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Functional Characterization of Citrus Polygalacturonase-inhibiting Protein

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ABSTRACT

A cDNA encoding a polygalacturonase-inhibiting protein gene (*SaiPGIPA*) was identified from the citrus cultivar Sainumphung (*Citrus* sp.), one of the most popular cultivars in northern Thailand. *SaiPGIPA* was expressed in *Escherichia coli* cells, and the functional properties of citrus PGIP were analyzed. The PGIP fusion protein inhibited by a maximum of about 60% of the endopolygalacturonase activity, and a mixture of the PGIP and fungal endopolygalacturonase released oligogalacturonides from polygalacturonic acid. The mixture containing the oligogalacturonides, endopolygalacturonase and PGIP induced expression of the PGIP gene and a chalcone synthase gene in citrus leaves. The mixture also induced resistance in cucumber leaves against *Colletotrichum lagenarium*.

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Key words : polygalacturonase-inhibiting protein, citrus, *Alternaria alternata*, polygalacturonase, Sainumphung, rough lemon.

INTRODUCTION

Polygalacturonase-inhibiting proteins (PGIPs) inhibit fungal polygalacturonases (PGs) and release pectic oligomers, which are able to elicit plant defense responses[7,14,27]. PGIPs have been detected in the cell walls of vegetative and fruit tissues, and the genes encoding the PGIPs have been cloned from several plants[10,11,18,20,26,29,30,31,33,35,36]. The most recent data on the structure and function of PGIP were provided by a series of studies on PGIPs from bean (*Phaseolus vulgaris* L.)[21,24]. Structural analysis of bean PGIP expressed in *Nicotiana benthamiana* identified a model of the $\beta\alpha\beta$ plant-specific leucine-rich repeats (LRR) fold with short helixes[21,24]. Further, site-directed mutagenesis and surface plasmon resonance analysis revealed that the residue Q253 of bean PGIP in the exposed β-strand/β-turn region is a major contributor to PGIP•PG binding[21].

Despite the progress in understanding PGIP in bean, no comparable molecular research has yet been done for the citrus PGIP, except for a partial protein purification of PGIP from sweet orange (*C. sinensis*) cv. Valencia[1]. Recently, we identified cDNA sequences encoding PGIP from rough lemon (*C. jambhiri* Lush.)[12]. Constitutive expression of PGIP was detected in fruits but not leaves, stems or roots by northern blots[12]. The rough lemon PGIP gene is inducible, and transcripts accumulated in leaves within 30 min after wounding or inoculation with nonpathogenic *Alternaria*[12]. Early induction of PGIP gene expression is probably due to polygalacturonase (PG) secreted by microbes because gene expression was not induced after inoculation with a PG-disrupted mutant of *A. citri*[12].

To understand more on the function of citrus PGIP, we constructed a prokaryotic expression system using the pRSET vector and *SaiPGIPA* gene of citrus cultivar Sainumphung (*Citrus* sp.), which is the most popular citrus cultivar in northern Thailand. This paper describes the functional characterization of the PGIP gene products, and a possible application of the mixture containing PG, PGIP and oligogalacturonides for inducing plant defense against pathogens.

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MATERIALS AND METHODS

Plant and fungal materials Citrus plants used in this study were cv. Sainumphung (*Citrus* sp.), a domestic cultivar of Thailand, and rough lemon (*C. jambhiri* Lush). Seeds and leaves of Sainumphung were kindly provided from the Thai Royal Project Panda Experimental Station, Chiang Mai, and Dr. P. Samitamane, Chiang Mai University, Thailand. Citrus seeds were germinated and grown for several weeks in vermiculite at 24°C. The seedlings were moved to pots and maintained in the greenhouse with a minimum temperature of at least 15°C. Young leaves (midrib length of 2-3 cm) were collected from the plants in pots with average heights of 100 to 150 cm for RNA and DNA isolation. Cucumber (*Cucumis sativus* L.) cv. Tokiwa of 3.5 to 4.2 leaf stages was used for inoculation with *Colletotrichum lagenarium*.

Spores of nonpathogenic *Alternaria alternata* strain O-94 and an anthracnose pathogen (*C. lagenarium*) of cucumber were used for inoculation tests. Strain O-94 was kindly provided from the Laboratory of Plant Pathology, Tottori University, Japan. Strain DCL-001 of *C. lagenarium*, the laboratory stock strain in the Central Research Laboratories, Dainippon Ink and Chemicals Co. Ltd., Japan, was used for inoculation test to cucumber.

Cloning of Sainumphung PGIP genes Total RNA from Sainumphung leaves was prepared 6 hr after inoculation with nonpathogenic *A. alternata* strain O-94 (10^5 spores/ml)[12], using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. Leaf tissue was frozen in liquid nitrogen and homogenized with a mortar and pestle, and 100 mg homogenate was used for total RNA purification. One μg of the total RNA was employed as the template for reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was carried out in a two-step process using the ThermoScript RT-PCR System (GibcoBRL, Bethesda, USA) according to the manufacturer's directions. cDNA was synthesized in the first step using total RNA and oligo-$(dT)_{20}$ at 55°C for 60 min. In the second step, PCR was performed using a pair of primers designed from 5′ and 3′-untranslated regions of cDNA sequence of rough lemon *PGIP1*[12], PGIP-OUT-F 5′-AGAAGCTGCAGCTTCACT-3′ and PGIP-OUT-R 5′-AAAGCTTCTCACCCATCC-3′ with cDNA as the template. The reaction mixture for PCR contained 100 ng cDNA template, 100 pmol each of the primers, 2.5 units of *Taq* polymerase, with supplied A501-3 reaction buffer and dNTP mixture (Takara, Shiga, Japan). PCR consisted of 1 cycle of 95°C for 2 min, 29 cycles of 55°C for 2 min, 72°C for 1 min, 95°C for 1 min and 1 cycle of 55°C for 2 min, 72°C for 1 min, 25°C for 10 min, using the TAKARA PJ 2000 thermocycler. PCR products were subcloned by pCR-Script SK(+) Cloning Kit (Stratagene, La Jolla, USA) following the manufacturer's instructions. Sequences of products from multiple clones were obtained from both strands by the dideoxy chain termination method[28] using an ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA) and an automated fluorescent DNA sequencer (Model 310, Applied Biosystems) following the manufacturer's instructions. DNA sequences were aligned with CLUSTAL W[32], and sequences were analyzed with Genetyx-Mac (Software Development Co., Tokyo, Japan).

Genomic DNA was isolated from young leaves of Sainumphung using the Phytopure Plant DNA Extraction Kit (Nucleon Biosciences, Coatbridge, Scotland), following the manufacturer's instructions. For genomic PCR and sequencing, 100 ng of genomic DNA and 100 pmol each of PGIP-OUT-F and PGIP-OUT-R primers were used for the PCR with conditions described earlier, and the sequences of the products from multiple clones were identified after subcloning with pCR-Script SK(+) vector as described earlier.

Detection of PGIP gene expression in Sainumphung leaves Either a spore suspension (10^5 spores/ml) of nonpathogenic *A. alternata* (O-94) or water (control) was sprayed on the lower surface of detached young leaves of Sainumphung as described previously[12,17,23]. Total RNA was purified from the leaves either immediately after inoculation or after 0.5, 2, 4, 6, 12 hr incubation in a moist chamber at 24°C in the dark. RT-PCR was performed as described earlier, and one tenth of the product was loaded on 1% agarose gel as described previously[12]. Rough lemon histone H4 (DDBJ/EMBL/GenBank accession number AB050889) was used as a control to ensure equal amounts of RNA template in every reaction[12]. Northern blots were performed as described previously[12,17].

The method described by Heitz *et al.*[15] was used with minor modifications for wounding leaves. Young leaves (midrib length of 2 to 3 cm) of Sainumphung were wounded at four places by crushing across the lateral veins of each leaflet with the edge of a spatula without making any visible breaks in the tissue. Total RNA was purified from the leaves either immediately after treatment or after 0.5, 2, 4, 6, 12 hr incubation in a moist chamber at 24°C in the dark. RT-PCR was performed as described earlier.

Expression of citrus PGIP gene in a prokaryotic system The open reading frame (981 bp) of Sainumphung *SaiPGIPA* or rough lemon *PGIPA*[12] was subcloned in frame in pRSET (b) vector (Invitrogen, Carlsbad, USA). A single colony of *E. coli* BL21-Gold (DE3) (Stratagene) transformed by the plasmid was incubated

into 2 ml LB medium containing ampicillin (50 μg/ml) with shaking (200 rpm) at 28°C overnight. These cells were added to LB medium with ampicillin (50 μg/ml) and further incubated until OD_{600} of the culture became 0.6–0.8. Isopropyl-1-thio-β-D-galactoside (IPTG) was then added to a final concentration of 1 mM, and the cells were incubated for different periods up to 4 hr at 28°C with shaking (200 rpm). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to confirm expression of the PGIP•His-tag fusion protein. Thirty μl of the cell culture was mixed with SDS-PAGE sample buffer, boiled and resolved on 10% (w/v) acrylamide gel following a gel-stain with 0.25% Coomassie brilliant blue (CBB) as described previously[16,17].

PGIP recombinant proteins were partially purified from the supernatant of the cell culture using ammonium sulfate precipitation. We also attempted to purify the proteins using His-tag affinity beads. In brief, *E. coli* cell culture (50 ml to 500 ml) incubated for 4 hr after IPTG addition or without IPTG addition was centrifuged for 5 min at 5000×g, and the supernatant was mixed with ammonium sulfate at a final concentration of 60% (w/v). The solution was incubated for 2 hr at 4°C, and the precipitated proteins were recovered by centrifugation at 10,000×g for 30 min. Precipitants were resuspended in 50 mM sodium acetate buffer (pH 5.0) (500 μl to 7 ml) and used as a partially purified PGIP preparation.

An attempt to purify PGIP•His-tag fusion proteins was made from either the partially purified PGIP preparation or the lysed cell preparation using MagExtractor-His-Tag Kit (Toyobo, Tokyo, Japan) following the manufacturer's instructions. One fifth of the purified proteins was run on SDS-PAGE and visualized using a silver staining kit (Wako, Tokyo, Japan) as described previously[16,17]. For the lysed *E. coli* cell preparation, *E. coli* cells were collected by centrifugation at 5000×g for 5 min from 50 ml cell culture with 4 hr incubation after IPTG addition, then were frozen at −85°C. The cells were lysed in the absorption buffer from a MagExtractor-His-Tag kit (Toyobo) and digested with lysozyme (at final concentration: 1 mg/ml) following the manufacturer's instructions.

Functional analysis of citrus PGIP Inhibitory activity of PGIP against fungal endoPG was estimated by the rate of inhibition of endoPG activity after the addition of different concentrations of partially purified PGIP preparation from supernatant of *E. coli* cell culture either with or without IPTG induction. A partially purified PGIP preparation from the *E. coli* clone transformed by the pRSET vector without any insertion was also used as the control. The endoPG produced by *Aspergillus niger*[19] was purified from a commercial pectinase preparation (Fulka, Tokyo, Japan) using a diethylaminoethyl Sepharose CL-6B (Tosoh, Tokyo, Japan) column with eluted buffer of 0.4 M NaCl and 50 mM Tris (pH 8.0) as described previously[16,17]. The endo nature of the purified PG was determined with a relative viscosity assay[16,17,34]. Purified endoPG was resuspended in 25 mM sodium acetate (pH 5.5) buffer. Fifty ng of the endoPG was mixed with 0.2% (w/v) of polygalacturonic acid (PGA) (Amersham Pharmacia Biotech, Buckingham, UK) and different concentrations of the partially purified PGIP preparation in 50 mM sodium acetate buffer (pH 5.0) prepared from *E. coli* cell culture with or without IPTG induction. The mixture (total 200 μl) was incubated for 2 hr at 37°C. The reducing groups released from PGA by endoPG were measured with a reducing group assay using 2-cyanoacetamide (Aldrich, St. Louis, USA) as described previously[13,16,17]. Protein concentrations were measured using CBB with bovine serum albumin as the standard[4].

Galacturonide fragments released in a mixture of endoPG, PGA, and PGIP were analyzed using thin layer chromatography (TLC) as described by Yao *et al.*[35] with minor modifications. Briefly, different combinations of endoPG (50 ng), PGA (1% (w/v)) and the partially purified PGIP preparation (30 μg) were mixed in 50 mM sodium acetate buffer (pH 5.0) (total 65 μl) and incubated for 4 or 24 hr at 37°C followed by boiling for 30 min. The samples were spotted and developed on a 10 cm×10 cm Silica gel 60 F_{254}TLC plate (MERCK, Darmstadt, Germany) in a solvent system of ethyl acetate, acetic acid, formic acid and water (9 : 3 : 1 : 4, v/v/v/v). Galacturonide fragments were detected by spraying 0.2% orcinal (Wako) in a solution of sulfuric acid and methanol (1 : 9, v/v) following incubation at 105°C for 5 min[35].

Use of a mixture containing galacturonide fragments, endoPG, and PGIP for induction of plant defense The effect of a reaction mixture containing galacturonide fragments, endoPG and PGIP on transcript accumulations of PGIP and chalcone synthase (CHS) genes[12,25] in citrus leaves and also on induction of disease resistance in cucumber was examined. The reaction mixture (total 200 ml) of endoPG (50 μg), 1% (w/v) of polygalacturonic acid (PGA) (Amersham Pharmacia Biotech) and the partially purified PGIP preparation (30 mg) in 50 mM sodium acetate buffer (pH 5.0) was prepared by incubation for 4 hr at 37°C as described earlier.

To examine the mixture on PGIP and CHS gene expression in Sainumphung leaves, a young leaf (midrib length of 2 to 3 cm) of Sainumphung was dipped into the reaction mixture (10 ml) in a 50 ml Corning tube (Corning, NY, USA) for 10 sec, air-dried at room temperature for 5 min, and incubated for 0.5, 2 or 4 hr in a moist chamber at 24°C in the dark. Young leaves were also dipped into 50 mM sodium acetate buffer (pH 5.0) as the control, and incubated as described earlier. Total RNA was purified immediately or after the incubation periods

as described earlier, and used for RT-PCR for PGIP gene amplification and northern blots for transcript detection of CHS gene[12,25] as described previously[12,17,23].

To test for induction of disease resistance in cucumber, the third leaf at the 3.5 to 4.2 leaf stages of cucumber (cv. Tokiwa) was dipped into the reaction mixture (50 ml) or 50 mM sodium acetate buffer (pH 5.0) (50 ml) for 30 sec. After 5 days, a spore suspension (10^5 spores/ml) of *C. lagenarium* was sprayed to the third and fourth leaves of cucumber plants grown in a greenhouse. The inoculated plants were incubated for 2 days in a moist chamber at 25°C, then continuously incubated for 7 days in a greenhouse. This test was done on four different plants for each treatment, and the number of lesions on the respective leaves was counted. Defense rate against control (sodium acetate treatment) was calculated as follows: defense rate (%) = {(average number of lesions on sodium acetate-treated plants − average number of lesions on mixture-treated plants)/(average number of lesions on sodium acetate-treated plants)} × 100.

RESULTS

Cloning of Sainumphung PGIP genes

RT-PCR using PGIP-OUT-F and PGIP-OUT-R primers designed from rough lemon *PGIP1*[12], amplified a 1154-bp cDNA encoding PGIP from total RNA of Sainumphung leaves inoculated with nonpathogenic *A. alternata* (O-94). The RT-PCR products were subcloned into a pCR script SK(+) vector, and the sequences of the inserts from multiple clones identified a PGIP-coding sequence designated as *SaiPGIPA* (DDBJ/EMBL/GenBank accession numbers of AB015356). Although we had sequenced multiple products of this RT-PCR, *SaiPGIPA* was the only sequence and is likely to be a single or major sequence of the RT-PCR products from leaves at 6 hr after inoculation with nonpathogenic *A. alternata*. *SaiPGIPA* consists of a 981-bp open reading frame with 327 deduced amino acids with an estimated mol wt of 36.2 kDa (Fig. 1). The deduced amino acid sequences of *SaiPGIPA* is

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A     1: MSNTSLLSLFFFLCLCISPSLSDLCNPNDKKVLLKFKKSLNNPYVLTSWNPKTDCCDWYC :60
B     1: MSNTSLLSLFFFLCLCISPSLSDLCNPNDKKVLLKFKKSLNNPYVLASWNPKTDCCDWYC :60
1     1: MSNTSLLSLFFFLCLCISPSLSDLCNPNDKKVLLKFKKSLNNPYVLASWNPKTDCCDWYC :60
2     1: MSNTSLLSLFFFLCLCISPSLSDLCNPNDKKVLLKFKKSLNNPYVLASWNPKTGCCDWYC :60
3     1: MSNTSLLSLFFFLCLCISPSLSDLCNPNDKKVLLKFKKSLNNPYVLASWNPKTDCCDWYC :60
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A    61: VTCDLTTNRINSLTIFAGDLPGQIPPEVGDLPYLETLMFHKLPSLTGPIQPAIAKLKNLK :120
B    61: VTCDLTTNRINSLTIFAGDLPGQIPPEVGDLPYLEILMFHKLPSLTGPIQPAIAKLKNLK :120
1    61: VTCDLTTNRINSLTIFAGDLPGQIPPEVGDLPYLETLMFHKLPSLTGPVQPAIAKLKNLK :120
2    61: VTCDLTTNRINSLTVFAGDLPGQIPPEVGDLPYLETLMFHKLPSLTGPIQPAIAKLKNLK :120
3    61: VTCDLTTNRINSLTIFAGDLPGQIPPEVGDLPYLETLMFHKLPSLTGPIQPAIAKLKNLK :120
         **************:******************** ************:************

A   121: TLRISWTNISGPVPDFISQLTNLTFLELSFNNLSGTIPGSLSKLQKLGALHLDRNKLTGS :180
B   121: TLRISWTNISGPVPDFIRQLTNLTFLELSFNNLSGTIPGSLSKLQKLGALHLDRNKLTGS :180
1   121: TLRISWTNISGPVPDFISQLTNLTFLELSFNNLSGTIPGSLSKLQKLGALHLDRNKLTGS :180
2   121: TLRISWTNISGPVPDFISQLTNLTFLELSFNNLSGTIPGSLSKLQKLGALHLDRNKLTGS :180
3   121: TLRISWTNISGPVPDFISQLTNLTFLELSFNNLSGTIPGSLSKLQKLGALHLDRNKLTGS :180
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A   181: IPESFGTFTGSIPDLYLSHNQLSGKIPASLGSMDFNTIDLSRNKLEGDASFLFGLNKTTQ :240
B   181: IPESFGTFTGSIPDLYLSHNQLSGKIPASLGSMDSNTIDLSRNKLEGDASFLFGLNKTTQ :240
1   181: IPESFGTFTGSIPDLYLSHNQLSGKIPASLGSMDFNTIDLSRNKLEGDASFLFGLNKTTQ :240
2   181: IPESFGTFTGSIPDLYLSHNQLSGKIPASLGSMDFNTIDLSRNKLEGDASFLFGLNKTTQ :240
3   181: IPESFGTFTGSIPDLYLSHNQLSGKIPASLGSMDFNTIDLSRNKLEGDASFLFGLNKTTQ :240
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A   241: RIDVSRNLLEFNLSKVEFPQSLTNLDLNHNKIFGSIPAQITSLENLGFLNVSYNRLCGPI :300
B   241: RIDVSRNLLEFNLSKVEFPQSLTNLDLNHNKIFGSIPAQITSLENLGFLNVSYNRLCGPI :300
1   241: RIDVSRNLLEFNLSKVEFPQSLTNLDLNHNKIFGSIPAQITSLENLGFLNVSYNRLCGPI :300
2   241: RIDVSRNLLEFNLSKVEFPQSLTNLDLNHNKIFGSIPAQITSLENLGFLNVSYNRLCGPI :300
3   241: RIDVSRNLLEFNLSKVEFPQSLTNLDLNHNEIFGSIPAQITSLENLGFLNVSYNRLCGPI :300
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A   301: PVGGKLQSFGYTEYFHNRCLCGAPLER                                    :327
B   301: PVGGKLQSFGYTEYFHNRCLCGAPLER                                    :327
1   301: PVGGKLQSFGYTEYFHNRCLCGAPLESCK                                  :329
2   301: PVGGKLQSFGYTEYFHNRCLCGAPLESCK                                  :329
3   301: PVGGKLQSFGYTEYFHNRCLCGAPLESCK                                  :329
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Fig. 1. Comparison of deduced amino acid sequences of the *SaiPGIP* gene family from citrus cultivar Sainumphung. PGIP sequences were aligned by CLUSTAL W[31], and identical residues are indicated with asterisks. A, B, 1, 2 and 3 indicate *SaiPGIPA*, *SaiPGIPB*, *SaiPGIP1*, *SaiPGIP2* and *SaiPGIP3*, respectively. Accession number for each gene is indicated in text.

97.2% identical to that of rough lemon *PGIP1*[12].

Genomic PCR of Sainumphung using the same primers amplified four additional sequences of 1154-bp PGIP genes. Sequencing of subcloned PCR products from multiple clones identified total of five different sequences (Fig. 1), and one of them was identical to that of *SaiPGIPA*, suggesting that the *SaiPGIPA* gene has no intron. Another *PGIP* sequence with a 981-bp ORF was designated as *SaiPGIPB*, and other with a 987-bp ORF were designated as *SaiPGIP1*, *SaiPGIP2*, and *SaiPGIP3* (DDBJ/EMBL/GenBank accession numbers of AB015643, AB064545, AB064546, and AB064547, respectively). These Sainumphung PGIP genes were 98.5% to 99.8% identical to each other at the amino acid sequence level (Fig. 1).

Transcript accumulation of Sainumphung PGIP genes

Northern blot could not detect transcripts of PGIP genes in total RNA isolated from Sainumphung leaves similar to the results obtained from rough lemon leaves[12] (data not shown). However, RT-PCR using primers PGIP-OUT-F and PGIP-OUT-R amplified PGIP cDNA, and the intensity of the band increased within 30 min after inoculation with nonpathogenic *A. alternata* (O-94) and with the wounding treatment in Sainumphung leaves (Fig. 2). The elevated intensity of the bands was maintained even after 12 hr, but the differences in the intensity of these bands from the fractions between 30 min and 12 hr after inoculation or wounding were difficult to quantify (Fig. 2). The bands were not amplified when the template RNA was degraded by addition of RNase in the reaction mixture or by RT-PCR without adding reverse transcriptase (data not shown). Northern blot also did not detect any signal from total RNA of pathogen- or wounding-induced leaves after any incubation period (data not shown).

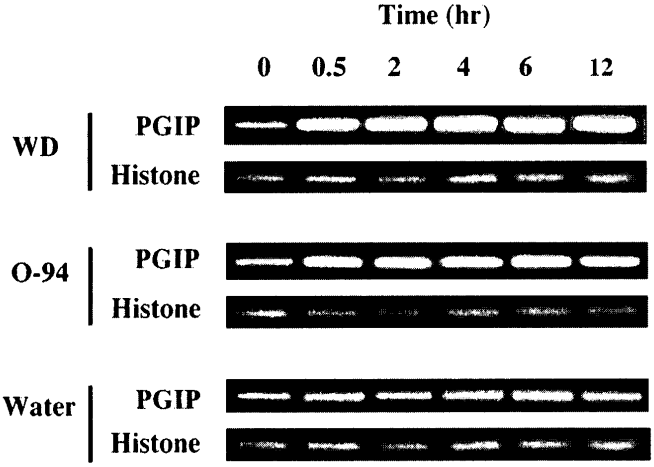

Fig. 2. RT-PCR detection of PGIP gene expression in Sainumphung leaves in response to wounding (WD) and inoculation with nonpathogenic *Alternaria alternata* (O-94). Wounding was done by crushing across the lateral veins of leaf tissue. Conidial suspension (10^5 spores/ml) of O-94 or water (Water) was sprayed on rough lemon leaves. RT-PCR was performed using ThermoScript RT-PCR System (GIBCO/BRL) with total RNA isolated from Sainumphung leaves at 0, 0.5, 2, 4, 6, or 12 hr after treatments. One tenth of the RT-PCR products was loaded on 1% agarose gel. Histone H4 (accession number AB050889)[12] was used for every reaction as control (Histone) of the equality of RNA template.

Prokaryotic expression of Sainumphung *SaiPGIPA*

Sainumphung *SaiPGIPA* or rough lemon *PGIPA* was expressed in *E. coli* cells using the pRSET expression vector. The mol wt of the His-tag fusion protein of Sainumphung and of rough lemon PGIP that appeared in the *E. coli* cell culture was 45 kDa on SDS-PAGE (Fig. 3). The fusion protein was detected 30 min after addition of IPTG, with the intensity of the band increasing gradually with extended incubation (Fig. 3). This protein did not appear without IPTG addition or in *E. coli* transformed with a mock vector (data not shown). However, a majority of the 45-kDa proteins detected in SDS-PAGE formed insoluble inclusion bodies, and they were not

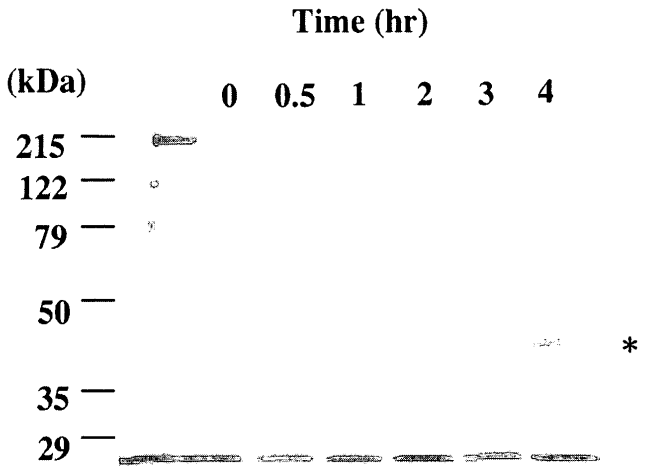

Fig. 3. Prokaryotic expression and purification of Sainumphung PGIP. Expression of PGIP•His-tag fusion protein in *E. coli* cell culture. The cells transformed with *SaiPGIPA* were incubated at 28°C for up to 4 hr after addition of IPTG. Thirty μl of cell culture was resolved on 10% (w/v) acrylaminde gel following gel-stain with Coomassie brilliant blue. Asterisk indicates the position of PGIP•His-tag fusion protein. The numbers on the left side indicate the position of protein size markers in kDa.

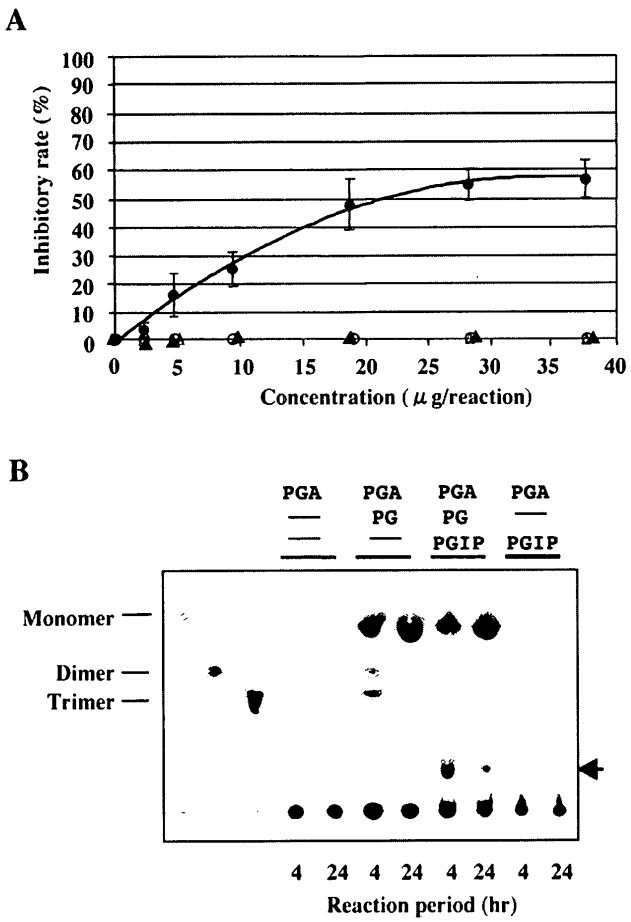

Fig. 4. Functional analysis of Sainumphung *SaiPGIPA* expression products. (A) Inhibition of endoPG activity by addition of partially purified PGIP preparations. Fungal endoPG (50 ng) was mixed with 0.2% (w/v) polygalacturonic acid (PGA) and different amounts of partially purified PGIP prepared from *SaiPGIPA*-transformed *E. coli* cell culture with (●) or without (○) IPTG induction in 50 mM sodium acetate buffer (pH 5.0). The preparations from *E. coli* transformed by mock vector with (▲) or without (△) IPTG were also used as the controls. The mixture (200 μl) was incubated for 2 hr at 37°C. Inhibition rates were calculated by comparing the activities of endoPG alone and endoPG with addition of the PGIP preparation. Protein concentrations were measured using Coomassie brilliant blue with BSA as the standard[4]. Each datum represents the mean of at least three replicates, and error bars represent standard deviations of the mean. (B) Detection of oligogalacturonides produced by endoPG and PGIP interactions on thin layer chromatography (TLC). Different combinations of polygalacturonic acid (PGA), endoPG, and PGIP mixture incubated for 4 or 24 hr at 37°C followed by boiling for 30 min were analyzed on TLC plate in a solvent system of ethyl acetate, acetic acid, formic acid and water (9 : 3 : 1 : 4, v/v/v/v). Respective sizes of galacturonide were detected by spraying 0.2% orcinal. Monomer, dimer and trimer of GA are in the left three lanes as size markers. Arrow indicates the oligogalacturonides on TLC.

solubilized (data not shown).

To examine the function of the fusion proteins, the physiologically active proteins were recovered from the supernatant fraction of the *E. coli* cell culture. PGIP recombinant proteins in the supernatant fraction of the 50-ml *E. coli* cell culture were precipitated by 60% ammonium sulfate, with further attempts to purify on a His-tag affinity column. The mol wt of the obtained protein was 32 kDa on SDS-PAGE and was detected as a single band (data not shown). However, the purification efficiency was very low, and an increase of culture volume (up to 500 ml) did not significantly change the yield of the purified products (data not shown). The band on SDS-PAGE could only be detected with a silver stain, not with CBB, even though one fifth of the purified sample from 50 ml of culture was loaded on the lane. The His-tag affinity column did not purify any protein from the lysed cell fraction of the *E. coli* culture (data not shown). These results were the same for both Sainumphung and rough lemon PGIP.

Because of the unreliable preparation of PGIP•His-tag fusion proteins using the His-tag affinity system, we used a partially purified PGIP preparation with 60% (w/v) ammonium sulfate precipitation for further analyses. EndoPG (50 ng) activity was inhibited by 54.8% by 28 μg of the partially purified PGIP preparation in 200 μl of reaction mixture. Further addition of the PGIP did not significantly increase the inhibitory rate (Fig. 4A). Protein from the *E. coli* cell culture prepared by the same method without addition of IPTG did not inhibit endoPG activity (Fig. 4A). Expression products of the pRSET vector without any insertion also did not affect endoPG activity (Fig. 4A).

Digestion products of PGA in the mixture of endoPG and the partially purified PGIP preparation were examined by TLC analysis. Oligomers of galacturonide were detected when PGA was incubated for 4 hr with both endoPG and the PGIP, while endoPG alone digested PGA mainly to monometric galacturonic acid with fewer dimers and trimers (Fig. 4B). PGIP alone could not digest PGA, and no digestion products developed on the TLC (Fig. 4B). Prolonged incubation (24 hr) with endoPG alone increased digestion of the dimers and trimers to monomers, while that with endoPG and the PGIP mixture decreased the oligomers but without complete digestion

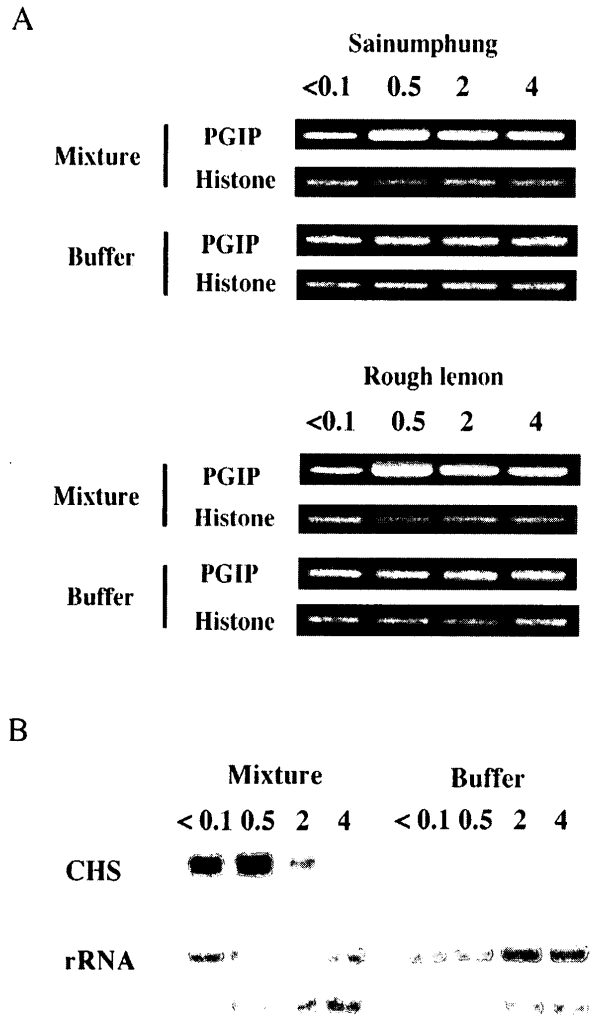

Fig. 5. Induction of PGIP and CHS gene expression in citrus leaves after dipping in mixture of oligogalacturonides, endoPG and PGIP. (A) RT-PCR examination of PGIP gene expression in Sainumphung and rough lemon leaves. Young leaves of Sainumphung or rough lemon were dipped into mixture of oligogalacturonides, endoPG and PGIP (Mixture) or 50 mM sodium acetate buffer (pH 5.0) (Buffer) for 10 sec, air-dried at room temperature for 5 min. Total RNA was purified immediately (<0.1) or 0.5, 2, 4 hr after drying at 24°C in the dark, and RT-PCR for PGIP gene amplification was employed as described in Fig. 2. (B) Northern blot analysis of CHS gene expression in Sainumphung leaves. Sainumphung leaves dipped in the mixture were incubated for 0.5, 2 or 4 hr at 24°C in the dark. Control leaf was also dipped into 50 mM sodium acetate buffer (pH 5.0) after incubation as just described. Total RNA was purified immediately after drying (<0.1) or after respective incubation periods described, and northern blotting used for transcript detection of *CHS* by the method described previously[12].

of the oligomers to monomers (Fig. 4B).

Mixture of oligogalacturonide fragments, endoPG, and PGIP induced plant defense

The reaction mixture containing oligogalacturonide fragments, endoPG and PGIP described in the previous section was examined the effect on citrus PGIP gene expression. At <0.1, 0.5, 2, or 4 hr after citrus leaves were dipped into the mixture and dried for 5 min, total RNA was isolated and used as the template for RT-PCR detection of PGIP gene expression. PGIP gene expression was induced within 30 min after this treatment in leaves of Sainumphung and rough lemon (Fig. 5A). Expression of *CHS*[25] was also determined with a northern blot of the total RNA of the mixture-treated Sainumphung leaves. The mixture containing galacturonide fragments, endoPG and PGIP induced expression of *CHS* immediately after the dipping treatment followed by drying for 5 min. The strongest expression was observed at 30 min after the treatment (Fig. 5B).

The mixture also induced a defense in cucumber leaves against the anthracnose pathogen. Dipping of the leaves into the mixture reduced the number of lesions caused by *C. lagenarium* inoculated 5 days after the treatment (Table 1). The average defense rate was 68.4% on the third leaf to which the mixture was applied (Table 1). An average 40.5% reduction in lesion number was also observed on the fourth leaf (Table 1).

DISCUSSION

We have identified PGIP genes from citrus cultivar Sainumphung using RT-PCR and genomic PCR. The regions containing PGIP genes in citrus genomes of different species and cultivars are likely to be highly conserved because the set of primers designed from 5′- and 3′-untranslated regions of rough lemon *PGIP1*[12] amplified the region from 18 different species or cultivars of citrus (Nalumpang *et al.*, unpublished data) including cv. Sainumphung. PCR amplification using genomic DNA identified multiple copies of the PGIP gene, indicating that PGIP genes exist as a gene family in Sainumphung as well as in rough lemon genomes[12]. A previous study of rough lemon *PGIP* revealed that transcripts contributed by multiple rough lemon *PGIP* were induced upon wounding or fungal attack[12]. Sainumphung PGIP gene was also inducible by wounding or microbe attack; however, we have not carried out an extensive analysis of individual *SaiPGIP* expression, except on the *SaiPGIPA* that was the dominant PGIP gene transcribed in Sainumphung leaves at 6 hr after inoculation with nonpathogenic *A. alternata* (O-94). Putative ORF sequences of other copies of Sainumphung PGIP genes designated as *SaiPGIPB*, *SaiPGIP1*, *SaiPGIP2* and *SaiPGIP3* are highly homol-

Table 1. Induction of resistance in cucumber to anthracnose pathogen after dipping into mixture containing pectic fragments, endoPG and PGIP

Treatment[a]	Plant	Third leaf		Fourth leaf	
		Number of lesions[b]	D.R.[c]	Number of lesions[b]	D.R.[c]
Mixture	1	65		82	
	2	29		11	
	3	79		107	
	4	53		46	
	Average	56.5		61.5	
Buffer	1	213		83	
	2	122		89	
	3	150		150	
	4	230		91	
	Average	178.8		103.3	
			68.4		40.5

a) Third leaf of cucumber (cv. Tokiwa) was dipped into mixture of pectic fragments, endoPG and PGIP in 50 mM sodium acetate buffer (Mixture) or 50 mM sodium acetate buffer (Buffer).

b) Spore suspension (10^5 spores/ml) of *Colletotrichum lagenarium* was sprayed on third and fourth leaves 5 days after dipping treatments. Lesions on inoculated leaves were counted after 2 days in a moist chamber and additional 7 days incubation in greenhouse.

c) Defense rate (D.R.) was calculated as follows: defense rate (%) = (Average number of lesions on buffer treated leaf/Average number of lesions on mixture-treated leaf)/(Average number of lesions on buffer-treated leaf) × 100. Average was calculated from number of lesions on four different plants.

ogous (98.5% to 99.8%), but they may have different expression patterns under individual regulation of their respective promoter regions, as in the case described for bean PGIP genes[2,9].

A large portion of the PGIP·His-tag fusion protein expressed in *E. coli* were inclusion bodies with an apparent mol wt of 45 kDa on SDS-PAGE. But we have identified PGIP activities in the supernatant fraction of an *E. coli* cell culture, and a protein with a mol wt of 32 kDa on SDS-PAGE was obtained from the fraction using a His-tag affinity column. However, it is not clear whether this 32-kDa protein is the functional PGIP or not. The estimated size of the PGIP calculated from the deduced amino acids of PGIP and the His-tag region of expression vector is 40 kDa; the apparent mol wt based on the mobility of the His-tag purified protein in the gel is different. Furthermore, the quantity of the 32-kDa protein was very low; only silver stain could show the existence of the protein. Thus, we could not use this His-tag purified fraction containing the 32-kDa protein for further analysis of PGIP function.

In this study, we examined the functional analysis of citrus PGIP using a partially purified PGIP prepared by ammonium sulfate precipitation. The partially purified PGIP preparation inhibited digestion activity of endoPG (50 ng) by a maximum of about 60%, while the same fraction prepared from an *E. coli* cell culture without IPTG induction did not have any inhibitory activity. EndoPG and PGIP are considered to interact in a 1 : 1 molecular ratio[21]. Thus, the inhibitory rate did not increase with the addition of more than 28 μg of the partially purified PGIP preparation, indicating that endoPG molecules in the 50 ng preparation was likely saturated by PGIP molecules in the 28 μg preparation in the 200 μl reaction mixture. It was a reasonable result that the PGIP did not inhibit endoPG activity completely and that 40% of the endoPG activity still remained after endoPG/PGIP complex formation, because hydrolysis of β-1,4 linkage of pectic saccharides is still necessary to digest PGA for releasing oligogalacturonides. The oligogalacturonides derived from PGA were produced only when endoPG and PGA were mixed with the partially purified PGIP preparation. The PGIP preparation alone or endoPG alone did not produce the oligomers, indicating that oligomer production was not due to contaminants from the partially purified PGIP preparation nor to intermediate products produced by digestion by endoPG alone. Oligogalacturonides observed in our system were stable. A prolonged incubation (24 hr) at 37°C or boiling for 30 min did not degrade the oligogalacturonides in the mixture. Cervone *et al.*[7] also demonstrated the high stability of the oligogalacturonides derived from PGA by purified PGIP from bean and fungal endoPG. These results led to the hypothesis that citrus PGIP also have a significant role for making oligogalacturonides similar to the cases described in other plant PGIP[7,30,35,36].

The elicitor activity of the oligogalacturonides on plant resistance has been suggested in a series of studies[7,14,27]. They are reported to initiate several reactions related to plant defense systems such as phytoalexin synthesis[8], oxidative burst[22], lignin synthesis[5] and induction of protease inhibitors[3]. Further, a treatment with endoPG alone was also reported to induce resistance, most likely from the interaction of the released oligosaccarides with PGIP in the cell wall[5,6,8]. Therefore, we examined the effect of a mixture of oligogalacturonides, endoPG or endoPG/PGIP complexes, instead of each alone, using the sample we used for the *in vitro* experiments for inhibition of endoPG activity and oligogalacturonide production with TLC. Our application of the mixture to citrus leaves by dipping without any addition of surfactant induced transcripts of PGIP and CHS genes within 30 min. The mixture also induced resistance in cucumber to *C. lage-*

narium. A significant reduction of lesions was observed on the mixture-treated third leaf as well as on untreated fourth leaf. Although high variability of the inhibitory rates, especially in the fourth leaf, was observed among the mixture-treated plants used as repeats, the induction of resistance was considered to be transmissible to other leaves as an acquired resistance. Based on our observations described in this study, the mixture of oligogalacturonides, endoPG or endoPG/PGIP complexes might have an agricultural application. They are easy to prepare without complicated purification steps, and multiple effects may be achieved from oligogalacturonides in the mixture and further production of oligogalacturonides from the plant cell wall by endoPG or the endoPG/PGIP complex in the mixture.

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