Overexpression of citrus polygalacturonase-inhibiting protein in citrus black rot pathogen

*Alternaria citri*

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Summary

The rough lemon (*Citrus jambhiri*) gene encoding polygalacturonase-inhibiting protein (*RlemPGIPA*) was overexpressed in the pathogenic fungus *Alternaria citri*. The overexpression mutant *AcOPI6* retained the ability to utilize pectin as a sole carbon source, and the overexpression of polygalacturonase-inhibiting protein did not have any effect on the growth of *AcOPI6* in potato dextrose and pectin medium. The pathogenicity of *AcOPI6* to cause a black rot symptom in citrus fruits was also unchanged. Polygalacturonase-inhibiting protein was secreted together with endopolygalacturonase into culture filtrates of *AcOPI6*, and oligogalacturonides were digested from polygalacturonic acid by both proteins in the culture filtrates. The reaction mixture containing oligogalacturonides possessed activity for induction of defense-related gene, *RlemLOX*, in rough lemon leaves.

Introduction

*Alternaria citri* is the pathogen causing the citrus storage disease Alternaria black rot; it infects a broad range of citrus cultivars at the stem end of the fruit and causes internal black discoloration and maceration of the fruit core around the central axis area during storage (Brown and MacCornack, 1972, 2000). We have been studying the role and transcription regulation of the endopolygalacturonase (endoPG; EC
3.2.1.15) that degrades pectic polysaccharides in the cell walls (Akimitsu et al., 2003, 2004; Ishiki et al., 1997, 1999, 2001, 2003; Katoh et al., 2006; Ohtani et al., 2003) during colonization with this pathogen.

The importance of endoPG in the virulence of macerating pathogens has been elucidated in several fungal models, including Aspergillus flavus on cotton bolls (Shieh et al., 1997), Botrytis cinerea on tomato (ten Have et al., 1998), A. citri on citrus (Ishiki et al., 2001), Claviceps purpurea on rye (Oeser et al., 2002), and Sclerotinia sclerotiorum on canola (oilseed rape) (Li et al., 2004). However, endoPG is also involved in the activation of plant defense responses by releasing oligogalacturonic fragments that possess elicitor activity (Hahn et al., 1989). Polygalacturonase-inhibiting protein (PGIP), which is present in the cell walls of a number of dicot plants (Hahn et al., 1989; Walton, 1994), decreases the rate of depolymerization of polygalacturonates by forming a complex with endoPG and also has a role in increasing the accumulation of oligogalacturonide elicitors (Cervone et al., 1987). PGIPs have been isolated (Favaron et al., 1994, 1997; Johnston et al., 1993; Lafitte et al., 1984; Pressey, 1996; Stotz et al., 1993, 1994; Yao et al., 1995) and the gene characterized in several different plants (Gotoh et al., 2002; Nalumpang et al., 2002; Stotz et al., 1994; Toubart et al., 1992; Yao et al., 1999). In previous studies, we cloned rough lemon cDNA encoding PGIP and expressed it in Escherichia coli cells to identify the functional properties of the rough lemon PGIP (Gotoh et al., 2002; Nalumpang et al., 2002). The rough lemon PGIP fusion protein inhibited endoPG activity by a maximum of about 60%, and a combination of the PGIP and fungal endoPG released oligogalacturonides from polygalacturonic acid, which possess activity for expression of defense-related genes in rough lemon leaves (Gotoh et al., 2002; Nalumpang et al., 2002).

Here we overexpressed the rough lemon PGIP gene in the fungal pathogen A. citri, and studied secretion of the plant gene products together with endoPG from the fungus in culture filtrates. The characteristics of the fungal mutant expressing the plant cell wall protein and the functions of the PGIP/endoPG complex in the culture filtrates are described.

Materials and methods

Fungal culture conditions and measurement of endoPG activity

A. citri Ellis & N. Pierce (ATCC 58171) (Cotty and Misaghi, 1984) and the RlemPGIPA overexpression mutant AcOPI6 were stored as glycerol stocks at –80 °C and were grown on V8 agar plates immediately before use for various experiments. They were grown in potato dextrose broth (PDB) or in pectin liquid or pectin agar medium containing 1% (w/v) pectin (P-9135; Sigma, St. Louis, MO, USA) (Ishiki et al., 2001). In the case of liquid pectin medium, they were grown in 200 mL flasks each containing 50 mL of the medium for 14 or 25 d at 24 °C, and the culture filtrates were filtered through four layers of gauze for protein gel blot analysis, induction of rough lemon defense-related genes, and endoPG enzyme assay; the mycelia mats were recovered for nucleotide isolations. EndoPG activity of the cultures was measured by a relative viscosity assay, and one unit was defined as the activity that reduces the viscosity of 1.5% (w/v) polygalacturonic acid solution (pH 5.0) at 37 °C for 5 min by 50%, as described previously (Ishiki et al., 1997).

Preparations of plasmid construct and RlemPGIPA overexpression mutant

The overexpression vector pAOEIK was produced by ligation of the cassette of trpC promoter (Lu et al., 1994) and trpC terminator (Mullaney et al., 1985) of Aspergillus nidulans, which were recovered from pSH75 (Kimura and Tsuge, 1993), into pCR-script SK Amp (+) (Stratagene, La Jolla, CA, USA) at BamHI and Xho I sites (Fig. 1). The full length RlemPGIPA sequence (Gotoh et al., 2002; Nalumpang et al., 2002) was amplified with the primer set EcoRI+PGIPAF (5′-GGAAATTCGAGCCTAACAGCTCATTGT-3′) and PGIPAR+EcoRI (5′-CGAATTCGAGCTCATTTTCGAGGCGGCG-3′), which were attached to restriction sites with a few additional nucleotides of EcoRI in respective 5′ terminals. Insertion of the amplified RlemPGIPA at the EcoRI site (Fig. 1) in pAOEIK, resulting in the transformation of pAOEIK-RlemPGIPA, was confirmed by PCR analysis with primer set PtrpCF2 (5′-TCTACGTCTTAAATGTCGAC-3′) and PGIPAR+EcoRI, following the sequence analysis of the product.

Protoplasts of A. citri were prepared with 10 mg/mL lysing enzymes (Sigma) and 30 mg/mL Kitalase (Wako, Tokyo, Japan) by the methods described previously (Ishiki et al., 2001). Co-transformation was performed with approximately 10⁷ protoplasts and 3 µg each of pAOEIK-RlemPGIPA and pSH75 (Kimura and Tsuge, 1993), which contains a cassette conferring hygromycin resistance. Following the transformation, the protoplasts were placed directly onto the regeneration medium containing 0.1% (w/v) Bacto yeast extract, 0.1% (w/v) Bacto casein digest, 1 M sucrose, and 0.8% (w/v) Bacto agar, and were incubated at 24 °C for 24 h. The plates were overlaid with 5 mL of the same medium containing 1.5% (w/v) Bacto agar and hygromycin B 100 µg/mL, and were incubated at 24 °C for 1 d. The colonies growing on the selective medium were moved to potato dextrose agar (PDA) containing 50 µg/mL hygromycin B and purified by single-spore isolation. Insertion of the construct was confirmed by genomic PCR with a primer set of

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A. digit  

Total RNA was isolated from mycelial mats grown at 24 °C for 14 d in PDB using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the method of Masunaka et al. (2000), or from rough lemon leaves after different periods of oligogalacturonide treatment, according to the method described previously (Gomi et al., 2002; Nalumpang et al., 2002).

Total RNA was loaded on 1% agarose-formaldehyde gels (5 or 10 μg/lane) and transferred to Hybond N+ membrane (GE Healthcare Bio-Sciences, Little Chalfont, UK) by capillary transfer. Total RNA on the membrane was stained with 0.02% (w/v) methylene blue in 0.3 M sodium acetate (pH 5.2) and destained in 20% (v/v) ethanol. The rRNAs were stained with methylene blue to confirm equal RNA loading. Hybridization was performed at 68 °C overnight with the PCR DIG probe (100 ng/mL) for RNA hybridization, and was visualized by anti-DIG antibody-alkaline phosphatase and CSPD (Roche) and exposing the membranes to X-ray films (Fujifilm RX-U, Tokyo, Japan).

For the RNA hybridization analysis, a RlemPGIPA PCR DIG probe with the entire region of RlemPGIPA was prepared with primers of EcoRI+PGIPAF and PGIPAR+EcoRI following the manufacturer’s instructions. The RlemLOX probe was described previously (Gomi et al., 2002). PCR was performed in a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with Taq DNA polymerase (2.5 units; Takara, Shiga, Japan) at 95 °C for one cycle, and 30 cycles of 2 min at 95 °C, 2 min at 50 °C, and 1 min at 72 °C, with 2 μM primer (each), 2.5 mM deoxynucleoside triphosphate (dNTP) mixture (each), 100 ng of genomic DNA from A. digit or plasmid containing RlemPGIPA, and the reaction buffer supplied with the Taq DNA polymerase (Takara).

Pathogenicity assay

To examine the pathogenicity of RlemPGIPA overexpression mutant AcOPi6 of A. digit, citrus fruits and peel segments were presterilized by soaking in 1% (v/v) sodium hypochlorite and then rinsed in sterilized water several times. The sterilized peel segments were used for the citrus peel assay as described previously (Isshiki et al., 2001; Katoh et al., 2006). Briefly, a piece (3 mm³) of mycelium on V8 juice agar was placed on the internal surface of peel segments (2 × 2 cm) from Washington navel orange (C. sinensis (L) Osbeck), and the peel segments were incubated in a sealed sterilized plastic plate at 24 °C for 5 d, with daily monitoring of mycelial growth.

A spore suspension (100 μL of 10⁶ spores/mL) was also injected directly into the central axis of sterilized citrus fruits by the method described previously (Isshiki et al., 2001; Katoh et al., 2006). Hassaku orange (C. hassaku hort. ex Tanaka) was used for this inoculation assay. The fruits were sterilized by soaking for 1–3 h in 1% (v/v) sodium hypochlorite and then rinsed in sterilized water before the inoculation. The inoculated fruits were then incubated in a sealed, sterilized plastic box at 24 °C for 3 weeks and sliced in half for observation of symptom.
development as described previously (Isshiki et al., 2001; Katoh et al., 2006).

Oligogalacturonide detection by thin layer chromatography (TLC)

Different combinations of mixtures (total 65 μL) of polygalacturonate acid (1% (w/v)) and culture filtrates (40 μL) or extracts (40 μL) of mycelial mats, from either A. citri wild type or AcOPI6, in 50 mM sodium acetate buffer (pH 5.0), were incubated for 4 h at 37 °C, boiled for 30 min, and analyzed on a Silica gel 60F254 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). Galecturonide fragments were detected by spraying 0.2% 5-methylresorcinol (Wako) in a solution of sulfuric acid and methanol (1:9, v/v), followed by incubation at 105 °C for 5 min using the methods described previously (Nalumpang et al., 2002; Yao et al., 1995). Extracts from mycelial mats were prepared by the methods described by Isshiki et al. (2001) with minor modifications. After removal of culture filtrates from pectin liquid medium (50 mL) containing 1% (w/v) pectin (Sigma) (Isshiki et al., 2001) for 14 d at 24 °C, the mycelium was homogenized with a mortar and pestle in liquid nitrogen. The homogenized mycelium was suspended in 50 mL of 100 mM acetate buffer (pH 5.0) containing 0.5 M NaCl, 0.02% (w/v) sodium azide, 0.3% (v/v) polyoxyethylene-sorbitan monolaurate (Tween 20), and one tablet of Complete Protease Inhibitor Cocktail Set (Roche). The suspended mycelium was incubated at 4 °C for 12 h. The preparation was centrifuged at 15,000 g for 10 min. The supernatant of the preparation was used as a mycelial mat extract, and also analyzed on Silica gel 60F254 TLC plates (Merck) as described above.

Protein gel blot analysis

Anti-RlemPGIPA antibodies were raised in mouse by injection of RlemPGIPA inclusion bodies (Nalumpang et al., 2002) produced by overexpression using a pRSET expression vector (Invitrogen, Carlsbad, CA, USA) in E. coli BL21-Gold (DE3) (Stratagene), as described previously (Ohtani et al., 2002). The inclusion bodies from E. coli were partially purified by centrifuging several times and then fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide) (Nalumpang et al., 2002); the band corresponding to the protein product was excised from the gel, homogenized in PBS, and injected into mice with four boosters at 1-week intervals (Ohtani et al., 2002).

For protein analysis, wild-type A. citri or AcOPI6 was incubated in pectin liquid medium containing 1% (w/v) pectin (Sigma) (Isshiki et al., 2001) for 14 d at 24 °C, and the culture filtrate (15 μL) was filtered through four layers of gauze, mixed with SDS-PAGE sample buffer (5 μL), boiled, and resolved on 12% (w/v) acrylamide gel as described previously (Isshiki et al., 1997, 2001). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane with an electrotransfer unit (LKB2117 Multiphor II) by using a transfer buffer composed of 99 mM Tris, 192 mM glycine, and 20% (v/v) methanol. The PVDF membrane was blocked with 0.3% (v/v) Tween 20 in phosphate-buffered saline (PBS-Tween) overnight at room temperature. The membrane was washed with PBS-Tween for 10 min, and incubated with anti-PGIP antibody at 1:2000 in PBS-Tween containing 5% (v/v) BSA for 1 h at room temperature. After additional washings with PBS-Tween, the membrane was treated with goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma) at a 1:10,000 dilution. Alkaline phosphatase was detected with 5-bromo-4-chloro-3-indolyl phosphate and nitrotriazolium blue in buffer containing 100 mM Tris (pH 9.5), 100 mM NaCl, and 10 mM MgCl2, as described previously (Akimitsu et al., 1992; Ohtani et al., 2002).

Analysis of rough lemon defense-related gene induction

Culture filtrates of wild-type A. citri or AcOPI6 in liquid pectin medium (50 mL) containing 1% (w/v) pectin (Sigma) (Isshiki et al., 2001) were incubated 14 d at 24 °C in 200 mL flasks, filtered through four layers of gauze, and filter-sterilized using a Millipore GS Filter Unit (22 μm; Millipore, Billerica, MA, USA). The culture filtrate (4 mL) from either wild-type A. citri or AcOPI6 was then mixed with polygalacturonate acid (1% (w/v)) in 50 mM sodium acetate buffer (pH 5.0) (total of 6.5 mL), and incubated for 4 h at 37 °C followed by boiling for 30 min. After incubation, the mixture was sprayed directly onto the lower surface of rough lemon leaves, and the leaves were incubated in a moist chamber at 24 °C for up to 24 h in the dark. Total RNA was extracted from the leaves at 0–24 h, following RNA hybridization analysis as described above.

Results

Generation of RlemPGIPA overexpression mutant

Hygromycin-resistant transformants of A. citri were generated by co-transformation with the vector pSH75 and RlemPGIPA overexpression vector pAOEIK-RlemPGIPA (Fig. 1). Among six transformants obtained, PCR analysis using primers of PtprCF2 and PGIPAR+EcoRI for amplification of a partial region of the vector sequence and the entire RlemPGIPA (Fig. 1A) detected amplificants of the expected size of 1073 bp from the genome of three transformants, indicating the integration of pAOEIK-RlemPGIPA into the genome, and one of the transformants, designated as AcOPI6, was used for this study (Fig. 1B).

To confirm overexpression of the RlemPGIPA gene, RlemPGIPA transcripts were detected by RNA hybridization. RNA gel blots confirmed that...
transcripts with the expected size of about 1000 bp were detected in the total RNA from AcOPI6 grown in PDB, while no signal was obtained from the total RNA from the wild type (Fig. 1C).

**Characterization of RlemPGIPA-overexpression mutant AcOPI6**

The RlemPGIPA overexpression mutant AcOPI6 was able to utilize pectin as a sole carbon source for growth. A mycelial block of AcOPI6 was placed on pectin medium or the internal surface of a sterilized piece of citrus peel. The growth of this mutant on both pectin liquid/agar medium and citrus peel was similar to that of the wild type strain (Fig. 2A and C). AcOPI6 sporulated as well as the corresponding wild types, and there were no differences in spore germination, appressorium formation, or infection hypha formation on cellulose membranes (data not shown). EndoPG activity, measured by the decrease in relative viscosity of 1.5% (w/v) solution (pH 5.0) of polygalacturonic acid, also showed normal endoPG production in the culture filtrates from AcOPI6, similar to that of the wild type (Fig. 2B). A spore suspension (1 x 10^6 spores/100 μL) of A. citri wild type or AcOPI6 was injected directly into the central axis of sterilized citrus fruits, but no obvious difference was found between the symptoms caused by the wild type or AcOPI6 (Fig. 2A). Three out of 6 transformants obtained were examined as the same manner, and all showed similar results, and one of the three transformants AcOPI6 was further examined.

To localize RlemPGIPA in AcOPI6, culture filtrates and mycelial mats from pectin liquid medium inoculated with either wild-type A. citri or AcOPI6 were analyzed immunologically using anti-RlemPGIPA polyclonal antibodies raised in mice by injections of RlemPGIPA fusion proteins expressed in E. coli. Protein gel blots confirmed that a single band with the expected size of about 70 kDa was detected in the culture filtrates from only AcOPI6, but not from the wild type (Fig. 3). The protein was also not detected in the extracts of mycelial mats either the wild type or AcOPI6 (Fig. 3).

**Examination of the function of expressed RlemPGIPA in culture filtrates of AcOPI6**

Products of polygalacturonic acid digested by the functional complex of overexpressed RlemPGIPA and secreted by endoPG into the culture filtrates from AcOPI6 were examined by TLC. Oligomers of four or more galacturonide residues were detected when polygalacturonic acid was incubated for 4h with the culture filtrates of AcOPI6 containing both RlemPGIPA and endoPG, while the oligomers were not detected when polygalacturonic acid was incubated for the same period with culture filtrates...
of the wild type containing only endoPG (Fig. 4). Neither were oligomers of galacturonide detected when polygalacturonic acid was incubated for 4 h with mycelial extracts from either wild type or AcOPI6 (Fig. 4).

**Induction of transcription of defense-related gene in rough lemon leaves**

The mixture containing oligogalacturonides generated by polygalacturonic acid and the culture filtrates with both RlemPGIPA and endoPG of RlemPGIPA overexpression mutant AcOPI6 were sprayed onto the lower surface of rough lemon leaves, and transcription of the defense-related gene lipoxynase (RlemLOX) was monitored by RNA gel blot analysis (Fig. 5). Transcription of RlemLOX was induced within 2 h, reached the maximum level at 12 h, and then declined gradually when leaves of rough lemon were treated with the mixture containing oligogalacturonides generated from polygalacturonic acid by culture filtrates or mycelial extracts of RlemPGIPA overexpression mutant AcOPI6 on TLC. (A) Different combinations of mixtures of culture filtrates from either A. citri wild type (WT) or AcOPI6 (OPI6), with/or without polygalacturonic acid (PGA) incubated for 4 h at 37 °C, followed by boiling for 30 min, were analyzed on TLC plates in a solvent system of ethyl acetate, acetic acid, formic acid, and water (9:3:1:4, v/v/v/v). The respective sizes of galacturonides were detected by spaying with 0.2% 5-methylresorcinol. Monomer (M), dimer (D) and trimer (T) of galacturonides are in the left three lanes as size markers. Arrow indicates the oligogalacturonides on TLC. (B) Different combinations of extracts of mycelial mats from either wild type (WT) or AcOPI6 (OPI6), with/or without polygalacturonic acid (PGA), were also analyzed on TLC plates, as described above.
by polygalacturonic acid and the culture filtrates of AcOPI6 (Fig. 5). In contrast, RlemLOX expression was detected at 12 h and reached the maximum level at 24 h when leaves of rough lemon were treated with polygalacturonic acid and the culture filtrates of the wild type not containing the oligogalacturonides (Fig. 5). These experiments were repeated independently three times with similar results.

Discussion

Cervone’s group (Di Matteo et al., 2006) generalized that PGIPs present in plant cell walls interact specifically with fungal or insect-derived endoPGs and are usually ineffective against enzymes of either bacterial or plant origin. The PGIP–endoPG interaction limits the enzymatic abilities of endoPG and leads to the accumulation of elicitor oligogalacturonides, which activate plant defense responses, such as synthesis of phytoalexins, lignin, and ethylene, production of reactive oxygen species, and induction of transcriptions of various defense-related genes (Ridley et al., 2001).

The functions of RlemPGIPA from rough lemon have already been identified as inhibition of endoPG activity, at a maximum of 60% compared to the normal activity without the PGIP addition with a saturation level of RlemPGIP and digestion of oligogalacturonides from polygalacturonic acid by addition of endoPG and the PGIP (Nalumpang et al., 2002). Because of the presence of an N-terminal signal peptide, indicating secretion of a protein from rough lemon cells, RlemPGIPA was expected to be located at the rough lemon cell walls, similar to the localization sites of other plant PGIPs (Gotoh et al., 2002; Nalumpang et al., 2002). The sorting function of the signal peptide of RlemPGIPA also worked in fungal cells of A. citri, and the RlemPGIPA expressed in AcOPI6 was detected only in the culture filtrates but not in the mycelial extracts. Although the endoPG activity in the culture filtrates from AcOPI6 was not different from that of the wild type, oligogalacturonides accumulated when the culture filtrates containing both endoPG and RlemPGIPA were reacted with polygalacturonic acid. Because the trpC promoter (Lu et al., 1994) of A. nidulans, used for the overexpression of RlemPGIPA, is a relatively mild promoter of transcription in fungal cells, endoPG activity will be inhibited, and more oligogalacturonides will be accumulated if the culture filtrates contain more PGIP from the overexpression mutant generated by the use of another vector carrying a stronger promoter.

The importance of PGIPs in plant defense has been elucidated by a series of studies. Overexpression of PGIP gene in Arabidopsis reduced symptoms and colonization by B. cinerea (Ferrari et al., 2003). Overexpression of pear PGIP in tomato and grapevine plants also significantly reduced endoPG activity as well as susceptibility to B. cinerea (Agüero et al., 2005; Powell et al., 2000). The overexpression of PGIP observed in these examples strengthened the plant defense by reduction of virulence factors of endoPG activity secreted by fungal pathogens, as well as the release of elicitor-active oligogalacturonides from pectic polysaccharides in the plant cell wall using a complex of the fungal pathogen-originated endoPG and the overexpressed PGIP in the cell wall. Thus, the reaction mixture containing elicitor-active oligogalacturonides produced by endoPG and RlemPGIP in AcOPI6 culture filtrates was sprayed onto the lower surface of rough lemon leaves, and induction of RlemLOX gene expression was monitored to determine its defense induction activity. As previously observed in the transcription induction of another defense-related

Figure 5. Induction of LOX gene expression in rough lemon leaves after spraying with oligogalacturonide mixtures. Reaction mixtures of culture filtrates from either A. citri wild type (WT) or AcOPI6 (OPI6), and polygalacturonic acid (PGA), were incubated for 4 h at 37 °C followed by boiling for 30 min. Rough lemon leaves sprayed with the respective mixtures were incubated for 2, 12, or 24 h at 24 °C in the dark. RNA hybridization used for transcript detection of RlemLOX was described previously (Gomi et al., 2002; Nalumpang et al., 2002). Equality of RNA loading in each lane was estimated by rRNA stained with methylene blue.
gene encoding a chalcone synthase in citrus leaves by oligogalacturonides produced by endoPG and RlemPGIP-fusion protein prepared in an E. coli expression system (Nalumpang et al., 2002), the mixture containing oligogalacturonides prepared from AcOPI6 culture filtrates also induced transcription of RlemLOX faster and more strongly than a control preparation containing no oligogalacturonides. Because the relationship between transcription of RlemLOX and rough lemon defense responses is well-defined (Gomi et al., 2002), induction of this gene by oligogalacturonides prepared from the AcOPI6 culture filtrates is considered to be induction of a rough lemon defense. These results suggest that overexpression of plant PGIP in a fungus is likely an effective way to produce endoPG–PGIP complex for a simple preparation of oligogalacturonides. Pathogenicity of AcOPI6 did not change because A. citri is a postharvest pathogen and can cause disease only in harvested fruits after detached from tree, in where induction of defense is likely less active than those in leaves. However, overexpression of PGIP with other fungal pathogens which can infect leaves will provide further information for the role of PGIP and endoPG complex in plant–pathogen interactions in future.

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References


