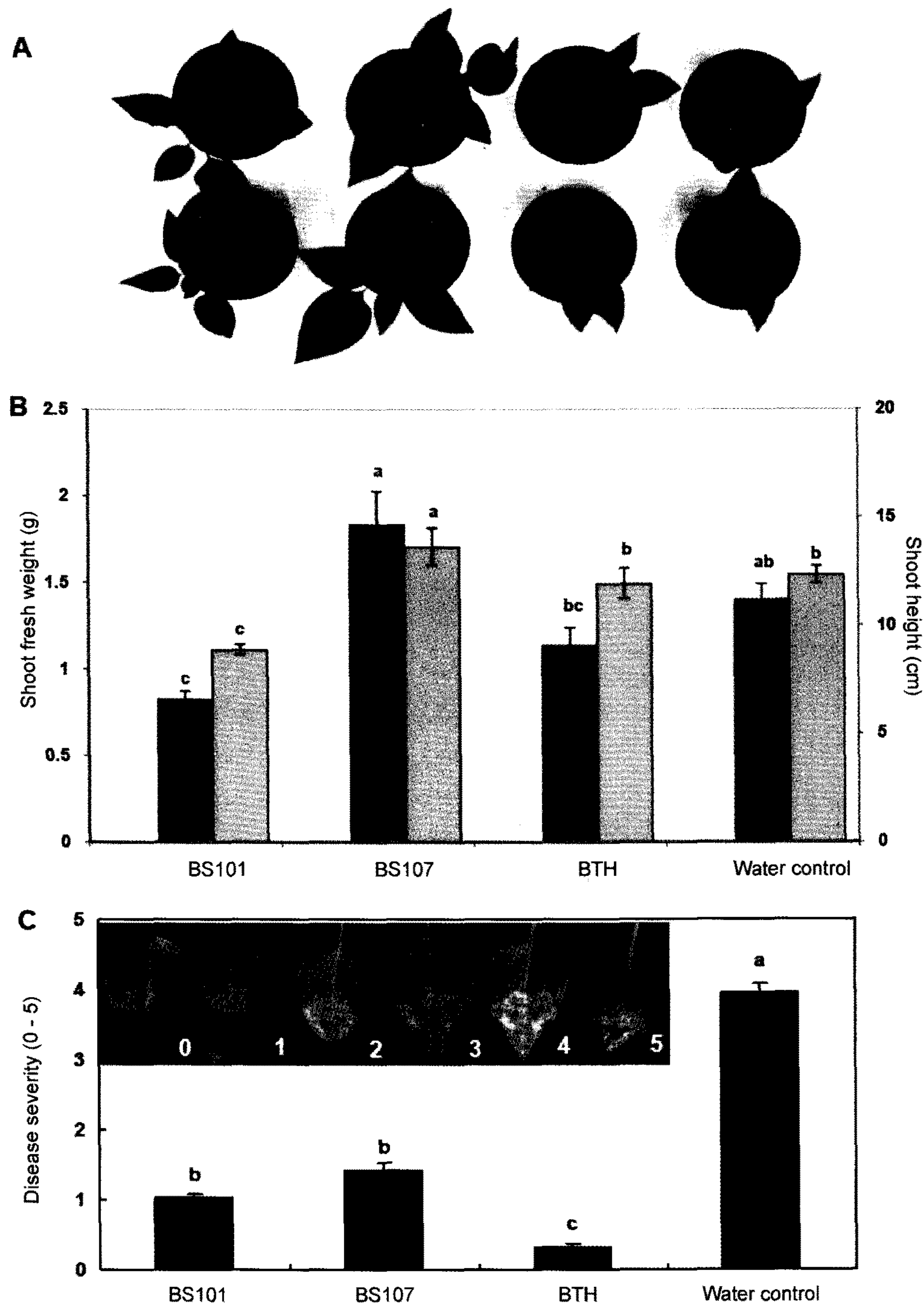

Fig. 1. Effect of *Bacillus cereus* strains BS101 and BS107 on ISR and plant growth. A) Representative photographs taken 12 days after spray-inoculation of *X. axonopodis* pv. vesicatoria (10^5 cfu/ml). As a positive control, plants were treated with 0.5 mM BTH. B) Shoot height (gray bar) and shoot fresh weight (black bar) were assessed 3 weeks after inoculation with strain BS101 or strain BS107 into pepper plants. C) Disease severity (0 - 5) as an indicator of ISR was measured 7 days after pathogen challenge. 0, no symptoms; 5, severe necrosis (inset indicates disease index). Different letters like a, b and c in (B) and (C) indicate statistically significant differences as compared to water-treated control plants ($P = 0.05$). Error bars indicate the standard error mean.

were used for non-stress treatments. Following inoculation with pathogen, plants were returned to the growth chamber and leaf tissue was harvested 0, 3, 6, 12, 24, 48, and 72 h after inoculation with *X. axonopodis* pv. vesicatoria, and then used for isolation of total RNA.

Isolation of total RNA, Reverse Transcriptase (RT)-PCR, and quantitative (Q)-RT-PCR. Total RNA was isolated

from inoculated leaf tissue according to the protocol of Kim et al. (2006). Total RNA was treated with 1 U of RNase-free DNase (Promega, USA) for 10 minutes (min) at 37 °C and then subjected to a second round of purification using the TRI reagent. First-strand cDNA synthesis was carried out in 20 l of AccuPower PCR PreMix (Bioneer, Korea) containing 1 g of DNase-treated total RNA, oligo (dT) primers and Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Invitrogen, USA). PCR reactions were carried out according to the manufacturer's instructions.

The candidate priming gene was analyzed using the following primers: 5'-ACTTGCAATTATGATCCACC-3' (CaPR1-F) and 5'-ACTCCAGTTACTGCACCATT-3' (CaPR1-R). Additional genes and the primer sets used to detect them were as follows: *CABGLU*, 5'-TTTTAGCTATGCTGGTAATCCGCG-3' and 5'-AAACCATGAGGACCAACAAAAGCG-3'; *CAChi2*, 5'-ATATTCCGAATGTCTAAAGTGGTAC-3' and 5'-ATTGGACGATGGAAGCCATCACCAG-3'; *CaPR4*, 5'-AACTGGGATTTGAGAACTGCCAGC-3' and 5'-ATCCAAGGTACATATAGAGCTTCC-3'; *CaPR10*, 5'-ATGTTGAAGGTGATGGTGGTGCTG-3' and 5'-TCCCTTAGAAGAACTGATACAACC-3'; *CaSIG4*, 5'-ACTTCCTTGCACAGATTTCAACTG-3' and 5'-AAGGGCCTTACAAACTGCACTTTC-3'; *CaLTP*, 5'-TTGCCTCCCTTATCTGCAGAATCG-3' and; 5'-TAATATAGAAGTGCAGCTTGGCAGG-3'; *CaPIN*-II, 5'-CTCGGAATTGTGATACAAGAATTGC-3' and 5'-AAGGTACGTACGGCTGCTTCTTTAC-3'; *CaAccOx*, 5'-AGAAAGCTGCAGAGGAAAGCAAAC-3' and 5'-TGAGATGCAACCGTTACTCCTATAC-3'; *Cadhn*, 5'-AGTGATCATTCTTTGCTTTATTCTTAC-3' and 5'-AACATTCATTCCCATGCTATC-3'.

As a control to ensure that equal amounts of RNA were analyzed in each experiment, we also analyzed *CaActin* using the primers 5'-TTGGACTCTGGTGATGGTGTG-30 and 50-AACATGGTTGAGCCACCACTG-3'. Candidate priming genes were amplified from 100 ng of cDNA by PCR using an annealing temperature of 55 °C.

Amplified PCR products were separated by 2% agarose gel electrophoresis. Q-RT-PCR was carried out using a Chromo4 real-time PCR system (BIO-RAD). Reaction mixtures (20 μl) contained 10 μl of 2×Brilliant SYBR Green QPCR master mix (BIO-RAD), cDNA and 100 pM each primer. The thermocycle parameters were as follows: initial polymerase activation, 10 min at 95 °C; then 40 cycles of 30 seconds (s) at 95 °C, 60 s at 55 °C and 30 s at 72 °C. Conditions were determined by comparing threshold values in a series of dilutions of the RT product, followed by a non-RT template control and a nontemplate control for each primer pair. Relative RNA levels were calibrated and normalized to the level of *CaACT1* mRNA (GenBank accession no. AY572427).

Statistical analysis. Analysis of variance for experimental datasets was performed using JMP software version 5.0 (SAS Institute Inc., USA). Significant effects of treatment were determined by the magnitude of the F value (P= 0.05). When a significant F test was obtained, separation of means was accomplished by Fisher's protected LSD at P= 0.05.

Results and Discussion

To better understand the mechanisms involved in ISR elicited by PGPR in crop plants, we collected 741 bacilli isolates from the root systems of crop plants, including pepper, tomato and Chinese cabbage grown in southern Korea (Ryu et al., 2005). Through secondary screening, we selected strains BS107 and BS101 for further analysis based on their capacity to reduce disease symptoms in pepper one week after leaf infiltration by *X. axonopodis* pv. vesicatoria (Fig. 1C). Disease severity in pepper plants that were subjected to root application of strain BS101 and BS107 was 1.0 and 1.5, respectively, while that of water-treated control plants was 4.0. To confirm the activation of ISR, spontaneous rifampicin-resistant bacteria of strains BS107 and BS101 were generated and assessed whether spatially separated from the challenge pathogen at the site of pathogen inoculation (Ryu et al., 2004). Bacterial colonies introduced into the root were not detected on the pepper leaf where disease symptoms appeared (data not shown) indicating that direct antibiosis between two strains BS107 and BS101 and *X. axonopodis* pv. vesicatoria was not occurred. As a positive control, root treatment with 0.5 mM BTH protected plants almost completely against *X. axonopodis* pv. vesicatoria (Fig. 1C). In addition to ISR, we evaluated whether the selected *Bacillus* spp. isolates increased plant growth. Application of strain BS107 increased shoot height and shoot fresh weight as compared to strain BS101 and water-treated controls (Fig. 1A, B). Thus, since one of our criteria to select target PGPR strains was plant growth-promoting properties and ISR, rather than general saprophytic properties, we chose strain BS107 for further analysis.

The growth of BTH-treated pepper plants was similar to water-treated control plants, which indicated that 0.5 mM BTH does not have a negative effect on plant fitness, as was previously reported for the chemical inducers BTH (known as Actigard® in USA and BION® in Europe) and DL-3-Aminobutyric acid (BABA) (Heil et al., 2000; van Hulten et al., 2006). As shown in the Fig. 1A and B, the capacity of plant growth promotion and ISR capacity were significantly increased following soil application of strain BS107 as compared to the application of strain BS101 and water

treatments. According to the theory of "allocation fitness cost", in many cases, ISR elicited in response to chemical elicitors requires "massive plant energy", which causes reductions in plant size and growth. BTH-treated barley exhibits reduced plant growth and decreased seed production in response to chemical elicitors, and the reduction in plant growth is more significant under nitrogen shortage conditions (Heil et al., 2000). The authors concluded that the reduction in plant growth was due to allocation fitness costs resulting from "metabolic competition between processes involved in plant growth and the synthesis of defense-related compounds" (Heil, 1999). Following the initial report of this phenomenon, many groups have observed similar effects. However, details of the underlying molecular and biochemical mechanisms are as yet unknown (Heil et al., 2001). PGPR often promote plant growth as well as elicit ISR in tomato, cucumber, pepper, tobacco and Arabidopsis (Kang et al., 2007; Murphy et al., 2003; Raupach and Kloepper, 1998; Ryu et al., 2007; Zhang et al., 2004). Recently, it was shown that two endophytes, *P. rhodesiae* PS4 and *P. ananatis* PS27, elicit ISR and increase shoot fresh weight under greenhouse conditions (Kang et al., 2007). Strains PS4 and PS27 also decrease disease severity caused by *X. axonopdis* pv. vesicatoria to 34% and 26%, respectively, of that of seen in water-treated control plants (Kang et al., 2007). Similar to the effects of strains PS4 and PS27, plants treated with strain BS107 exhibited mild clorosis or no symptoms 7 days after pathogen infiltration of the leaf, whereas plants treated with water exhibited severe necrosis (Fig. 1A, C). Reducing the concentration of BTH to 0.5 mM, a concentration that was previously shown to elicit ISR, resulted in a decreased effect on reduction of plant growth, as compared to previous results (data not shown). The observation that PGPR application enhanced plant growth and ISR cannot be explained based on the concept of allocation fitness cost. However, to date, an explanation for this effect of PGPR on plant growth has been elusive, despite several reports on the molecular mechanism of ISR (Kloepper et al., 2007; van Hulten et al., 2006). For example, recently, the effects of a commercial preparation consisting of *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a, termed BioYield®, on photoperiod-dependent plant growth and ISR in pepper were assessed. In this pioneering experiment, BioYield® promoted plant growth only in early January, which indicated that different periods of daytime (photoperiods) affect the ability of PGPR to induce plant growth. The authors also assessed the capacity of PGPR to elicit ISR against *X. axonopdis* pv. vesicatoria in pepper and tomato (Kloepper et al., 2007). Interestingly, a short photoperiod (6 h of light) abolished the plant growth-promoting capacity of PGPR, but had no effect on its ability to elicit ISR. Thus, it appears that the induction of plant growth-specific biochemical and signaling pathways by PGPR is sensitive to photoperiod. Recent studies using Arabidopsis revealed that pre-treatment with relatively low concentrations (5 and 10 μg/L) of BABA, referred to as priming, resulted in a weak effect on plant growth and seed production as compared to the direct induction of ISR with high doses (40 and 60 μg/L) of BABA. Treatment with high doses of BABA also resulted in significant reductions in fitness parameters, which indicates that the priming of ISR maintains plant fitness when pathogens attack. In addition to chemical-induced priming of ISR, priming effects can also be elicited by beneficial bacterial (Akram et al., 2008; Kim et al., 2004). In tomato, *P. putida* BTP1 treatment resulted in the accumulation of systemic phytoalexin only after pathogen challenge (Akram et al., 2008). Subtractive hybridization of total mRNA from cucumber plants subjected to root colonization by *P. chlororaphis* O6 and control plants treated with water before and after challenge with a fungal pathogen, *C. cassiicola*, revealed that six distinct genes, including the genes for a hypersensitive-induced reaction protein and a signal recognition particle receptor, were expressed more rapidly and at higher levels only after pathogen inoculation in strain O6-treated plants as compared to water-treated control plants (Kim et al., 2004). More recently, it was shown that *P. fluorescens* WCS374r elicits ISR against *M. oryzae* in rice, and that this effect is dependent on the priming of pseudobacin secretion by strain WCS374r (de Vleesschauwer et al., 2008).

Candidate marker genes for ISR priming by PGPR have yet to be identified in solanaceous plants. To identify ISR priming genes in pepper, we examined pepper defense-related genes that have recently been characterized in molecular and biochemical studies of compatible and incompatible interactions. We examined the expression levels of *CaPR1*, *CaBPR1* (Kim et al., 2000), *CaPR4* (Park et al., 2001), *CaPR10* (Park et al., 2004b), *CaTin1*, *CaTin1-2* (Shin et al., 2003), *CaCYP* (Kim et al., 2006), *CaPF1* (Yi et al., 2004), *CaGLP1* (Park et al., 2004), and *CaALaAT* (Kim et al., 2005). *CaPR1*, *CaPR4*, and *CaGLP1* were identified as candidate priming genes following challenge by avirulent pathogen infiltration in pepper (Kim et al., 2000; Park et al., 2001, 2004a). The expression of *CaBPR1* mRNA is strongly induced by the incompatible interaction of pepper plants with *X. axonopodis* pv. vesicatoria. *CaBPR1* mRNA expression in *X. axonopodis* pv. vesicatoria- and *Phytophthora capsici*-infected leaves is involved in the resistance response mediated through ethylene biosynthesis The expression of *CaBPR1* mRNA is induced by treatment with BABA or salicylic acid (SA), but not by wounding or treatment with jasmonic acid (JA) alone. As described by Park et al. (2004a), *CaGLP1* (PR-16) is induced in pepper

leaves infected with *X. axonopodis* pv. vesicatoria and Tobacco mosaic virus (TMV_0), and by SA and ethylene treatment, but not by JA. *CaCYP1* is involved in the SA-dependent defense pathway and is induced by SA and abscisic acid (ABA). Using a virus-induced gene silencing (VIGS)-based reverse genetics approach, gene silencing of *CaCYP1* in pepper plants was shown to enhance susceptibility to *X. axonopodis* pv. vesicatoria and reduce the expression of the defense related genes *CaLTP1*, *CaSIG4* and *Cadhn*. The expression of *CaAlaAT1* in pepper plants is increased by the incompatible interaction of pepper with TMV-P_0 and *X. axonopodis* pv. vesicatoria, similar to

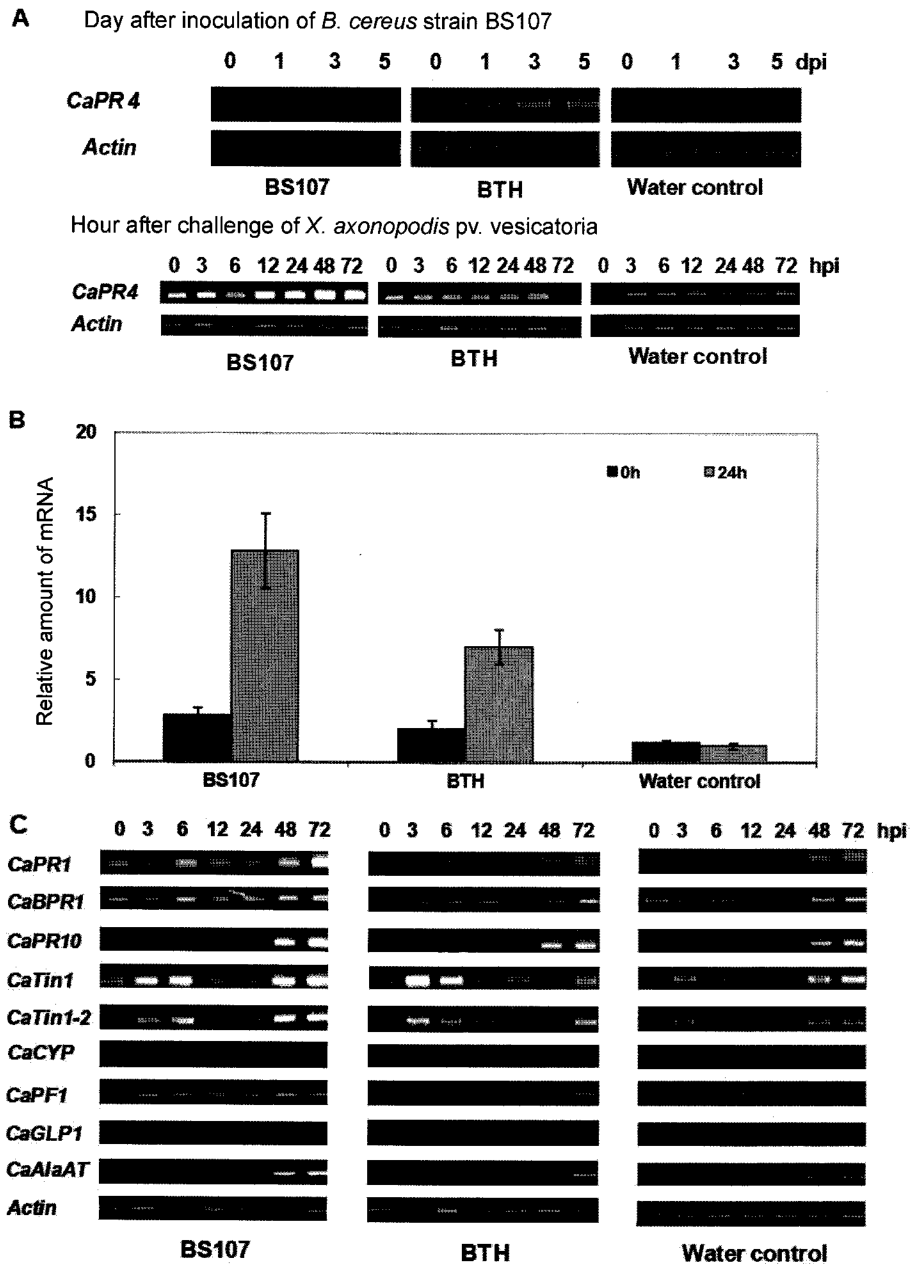

Fig. 2. Elicitation of defense-related gene expression by strain BS107 in pepper following bacteria inoculation and pathogen challenge. A) *CaPR4* expression 0, 1, 3, and 5 days after strain BS107 inoculation on the pepper roots (upper panel); *CaPR4* expression 0, 3, 6, 12, 24, 48, and 72 hours after leaf infiltration of *X. axonopodis* pv. vesicatoria 7 days after BS107 treatment (bottom panel) B) Validation experiment of the *CaPR4* gene expression 0 and 24 hours after leaf infiltration of *X. axonopodis* pv. vesicatoria 7 days after BS107 treatment quantitative RT-PCR. Relative expression was calculated and then normalized to *CaActin* expression, which was set as 100%. C) Expression of the selected pepper defense related-genes, *CaPR1*, *CaBPR1*, *CaPR10*, *CaTin1*, *CaTin1-2*, *CaPF1*, *CaCYP*, *CaGLP1*, and *CaALaAT* was analyzed by RT-PCR. Amplified products were separated by gel electrophoresis and visualized by ethidium bromide staining. As a positive control, plants were treated with 0.5 mM BTH.

CaGLP1. *CaAlaAT1* encodes a putative alanine aminotransferase and is involved in leaf senescence. Expression of *CaAlaAT1* is triggered by SA and ethylene, but not by methyl jasmonate (MeJA). The expression of *CaPR4* in pepper plants is induced by MeJA, ethephone and wounding, but not by SA. *CaPR4* expression is also enhanced during the defense response to TMV-P_0. The ERF/AP2 transcription factor *CaPF1* in pepper plants responds to both biotic and abiotic stresses induced by MeJA treatment, ethephon and cold stress. Moreover, *CaPF1* transgenic Arabidopsis exhibits enhanced defense responses against *P. syringae* pv. tomato DC3000 and increased tolerance to cold stress. The expression of *CaTin1* and *CaTin1-2* is increased during the induction of SAR to TMV-P_0 and *X. axonopodis* pv. vesicatoria infection. *CaTin1* and *CaTin1-2* in pepper plants share a bidirectional promoter, and exhibit 80.4% similarity and 58.0% identity at the amino acid level. Both genes respond only to ethylene treatment, not to SA, MeJA, ABA or NaCl. A hot pepper plant cDNA clone, encoding CaPR-10, and the plant transcription factor CaWRKY are induced by the incompatible interaction with TMV-P_0 and *X. axonopodis* pv. vesicatoria, but not by the compatible interaction. *CaWRKY*, *CaPR-10*, and *CaPKc1*, which is expressed during hypersensitive responses (HR) in pepper leaves in response to infection with *Tobacco mosaic virus* (TMV)-P_0, are responsive to SA, JA, ethylene (ET), wounding and sodium stress (Kim et al., 2005; Park et al., 2002, 2004).

To understand mechanism on strain BS107-mediated ISR on pepper, we focused on priming effect of the defense-related genes after pathogen challenge. In the current experiment, we assessed *CaPR4* expression as a marker gene for induction of plant defense after strain BS107 inoculation and after pathogen challenge with *X. axonopodis* pv. vesicatoria (Fig. 2A). The inoculation of strain BS107 on the root did not alter the transcription of *CaPR4* gene while 0.5 mM BTH treatment significantly increased the expression compared to water control treatment (Fig. 2A). To confirm these results, we used Q-RT-PCR to analyze the priming of *CaPR4* expression by strain BS107 (Fig. 2B). Following normalization of the expression levels of each gene to constitutively expressed *CaActin*, we observed that the expression of *CaPR4* 24 h after pathogen challenge in pepper plants treated with BS107, 0.5 mM BTH and water increased 4.52-, 3.48- and 0.80-fold, respectively, as compared to 0 h (Fig. 2B). These results strongly indicated that the expression of *CaPR4* is primed by treatment with either BS107 or BTH. When compared to water-treated plants 24 h post-challenge, BS107 treated pepper roots exhibited a 12-fold increase in *CaPR4* expression, while BTH-treated plants exhibited a 7-fold increase (Fig. 2B). These results point to *CaPR4* as a novel priming gene involved in PGPR-elicited ISR in pepper. These results strongly indicated that *CaPR4* expression in pepper is primed by BS107 treatment.

Among the nine genes that we selected besides *CaPR4* gene, we detected slightly stronger and more rapid transcription of *CaPR1* following pathogen challenge (Fig. 2C) in BS107-treated plants as compared to water control, whereas the expression pattern of *CaPR10* was similar under all treatment conditions. These results suggested that *CaPR1* and *CaPR4* are candidate priming genes involved in ISR elicited by strain BS107 in pepper. The expression of *CaTin1* and *CaTin1-2* was strongly induced in plants treated with strain BS107 and BTH as compared to water-treated control plants 3 h after pathogen challenge. After 12 h, the expression of *CaTin1* and *CaTin1-2* was reduced to pre-challenge levels. The expression of *CaBPR1* in BS107-treated plants was slightly increased 3 h after pathogen challenge, whereas the expression pattern of *CaBPR1* was

Table 1. Pepper defense-related genes analyzed in the current study

Genes \ Treatment	SA	JA	ET	ABA	H_2O_2/ MV	NaCl	Wound	Site of expression[a]	Time (h)	References
CaCYP1	++		–	++				L	24	Kim et al. 2006
CaAlaAT1	+++	–	+++					L	12	Kim et al. 2005
CaGLP1	+++	–	++			–	–	L	6	Park et al. 2003
CaPF1		+++	+++			++		L	6	Yi et al. 2004
CaPR4	–	++	++				++	F,L,R	6	Park et al. 2000
CaTin1	–	–	+					L	8	Shin et al. 2003
CaTin1-2	–	–	+	–	++	–		L	6	Shin et al. 2003
CaBPR1	+	–	+++				–	R,F,GF		Kim et al. 1999
Ca-COX-1	+	++	+		+		+	L	6	Kim et al. 2002
CaPR10	++	+++	+++		+++	++		L		Park et al. 2002

+,– Gene expression was measured at 6 and 24 h after chemical treatment. +, weak expression; +++, strong expression; –, not expressed.
[a] Gene expression in the indicated part of the plant. F, fruit; L, leaf; R, root; S, stem; RF, red fruit; GF, green fruit, SA, salicylic acid; JA, jasmonic acid; ET, ethylene; ABA, abscisic acid; MV, methyl viologen.

similar in plants treated with BTH and water treatments. Genes involved in the SA-dependent signaling pathway (*CaCYP1, CaAlaAT1* and *CaGLP1*) were expressed at similar levels in BS107-, BTH-, and water-treated plants. The expression of *CaPF1*, which is involved in JA- and ET-mediated signaling pathways, did not differ among treatment conditions. These results indicated that the priming of *CaPR4, CaTin1* and *CaTin1-2* expression by strain BS107 plays a role in ISR against pathogen in pepper, and that the ET-mediated response pathway is involved in the induction of ISR by strain BS107. Similary, in *P. chlororaphois* O6 pretreated plants, galactinol content increased 12 hours earlier following inoculation with *C. cassiicola* as compared to water pretreated (control) plants, whereas there was no significant difference between control and *P. chlororaphois* O6-treated plants in the absence of *C. cassiicola* challenge (Kim et al., 2008). The rhizobacterium *P. fluorescence* WCS374r induces systemic resistance in rice against *Magnaporthe oryzae* (De Vleesschauwer et al., 2008). Root colonization by *P. fluorescence* WCS374r results in a more rapid accumulation of hydrogen peroxide induced by increased pseudobactin (*Psb374*) than in control plants at sites of pathogen entry (De Vleesschauwer et al., 2008). Recent reports obtained from transcriptome analysis of Arabidopsis indicate that PGPR prime host plants to respond to pathogens before a direct attack (Verhagen et al., 2004).

Conclusion

The root-colonizing *Bacillus cereus* BS107 that was selected through massive screening for bacteria to have ISR capacity against a bacteria pathogen, *X. axonopodis* pv. vesicatoria as well as augmenting plant growth primed defense-related genes on pepper resulting that the transcriptional expression of defense genes such as *CaPR4*, *CaPR1*, and *CaTin1* did not changed after strain BS107 treatment on the pepper root but was strongly and rapidly upregulated subsequent challenge by the pathogen on the leaf. Our results indicate that priming of defense genes acts critical role on *Bacillus* spp.-elicited ISR on crop plant.

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