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Short Communication

Spatial distribution of polygalacturonase-inhibiting proteins in *Arabidopsis* and their expression induced by *Stemphylium solani* infection

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ABSTRACT

Disease-induced polygalacturonase-inhibiting proteins (PGIPs) are the major defense proteins which play an important role in resistance to infection of pathogens. To date, the AtPGIP expression in *Arabidopsis* induced by *Stemphylium solani* (*S. solani*) was not described. Here the distribution of AtPGIPs and their expression induced by *S. solani* infection in *Arabidopsis* was reported. Notably, immunofluorescence localization showed that the AtPGIPs were distributed in leaves, petioles, stems and roots of 5 week old *Arabidopsis*, but they were mainly localized in epidermis, vascular bundles and vascular cylinder. Further studies indicated that the transcription level of *AtPGIP1* and *AtPGIP2* was both up-regulated in response to infection with *S. solani* which caused hypersensitive cell death, but the transcription level of *AtPGIP2* was less induced than *AtPGIP1*. Consistently, the bulk AtPGIPs of *Arabidopsis* showed a higher activity in leaves infected by *S. solani*. Taken together, our preliminary results showed that AtPGIPs were spatially distributed and AtPGIP expression might take part in resistance to infection of *S. solani*. This study might highlight the potential importance of AtPGIPs and plant disease resistance.

1. Introduction

During pathogenesis, endopolygalacturonases (PGs) are the first enzymes to be secreted by fungal pathogens when they encounter plant cell wall (Jones and Jones, 1997; Sexton et al., 2000). PGs degrade plant cell wall which allows other hydrolyzation enzymes to degrade cell wall more easily (Karr and Albersheim, 1970; Reymond et al., 1994). Polygalacturonase-inhibiting proteins (PGIPs) are present in the cell walls and play a key role in plant disease resistance (Prabhu et al., 2012; Sathiyaraj et al., 2010; Schacht et al., 2011; Veronico et al., 2011). PGIPs specifically trigger their effect through inhibiting PGs activity, retarding the pathogens invasion, preventing pathogen spread, and also involving in defence responses in plants, such as hypersensitive response (Ferrari et al., 2006; Janni et al., 2008; Kortekamp, 2006; Spinelli et al., 2009).

PGIPs are an important family of defence proteins, which belong to a large super family of leucine-rich repeat (LRR) proteins (De Lorenzo and Ferrari, 2002; Huang et al., 2011; Joubert et al., 2006; Maulik et al., 2012; Shanmugam, 2005). These proteins have consequently acquired considerable importance in recent years. PGIPs and the products of many plant resistance genes share a LRR structure, and the plants use the LRR for their "immune" function and recognition of non-self-molecules (De Lorenzo and Ferrari, 2002; Di Matteo et al., 2003; Huang et al., 2011; Ilag et al., 2000; Jones, 2001). The overexpression of a bean PGIP in transgenic wheat confers increased resistance to the fungal pathogen (Janni et al., 2008). Overexpression of *AtPGIP1* and *AtPGIP2* in *Arabidopsis* significantly reduces *Botrytis cinerea* disease symptoms (Ferrari et al., 2003). Interestingly, antisense expression of the *AtPGIP1* gene reduces PGIPs accumulation and enhances susceptibility to *Botrytis cinerea* (Ferrari et al., 2006). Thus, these results indicated that AtPGIP1 and AtPGIP2 are required for disease resistance.

The internal transcribed spacer (ITS) regions are non-coding sequences interspaced among highly conserved fungal rDNA and have been shown to have a high heterogeneity among different fungal genera and species (Chambers et al., 1986; Iwen et al., 2002; Sharma et al., 2012). The sequence variation within the ITS region allowed reliable and faster discrimination of the isolates at both the genus and species level (Centis et al., 1996; Gardes and Bruns, 1993). Thus, we used the ITS region for molecular indentification of the plant pathogenic fungi as *S. solani*.

Leaf blight caused by *S. solani* is major fungal disease in China where it has caused severe crop losses. *Arabidopsis* may be the best model system for basic research of plant disease resistance. To date, there is little known about the AtPGIP expression in *Arabidopsis* induced by *S. solani*. So, the aims of this project are sought to report the constitutive

Abbreviations: ITS, internal transcribed spacer; LRR, Leucine-rich repeats; PDA, potato dextrose agar; PGA, polygalacturonic acid; PGs, polygalacturonase; PGIPs, polygalacturonase-inhibiting protein; RT-PCR, reverse transcriptase polymerase chain reaction; qRT-PCR, quantitative real-time polymerase chain reaction; *S. solani*, *Stemphylium solani*.

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distribution of AtPGIPs and study the PGIP expression in leaves of *Arabidopsis* during *S. solani* infection. Our preliminary result showed that AtPGIPs were spatially distributed and AtPGIP expression might take part in resistance to infection of *S. solani*. This study might highlight the potential importance of AtPGIPs and plant disease resistance.

2. Materials and methods

2.1. Amplification of ITS region

Genomic DNA was extracted from mycelium by a CTAB procedure and quantified by UV spectrophotometry. Sequences for the primers ITS1 and ITS4 region were 5′-TCCGTAGGTGAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′, respectively (Gardes and Bruns, 1993). Amplification was performed using the following program: 94 °C for 3 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 5 min. Amplification products were electrophoresed in 1.4% agarose gels, stained with ethidium bromide and photographed to verify the fragment size. The amplified products obtained with primer pair ITS1 and ITS4 were sequenced. The sequence was used to identify the fungi with the help of the BLAST program (www.ncbi.nlm.nih.gov/BLAST).

2.2. Plant materials and fungal inoculation

Arabidopsis (*Arabidopsis thaliana*) L. Heynh. Ecotype Columbia was cultivated in a greenhouse under conditions of 14 h light (120 mmol/(m^2.s))/10 h dark cycle, 23 °C. Five week old plants were infected with spores *S. solani* grown on potato dextrose agar (PDA) for 7– 10 days at 24 °C with a 12 h photoperiod before spore collection. For disease treatment, leaves were inoculated with 5×10^4 conidial spores of *S. solani* per milliliter. The tip of 20 μL pipettors was gently applied to the surface of the leaves to induce small wounds without punching out tissue. About 1 wound of 1 leaf tissue was produced. A 5 μL droplet of spore was spotted onto each wound and leaves were incubated at 23 °C with a 14/10 h photoperiod. As controls, leaves were mock-inoculated in the same way with sterile non-inoculated medium. Leaves were harvested at different time points, immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.3. Antigen preparation and antibody production

The sequence encoding for the part-length *AtPGIP1* was amplified with primer P1 (5′-GCGCCATGGGTGGGCACCAACTACCTA-3′) containing *Nhe* restriction site and primer P2 (5′-GCGAAGCTTTTAATTCACTTCTCCGT-3′) containing *HindIII* restriction site. The amplification products were cloned into pGEM®-T Easy Vector (Promega, Madison, WI, USA), sequenced and digested. The digested fragment was ligated into bacterial expression vector pET-28a (Invitrogen, Carlsbad, CA, USA) pre-digested with *Nhe* and *HindIII* (Fig. S1). The recombinant plasmid contained the part-length of *AtPGIP1* sequence fused upstream of the gene encoding 6× His. Correct insertion of the fragment was confirmed by DNA sequencing. The recombinant plasmid was subsequently introduced into *E. coli* BL21 (DE3) strain (Invitrogen). When the OD600 of the *E. coli* BL21 (DE3) harboring the recombinant plasmid reached 0.6, IPTG (1 mmol/L) was added into the culture and the culture continued growth at 37 °C for 4 h to induce the fusion protein expression. The fusion protein was analyzed by SDS-PAGE along with *E. coli* BL21 (DE3) harboring pET-28a as controls. To purify the fusion protein, we used the Ni-NTA column (Fig. S1). Polyclonal antiserum against antigen was obtained from New Zealand white rabbits. Blood (0.5 mL) was collected and used to check the titer of antiserum by ELISA and then the antiserum was harvested from the arteriae carotis. ELISA showed that the antibody titer is quite high (data not shown). Bound antibodies were detected with peroxidase-conjugated secondary antibodies.

2.4. Fluorescence microscopy

Five-week-old *Arabidopsis* young leaves, stems, petioles and roots were collected and cut to pieces, then immediately fixed in 4% formaldehyde solution and then embedded in paraffin. After fixation and embedding, tissue sections were cut with a microtome (Leica, Wetzlar, Germany), placed onto poly-lysine at 37 °C. Samples were deparaffinized by washing in xylene series and rehydrated in an ethanol series, washed in PBS, and blocked in PBS containing 5% bovine serum albumin (BSA). Then, sections were incubated with undiluted primary antibody overnight at 4 °C, using a coverslip and a humidity chamber. Rabbit polyclonal antibodies raised against the N-terminal peptide containing 108 amino acids of AtPGIP1 were used as primary antibody. After several rinses with PBS containing 0.05% Tween-20 (PBS-T), and then incubated for 2 h at room temperature with FITC-conjugated goat anti-rabbit IgG antibody diluted 1:3000. Washing was performed for 15 min in PBS-T at room temperature and the slides were rinsed with PBS before scan.

Anti-PGIP antiserum was omitted and the fluorescent anti-rabbit IgG-FITC conjugate was applied in negative control. Immunofluorescence analysis was performed on 10 μm semi-thin section. For detection, samples were examined with a Leica TCS-NT confocal laser microscope. In the confocal system, Alexa fluorescence was excited at 488 nm and emission read in the 515–545 nm interval with a green filter (BP530/30). Controls were made by replacing primary antibody with PBS.

Anti-AtPGIP1 rabbit polyclonal antibody was used in fluorescence microscopy analysis. Amino acid sequence of AtPGIP1 antigen is 72.22% identical with the antigen of AtPGIP2 (Fig. S1). Even though the signals that we detect belong to AtPGIP1, we need to take into account that we might be detecting AtPGIP2 as well. Accordingly, AtPGIPs are mentioned in the microscopy.

2.5. Semi-quantitative reverse transcription PCR

Total RNA was isolated from *Arabidopsis* leaves using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the user manual and quantified by UV spectrophotometry. cDNA synthesis and semi-quantitative reverse transcription PCR (RT-PCR) was carried out as described by (Szankowski et al. (2003). The primers were designed (Table S1).

2.6. Quantitative real-time polymerase chain reaction

RNA extraction and cDNA synthesis from *Arabidopsis* was performed as described in the kit of quantitative real-time polymerase chain reaction (qRT-PCR; Takara, Kyoto, Japan). qRT-PCR was performed on an iCycler iQ™ Real-Time PCR system, using SYBR green (Takara) method. For detection of *AtPGIP1*, *AtPGIP2* and housekeeping *UBQ5* gene, forward and reverse primers were designed (Table S1). Each PCR reaction (20 μL) contained 200 nM primer and 0.5 μL cDNA. The thermal cycling conditions were 95 °C for 10 s followed by 95 °C for 5 s, 60 °C for 20 s for 40 cycles, followed by melt cycle from 50 to 96 °C. The genes were monitored by qRT-PCR at different time point. Melting point analysis of the *AtPGIP1*, *AtPGIP2* and housekeeping gene products resulted in a single peak in all samples, indicating the presence of a single PCR product. Data are represented as mean ± SD of the values obtained from triplicate experiments.

2.7. AtPGIPs activity assay

Total proteins were prepared by homogenizing tissues (2 mL/g tissue) in 2–5 volumes of 1 M NaCl, 0.1 M sodium acetate, pH 6.0, 1% polyvinylpyrrolidone-40, 0.2% NaBisulfate, and homogenates were incubated for 1 h at 4 °C and centrifuged for 15 min at 15,000*g* (Tamuraa et al., 2004). The supernatant was precipitated with 2 volumes cold acetone. Protein concentration was determined in parallel by the method of Bradford (Bradford, 1976). Approximately 50 μg of total proteins was made for activity assay.