Characterization of the Genes Involved in
Induced Systemic Resistance in Cucumber Plants

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Abstract - Root colonization by a rhizobacterium, *Pseudomonas chlororaphis* O6, elicited induced systemic resistance (ISR) in the leaves of cucumber plants against fungal and bacterial pathogens. To understand the role of unique genes during strain O6-mediated ISR, a suppressive subtractive hybridization method was undertaken and led to isolation of twenty-five distinct genes. The transcriptional levels of all the genes showed an increase much earlier under O6 treatment than in water control plants only after challenge with pathogen, while no difference detected on the plants without pathogen challenge. This suggests that O6-mediated ISR is associated with the priming phenomenon, an enhanced capacity for the rapid and effective activation of cellular defense responses after challenge inoculation.

Key words - *Corynespora cassiicola*, Cucumber, Powdery mildew, Priming, *Pseudomonas chlororaphis* O6, *Pseudomonas syringae* pv. *lachrymans*, Root colonization

Introduction

Plants have evolved various mechanisms by which they defend themselves against pathogens. Besides their constitutively preformed barriers, plants can induce resistance against pathogen attack upon appropriate stimulation experienced by the plant prior to contact with the pathogen (s). To date, two types of induced resistance have been defined based on differences in the stimulation: systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Ryals *et al.*, 1996; van Loon *et al.*, 1998). SAR is known to be elicited by necrosis-inducing pathogens or by chemicals such as salicylic acid (SA) and its analogues (Ryals *et al.*, 1996). SAR is characterized by an early increase in endogenously synthesized SA, and the concomitant activation of a set of SAR genes (Ryals *et al.*, 1996; Sticher *et al.*, 1997). Similarly, ISR elicited by root-colonizing bacteria (rhizobacteria) capable of reducing disease in above-ground plant parts through a plant-mediated mechanism has been demonstrated in many plant species. A trait of ISR is effective against a broad spectrum of plant pathogens, including oomycetes, fungi, bacteria and viruses (van

Loon *et al.*, 1998). These large numbers of rhizobacteria thrive on the surface or inside of the plant root where nutrients (plant exudates) are provided. Among rhizobacteria, *Pseudomonas chlororaphis* O6 which was isolated from the roots of field-grown wheat and reported to produce phenazines, also elicited ISR in tobacco by showing protection against two foliar bacterial pathogens, *P. syringae* pv. *tabaci* and *Erwinia carotovora* subsp. *carotovora* (Radtke *et al.*, 1994; Spencer *et al.*, 2003), and in cucumber against the leaf spot fungus *Corynespora cassiicola* (Kim *et al.*, 2004).

Recently, to identify ISR-related genes, Verhagen *et al.* (2004) surveyed the transcriptional response of over 8,000 Arabidopsis genes during rhizobacteria-mediated ISR. None of the 8,000 genes tested showed a consistent change in expression in response to effective colonization of the roots by WCS417r in the leaves, indicating that the onset of ISR in the leaves is not associated with detectable changes in gene expression. After challenge inoculation of the bacterial speck pathogen *P. syringae* pv. *tomato* DC3000 on the WCS417r-induced plants, interestingly, 81 genes showed an augmented expression patterns in the leaves (Verhagen *et al.*, 2004). The result suggests that these genes were primed to respond faster or more strongly when expose to pathogen attack. The capacity for augmented defense expression is called "priming",

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which becomes apparent only after challenge inoculation on the distal, untreated plant parts, and the phenomenon has been demonstrated in different plant species against pathogens, insects, and even abiotic stresses (Conrath et al., 2002). Here we demonstrate that P. chlororaphis O6-mediated ISR is also associated with several genes that showed significantly faster induction in cucumber leaves than in a water-treated control after challenge inoculation with C. cassiicola.

Materials and Methods

Rhizobacterial inoculation on cucumber seeds and the plant growth conditions

The rhizobacterium P. chlororaphis O6 was grown on King's medium B (KB) agar plates for 24h at 28℃. Subsequently, bacterial cells were collected, resuspended in 0.02 M phosphate buffer (pH 7.0), and an equal volume of 2% methylcellulose (Sigma) was then added. The bacterial suspension was mixed with cucumber seeds (Cucumis sativus L., variety Baekseong), resulting in 5×10^8 to 1×10^{10} CFU per seed, and then air-dried overnight in a laminar flow hood (Raupach and Kloepper, 1998). Prior to mixing with strain O6, the cucumber seeds were surface-sterilized with a solution of 10% hydrogen peroxide to eliminate seed-infesting organisms. Each cucumber seed coated with strain O6 was planted to a depth approximately 1cm in a 10cm³ polyvinyl pot containing 500cm³ of sterilized soil-less growing medium (peat moss 7 : vermiculite 3). Plants were cultivated in a growth chamber with a 10h light (25℃)/ 14h dark (20℃) cycle at 70% relative humidity with daily watering. One week after sowing when cotyledons were sprouted, a 20mℓ of the rhizobacterial suspension (1×10^8) in sterile water was once applied around the seedling per pot.

Disease assessment on cucumber plants

Four-week-old cucumbers in pots were challenge-inoculated with Corynespora cassiicola by spraying a conidial suspension of the fungus at a concentration of 5×10^4 spores/ml of sterile water, and were kept in a growth chamber at 100% relative humidity for 12h. The fungus was grown on Czapek Solution Agar (CSA; Difco) plates for 2 weeks at 28℃ in an incubator; aerial mycelia were removed by gently rubbing the surface with a writing brush in tap water, and the samples were then air-dried in a laminar flow hood. To produce the conidia, the plates were placed in an incubator for 1 week at 28℃ with illumination by fluorescent lamps (about 2,000

Lux). Four to six days after challenge inoculation, disease severity was assessed by determining the number of target leaf spots per leaf. Four-week-old cucumbers were challenge-inoculated with Pseudomonas syringae pv. lachrymans by spraying the bacterial suspension of 1×10^8cfu/mℓ. One to two days after challenge inoculation, disease incidence was rated by counting the number of symptomatic leaf spots per inoculated leaf. The bacterial cells were grown at 28℃ with shaking at 200rpm in Luria-Bertani (LB) broth to OD600nm = 2.0, centrifuged, and resuspended in sterile water.

Subtractive hybridization and differential screening

A full-length cDNA library was constructed using 5μg of poly (A)⁺ RNA extracted from the O6-colonized cucumber leaves 12h after inoculation with C. cassiicola. Total RNA was isolated from cucumber leaves by the Phenol/SDS/LiCl method (Zhu et al., 1998), and then Poly (A)⁺ RNA was extracted from the total RNAs according to the Oligotex mRNA batch procedure (Quiagen). The enriched cDNA library was constructed using a cDNA Synthesis Kit/ ZAP-cDNA® Synthesis Kit/ ZAP-cDNA® Gigapack® II Gold Cloning Kit (STRATAGENE) according the protocol of the manufacturer. Subtractive hybridization was performed using the CLONETECH PCR-Select™ cDNA Subtraction Kit. For subtraction, double-stranded cDNAs were synthesized separately from Poly (A)⁺ RNAs extracted from C. cassiicola-infected cucumber leaves for 12h with and without strain O6 colonization. Differential screening between colonized and uncolonized plants was performed using Hybond-N⁺ membrane (Amersham Pharmacia), PCR-Select Differential Screening Kit (CLONTECH), and ExpressHyb Hybridization Solution (CLONTECH). Candidate cDNAs in pBluscript II plasmid were excised from the candidate plaques according to the single-clone excision protocol (STRATAGENE), and expression of the candidate gene was then analyzed by Northern blotting or by real-time RT-PCR.

Northern blot analysis

Twenty micrograms of total RNA, which had been extracted from the test plants after inoculation with the corresponding pathogen by the Phenol/SDS/LiCl method, were separated on a 1.2% formaldehyde/ agarose gel and transferred onto a nylon membrane. The RNA blot was probed with the P³²-labeled cDNA probe and washed at high stringency. Hybridization and P³²-labeling of cDNA were performed according to the standard procedures (Sambrook et al., 1989).

Table 1. Induced systemic resistance elicited in the leaves of cucumber plants by root colonization of *Pseudomonas chlororaphis* O6 against the foliar target leaf spot caused by *Corynespora cassiicola* and against the angular leaf spot caused by *Pseudomonas syringae* pv. *lachrymans*.[a]

Challenge inoculation with	Total lesion number per a leaf		Percent protection
	O6(-)	O6 (+)	
C. cassiicola	77.3 ± 21.7	19.3 ± 12.4	75.0
P. s. pv. *lachrymans*	69.5 ± 23.5	17.4 ± 14.8	74.9

[a] Percent protection of the disease was estimated 4 to 6 days after challenge inoculation by counting the total number of lesions produced on the fully expanded 2nd leaf with (O6+) and without (O6-) the rhizobacterial colonization. Each experiment was repeated three times. One representative trial included ten plants for each treatment. The data are displayed as mean ± standard deviation.

Results and Discussion

Plants develop an enhanced defensive capacity that is so called induced systemic resistance (ISR) after colonization of the roots by selected strains of nonpathogenic rhizobacteria. Root colonization of the rhizobacterium *P. chlororaphis* O6 elicited ISR in tobacco by showing protection against two foliar bacterial pathogens, *P. syringae* pv. *tabaci* and *Erwinia carotovora* subsp. *carotovora* (Spencer *et al.*, 2003), in cucumber against the leaf spot fungus *Corynespora cassiicola* (Kim *et al.*, 2004), and in cucumber against a bacterial leaf spot pathogen *P. syringae* pv. *lachrymans* (Table 1). The average protection rate accomplished by the *P. chlororaphis* O6 was approximately 75% (Table 1). Root colonization by the strain O6 also significantly decreased the number and size of naturally occurring powdery mildew spots compared to controls where the plants were not colonized by strain O6 (Fig.1). Initial onset of disease was delayed, and final incidence was reduced in the O6 colonized plants. We did not intend to inoculate the fungus but naturally occurred by an accident in a greenhouse. These results suggest together with previous results (Kim *et al.*, 2004; Spencer *et al.*, 2003) that *P. chlororaphis* O6 elicit ISR which is effective against a broad spectrum of plant pathogens.

To detect specific genes involved in the *P. chlororaphis* O6-mediated ISR, we employed a suppressive subtractive hybridization method using mRNAs extracted from *C. cassiicola*-inoculated cucumber leaves with or without pre-treatment of *P. chlororaphis* O6 on the roots, respectively. This result led to isolation of twenty-five distinct genes. The selected clones were designated as *CsISR1* to *CsISR25* (corresponding to *Cucumis sativus* Induced Systemic Resistance genes). Expression patterns by pre-treatment of *P. chlororaphis* O6 and pathogen challenge with *C. cassiicola*, and putative functions of the genes are listed in Fig. 2. The twenty-five isolated genes showed no consistent change in expression in the leaves in response to effective root-colonization by strain O6, since no differences were detected in the results of Northern blot between O6- and O6+ without pathogen challenge (0h) (Fig. 2). After challenge inoculation with *C. cassiicola* on the ISR-expressing cucumber leaves, all of the twenty-five genes showed augmented
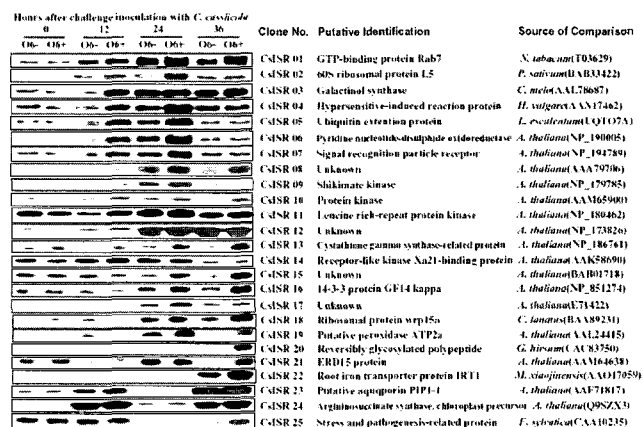

Fig. 2. Specific genes involved in *P. chlororaphis* O6-mediated induced systemic resistance in cucumber plants. The genes were obtained by subtractive hybridization and differential screening of a cDNA library constructed using mRNA extracted from leaf tissues treated with strain O6 on roots 12h after challenge inoculation with *C. cassiicola*. Northern blot analysis of each gene is shown using total RNA extracted from the cucumber leaves 0, 12, 24, and 36 h after challenge inoculation with (O6+) and without (O6-) colonization. Inferred properties of proteins encoded by the genes are described on the right.

Fig. 1. Protection of powdery mildew disease by *P. chlororaphis* O6-mediated induced systemic resistance in cucumber plants. The disease was naturally occurred in a greenhouse by a powdery mildew fungus on cucumber leaves with (B) and without (A) root colonization by the strain O6.

expression patterns that indicated a much faster response in time after pathogen attack (Fig. 2). For an example, the transcript of the *CsISR3* clone was accumulated after challenge inoculation, showing the highest accumulation at 24h without strain O6-mediated ISR (O6-). However, the transcript accumulated approximately 12h faster in roots colonized with O6 (O6+) than in control leaves (O6-), showing nearly the maximum level at 12h after challenge inoculation (Fig. 2). Therefore, increased accumulation of the gene transcript occurred approximately 12 hours earlier in the O6-colonized, challenge-inoculated leaves compared with only challenge-inoculated leaves.

Plants are constantly attacked by a multitude of disease-causing organisms in nature, including bacteria, fungi, viruses, and nematodes. Plants resist such pathogen attacks using a broad range of defense mechanisms. These include preexisting physical and chemical barriers, as well as inducible defense responses that become activated after pathogen infection. Since Wei *et al.* (1991) and van Peer *et al.* (1991) reported that some selected strains of nonpathogenic rhizobacteria activate plant defense responses against fungal pathogens in cucumber and carnation, many previous studies have reported this elicitation of plant defense, referred to as ISR. Application of the rhizobacterium *P. chlororaphis* O6 to the roots elicited ISR in cucumber and tobacco against different fungal and bacterial pathogens (Table 1) (Kim *et al.*, 2004; Spencer *et al.*, 2003). ISR-expressing plants are shown to be primed for augmented expression of a specific set of pathogen-responsive genes (Conrath *et al.*, 2002; Verhagen *et al.*, 2004). The priming process provides a plant with an enhanced capacity for rapid and effective activation of cellular defense responses that are induced only after contact with a pathogen, and the nature of the ISR elicited by rhizobacterial colonization can be explained by this process.

Acknowledgements

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