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Isolation and Characterization of the Salicylic Acid Induced Gene in *Rehmannia glutinosa* by Differential Display

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Rehmannia glutinosa **is a perennial medicinal plant belonging to the family** ***Scrophulariaceae*** **with more than 300 species known in the world, especially in temperate regions. Its roots have been used widely in Korea for medicinal purposes. However, it is commonly infected by various pathogens during storage, causing great damage to the roots, and impedes the intensive farming of the crop. Therefore, an attempt has been made to isolate and screen a resistance gene against the pathogen** ***Fusarium oxysporum*** **using differential display. We treated salicylic acid (SA), and isolated a resistance gene that responds to SA. As a result, we found that SA was involved in plant defense mechanism in pathogenicity tests with SA treated and non-treted plants, and we isolated a partial PR-la gene through differential display polymerase chain reaction (DD-PCR) method.**

KEYWORDS: Differential display, *Fusarium oxysporum*, PR1-a gene, *Rehmannia glutinosa*, Salicylic acid

Rehmannia glutinosa is a perennial medicinal plant belonging to Scrophulariaceae with ca. 300 species known in the world, especially in temperate regions. Its roots has been used widely in Korea for a long time for medicinal purposes. *R. glutinosa* contains iridoid, catalpol, leonuride, stachyose, sucrose, mannitol, and amino acids, and it is used for hematic, antifebriel, and detoxification purposes, for general robustness, as a cardiotonic drug and in diabetes treatment. *R. glutinosa* shows weak fertility and it mainly proliferates through root branching and rootlet growth. However, roots for proliferation are usually infected with various pathogens during storage, and these infections cause great damage to the yield of this crop (Hiraga *et al.*, 2000). Among the various pathogens infected, *Fusarium oxysporum* is the most important pathogen that causes root disease. Therefore, it is necessary to breed disease resistance against this pathogen and in recent years research work on Pathogenesis-Related (PR) proteins related with disease resistance have been emphasized. Various PR-proteins show anti-fungal activities and these PR-proteins expressed in some transformed plants show disease resistance (Pieterse *et al.*, 1998).

Salicylic acid seems to be involved in both the hypersensitive response and in systemic acquired resistance (Chamnongpol *et al.*, 1998; Horvath and Chua, 1996; Ivashuta *et al.*, 1999; McKendree *et al.*, 1995). Salicylic acid and isonicotinic acid are true systemic acquired resistance (SAR) activator because not only do they induce resistance to the same spectrum of pathogens and expression of the same genes as do pathogens, but also these chemicals have no anti- microbial activity (Lawton *et al.*, 1995; Seehaus and Tenhaken, 1998). This area of research, has tremendous commercial potential, and therefore the search for SAR inducing compounds is likely to continue and actually to increase (Chen *et al.*, 1993; Malamy *et al.*, 1990; Murray and Thompson, 1980).

Defensive proteins are drawing much attention from plant breeders as a tool for genetically modified crops because of their protective activities against microbes. Several workers have focused on the isolation, characterization, and regulation of expression of PR-proteins since the discovery that several of the PR-proteins have antimicrobial or insecticidal activity and can delay the progression of diseases caused by several pathogens belonging to diverse genera. Recently differential display was developed as a molecular analytical tool which allows a systematic, sensitive non-biased detection of alteration in gene expression at the level of mRNA (Yun *et al.*, 1999). The essence of this method is to systematically amplify messenger RNA 3' termini using a pair of specifically designed primers, which consists of an anchored oligo-dT, and short primer with an arbitrary sequence. Multiple amplified cDNAs defined by a given pair of primers are then distributed on a denaturing polyacrylamide gel. Side-by-side comparison of mRNA species from two or more related samples allows identification of both up and down regulated genes of interest. By changing primers from both directions, statistically most of the expressed genes in a cell may be visualized with these methods (Molina *et al.*, 1998; Murray and Thompson, 1980). Therefore, this study was carried out to isolate a gene that is resistant to the pathogen *Fusarium oxysporum* using differential dis-

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play (James *et al.*, 1984; Yun *et al.*, 1999).

Materials and Methods

Plant material and treatment. *Rehmannia glutinosa* was obtained through the courtesy of C. Y. Yu. (Kangwon National University). *In vitro* plants were subcultured on Murshige and Skoog vitamin powder medium with 3% sucrose and maintained at 22°C under 16/8 light/dark cycle. After four weeks, plants were transferred to Hoagland solution at 22°C under 16/8 light/dark cycles in a growth chamber. The treatment of *R. glutinosa* (four week old plants) was carried out. For treatments, SA were adjusted to the desired final concentration (1 mM) by adding small volumes of concentrated stock. The SA solution was prepared in 10% acetone and adjusted to pH 6.8. Prepared SA solution was inoculated to *R. glutinosa* with brush. During the treatment, the plants were maintained at 22°C under standard growth conditions.

Pathogenicity test. A pathogenicity test was conducted to determine the development of *Fusarium oxysporum.* The test pathogen was isolated from infected plants. Each isolate was grown in a 250 m*l* flask containing 100 m*l* of potato dextrose broth (PDB) with shaking (150 rpm) at 26°C under constant light. After 7 days, fungal cultures were filtered through four layers of cheese cloth. Final cell density was maintained to 10^6 CFU/m*l*. SA treated *R. glutinosa* and non-treated *R. glutinosa* was inoculated with liquid culture of *F. oxysporum* in Hoagland solution medium. Disease incidence was evaluated 4 weeks after the inoculation by counting the number of diseased plants. Control plants were grown under the same conditions without the pathogen.

Isolation of total RNA and mRNA purification. Isolation of total RNA from control plants was performed with plantlets culture *in vitro*. Isolation of RNA from SA treated plantlets was performed 24 hrs after the SA inoculation according to the modified procedure described by R. Meier and Murray *et al.* method (Nelson *et al.*, 1983).

PCR cDNA library construction.
First strand DNA synthesis: RAV-2 reverse transcriptase was used for first strand cDNA synthesis. The initiation of the first strand synthesis depends upon hybridization of a primer to the mRNA, usually at the poly A tail. The reaction conditions for first strand synthesis have been optimized for yield and size of the cDNA. Successful cDNA synthesis demands an RNase-free environment. To a 0.5 m*l* micro centrifuge tube, 2 μg of purified mRNA was mixed with 2 μl of 5× first strand synthesis buffer, 1 μl of 2.5 mM dNTP mixture, 1 μl of oligo dT-RA primer, 1 μl of RAV-2 reverse transcriptase and 3 μl of DEPC-treated H_2O and was finally brought to 10 μl and incubated at 30°C for 15 min, at 42°C for 1 hr, and 80°C for 5 min in continuation.
2nd strand cDNA synthesis: Pipetting into the first stand reaction tube was followed with 1st strand cDNA synthesis reaction 10 μl, 10 μl of 5× second strand synthesis buffer (TAKARA), and 20.5 μl of DEPC-treated H_2O. Then 6.5 μl of *E. coli* DNA polymerase and 1.0 μl of *E. coli* RNase H/*E. coli* DNA liagse mixture were added. The reaction was incubated at 12°C for 1 hr, at 22°C for 1 hr, and 70°C for 10 min. Following that, 2 μl of T4 DNA polymerase was added by mixing gently. Again the reaction mixture was incubated at 37°C for 10 min, then 4 μl of stop solution was added with gentle tapping, and the reaction was stopped.
Cassette adaptor: The previous reaction was resuspended with 5 μl of DEPC-treated dH_2O. The cDNA was ligated with the adaptor by adding 2 μl of Ca, Ra Cassette Adaptor and 6 μl of ligation solution II was added, with gentle tapping, then 12 μl of ligation solution I was immediately added. The mixture was incubated at 16°C for 30 min. The DNA precipitation procedure was the same as described earlier.
Amplification of cDNA: The 30 μl of double strand cDNA was amplified with cDNA synthesis Kit (TAKARA, Code 6120) in a DNA thermocycler. The reaction profile consisted of 35 cycles at 94°C for 1 min, at 60°C for 30 sec, and at 72°C for 3 min. The reaction was extended at 72°C for 10 min and the amplified products were stored at 4°C.

Differential display. The differential display protocol was followed as described by Casselman *et al.*, Ivashuta *et al.* and Liang *et al.* (Cornelissen and Melchers, 1993; Liang and Pardee, 1992; Nelson *et al.*, 1983). DD-PCR reactions were prepared for each 20 μl PCR reaction by mixing 1× PCR reaction buffer, 100 μM of dNTPs mix, 0.5 μM of T11 primer, 2 μl cDNA, 1 U Taq DNA polymerase, and 0.5 μM arbitrary primers. The PCT-100™ (MJ research Inc., USA) PCR system from Perkin Elmer was used for the reaction. The program was designed as follows : For the first cycle, samples were incubated at 94°C for 2 min, 40°C for 1 min, and 72°C for 1 min. The remaining 39 cycles were identical except that samples were kept at 94°C for only 30 seconds for denaturation. After 40 cycles, samples were incubated at 72°C for 10 min and kept at 4°C. The PCR product was loaded on 6% polyacrylamide gel and stained with silver.

cDNA Southern blotting. The cDNA was loaded on 1% agarose gel with ethidium bromide. The gel was depurinated in 0.25 M HCl for 10 minutes. After rinsing twice with dH_2O, the gel was denatured in a denaturing buffer (0.5 N NaOH, 1.5 M NaCl) for 30 minutes, and

Table 1. Results of pathogenicity tests on *Rehmannia glutinosa* with SA treatment or non-treatment

Treatments	Disease ratings[1]			Average[2]
	I	II	III	
Fusarium oxysporum R-3 inoculated	3.0	3.0	1.3	2.4b
F. oxysporum R-10 inoculated	4.0	3.3	2.6	3.3c
SA treated and *F. oxysporum* R-3 inoculated	2.0	1.3	1.6	1.6a
SA treated and *F. oxysporum* R-10 inoculated	1.6	1.0	2.0	1.5a

[1]Disease ratings were based on 0~5 scales; 0 = no disease, and 5 = death of seedlings.
[2]Pathogenicity tests were conducted three times with three replications each, and the average values were obtained. Values with t he same character are not significantly different at $P = 0.05$.

stored in 0.4 N NaOH until transfer. The transfer was performed on a nylon membrane for 16 hours using a 0.4 N NaOH transfer solution, and finally, the membrane was washed with 2× SSC and dried for 1 hour between 3 MM papers. The membrane was incubated with dUTP labeled fragment 116-2 (unique cDNA fragment by differential display) at 65°C for 16 hours. The signals were detected by exposing the membrane to X-ray film (Agfa CP-BU, Belgium) for 30 minutes using Chemiluminescent Detection with Alkaline Phosphatase (CSPD).

Blast search. Partial nucleotide sequences of the clones obtained from the modified differential display were determined using an automated DNA sequencer. Analyses of the nucleotide sequences and their deduced amino acid sequence were performed using the DNA star program (Lasergene). The homology search was performed using BLAST programs to check GENEBANK, EMBL, and Swiss Prot database.

Results and Discussion

Rehmannia glutinosa has been used as herbal Chinese medicine in the Orient. In recent years, as the area of cultivation of *R. glutinosa* increased, it has been plagued by several different pathogens. Among these, root rots casued by *F. oxysporum* has been the most devastating disease. We isolated *F. oxysporum* R-3 and R-10 from the rotten

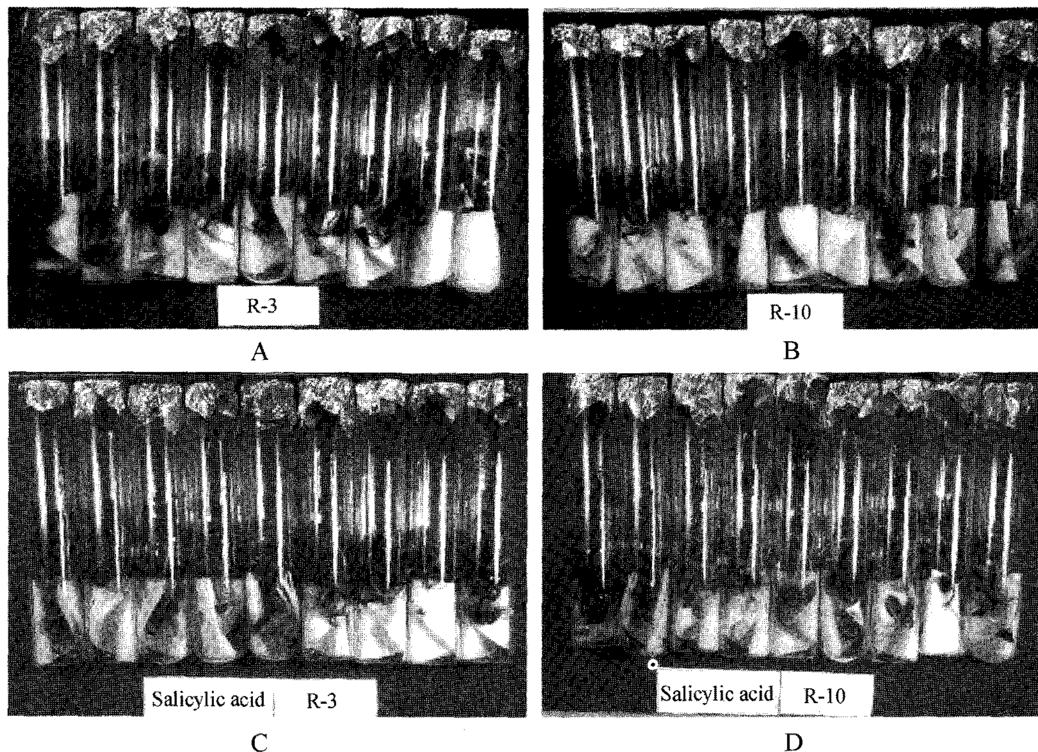

Fig. 1. Results of pathogenicity tests of *Rehmannia glutinosa* non-treated (upper panel) or treated (lower panel) with salicylic acid (SA) and infected with *Fusarium oxysporum*. (A) SA-non-treated and infected with *F. oxysporum* R-3, (B) SA-non-treated and infected with *F. oxysporum* R-10, (C) SA-treated and infected with *F. oxysporum* R-3, (D) SA-treated and infected with *F. oxysporum* R-10.

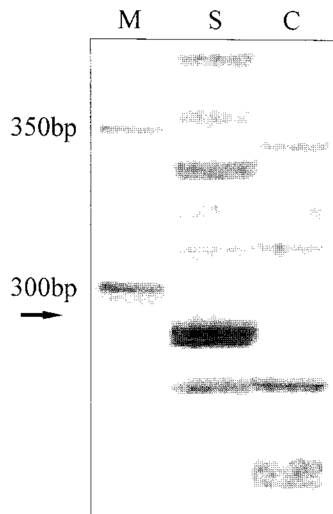

Fig. 2. Silver staining of typical differential display gel. The arrows indicate differentially expressed 116-2 fragments (286 bp). (M) molecular marker. (S) application of 1 mM salicylic acid. (C) untreated control plants.

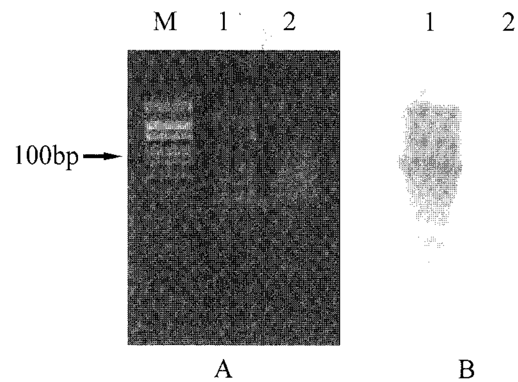

Fig. 4. Electrophoresis of total PCR cDNA on an 0.8% 1X TBE agarose gel (A), and cDNA southern blotting with differential display fragment of 116-2 (B). Lanes: (A) M. DNA molecular marker. 1. Treated with SA plant cDNA. 2. Control plant cDNA. (B) 1. Treated with SA plant. 2. Control plant.

roots of *R. glutinosa* and their pathogenicity were determined.

In the pathogenicity test, it was found that SA-non-treated plants were severely infected by the pathogen showing typical root-rot symptoms, whereas the SA-treated plants were found healthier when compared to non-treated plants (Table 1, Fig. 1). This might be due to the possibility that SA induced the gene that responded to defend against the pathogen attack.

We used differential display to identify genes expressed in salicylic acid treated plants and control plants. Gene expression was monitored on the first day after applying with salicylic acid. We identified and cloned numerous unique cDNA fragments by differential display (Fig. 2). About 30 bands were cut out of the differential display gels. A few of these bands failed to re-amplify. However, most gave a clear band after the second PCR reaction. Among the thirty bands expressed, most of the bands were false bands, and three of the expressed clones were throught to be induced by the chemicals or the pathogens. However, in cDNA southern analysis, only one clone was found to be related to the resistance.

The fragment of differentially expressed cDNA was re-amplified and the re-amplifed products were loaded with 1% agarose gel and the desired band was excised and the cDNA was purified with an intron purification kit (Intron, Korea). Each re-amplified PCR product was ligated into cloning vector following the manufacturer's instructions (Invitrogen BV). Competent *E. coli* cells transformed with 2 *μl* of each ligation reaction were plated onto LB agar medium with 100 *μ*g/m*l* ampicillin. Finally, the plasmid was extracted with a Bioneer plasmid extraction kit™ (Bioneer, Korea). The insert was amplified using M13 primer. The partial nucleotide sequence was deduced to amino acid sequence and applied to BLAST analyses to examine their homology with genes previously reported to the database (Baillieul *et al.*, 1995). Clone 116-2 showed strong homology with PR-1a protein (Fig. 3). The induction of PR-1a expression is closely correlated with the onset of SAR, a defense response which is effective against a variety of fungal, viral, and bacterial pathogens

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>gi|1469931|gb|U49241.1|NGU49241
         cds

PR-1a: 31   SGDFMTAAKAYEM*CYEKQTTDHGSNHCAQGQYCGHYTQYYWRNSYRYGCARYQCNNGGY 210
            SGDFMTAAKAYEM     EKQYYDHGSNHCAQGQYCGHYTQYYWRNSVRVGCARYQCNNGGY
116-2: 282  SGDFMTAAKAYEMWVDEKQTTDHGSNHCAQGQYCGHYTQYYWRNSYRYGCARYQCNNGGY 461

PR-1a: 211  AYSCNYDPLGNFYGQSPY*FK* 276
              YSCNYDP GNFYGQSPY*FK*
116-2: 462  YYSCNYDPPGNFYGQSPY*FK* 527
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Fig. 3. Alignment of the translated fragment 116-2 to a PR-1a protein from *Rehmannia glutinosa*. 286bp 116-2 fragment was amplified by arbitray primer on both sides. *Nicotiana glutinosa* pathogenesis-related protein 1 mRNA, complete. Length = 684, Score = 194 bits (419), Expect = 3e-48, Identification = 77/82 (93%), Positives 77/82(93%), Frame = +1/+3.

(Yun *et al*., 1999). While PR-1a has been postulated to be involved in SAR, and is the most highly expressed of the PR proteins, evidence for its role is lacking. cDNA southern analysis showed that PR-1 mRNA were induced in salicylic acid - treated leaves (Fig. 4). It demonstrated that PR-1 gene was induced by salicylic acid treatment in *R. glutinosa*.

An ever-increasing body of evidence suggests that salicylic acid plays an important role in the activation of defense responses against pathogen attack in plants. During the past several years, different studies have been initiated to elucidate the mechanism of SA action in plant disease resistance. However, the precise role of SA has yet to be determined. The function of SA as a primary long distance signal in SAR is questionable (Rauscher *et al*., 1999; Sato *et al*., 1996). It must be concluded that the high extent of sequence conservation of the plant PR-1a proteins from different plant families is remarkable, but so far does not offer any clue concerning their mode of action. To define their function in plant, new strategies need to be devised (Hasegawa *et al*., 1982; Jia *et al*., 1989; Lawton *et al*., 1996). We also constructed a PCR cDNA library. It was very useful in searching for a gene using Ca and Ra primer. From this work, we have isolated the gene PR-1a mRNA from SA treated *R. glutinosa* and due to its induction test plant showed resistance against the pathogen.

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