Endopolygalacturonase Is Essential for Citrus Black Rot Caused by *Alternaria citri* but Not Brown Spot Caused by *Alternaria alternata*

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Alternaria citri, the cause of Alternaria black rot, and *Alternaria alternata* rough lemon pathotype, the cause of Alternaria brown spot, are morphologically indistinguishable pathogens of citrus: one causes rot by macerating tissues and the other causes necrotic spots by producing a host-selective toxin. To evaluate the role of endopolygalacturonase (endoPG) in pathogenicity of these two *Alternaria* spp. pathogens, their genes for endoPG were mutated by gene targeting. The endoPGs produced by these fungi have similar biochemical properties, and the genes are highly similar (99.6% nucleotide identity). The phenotypes of the mutants, however, are completely different. An endoPG mutant of *A. citri* was significantly reduced in its ability to cause black rot symptoms on citrus as well as in the maceration of potato tissue and could not colonize citrus peel segments. In contrast, an endoPG mutant of *A. alternata* was unchanged in pathogenicity. The results indicate that a cell wall-degrading enzyme can play different roles in the pathogenicity of fungal pathogens. The role of a cell wall-degrading enzyme depends upon the type of disease but not the taxonomy of the fungus.

Additional keywords: ACR-toxin, host-specific toxin, pectinase.

The plant cell wall is a potential barrier to the penetration and spread of phytopathogenic bacteria and fungi, and many plant pathogens make extracellular enzymes that can degrade cell wall polymers. Cell wall-degrading enzymes and their genes have been studied for their possible role in many aspects of pathogenicity, including penetration, maceration, nutrient acquisition, plant defense induction, and symptom expression (Cooper 1983; Cooper 1984; Walton 1994). Despite extensive knockout experiments by the targeted gene disruption of particular cell wall-degrading enzyme genes of fungal pathogens (e.g., Scott-Craig et al. 1990), conclusive establishment for the function of cell wall-degrading enzymes is still ambiguous. The evaluation of a single enzyme in pathogenicity is usually difficult because of the retention of certain residual enzyme activity in the disruption mutants or

because of the possible existence of other pathogenicity factors that can compensate for the lack of a single enzyme.

Extensive studies of pectinases such as pectate lyase have been performed with the bacterial pathogens *Erwinia carotovora*, *Erwinia chrysanthemi*, and *Pseudomonas viridiflava*, all of which can macerate plant tissues (e.g., Barras et al. 1994; Collmer and Keen 1986). The role of fungal pectinases in pathogenesis also has been elucidated in many biotrophic and necrotrophic parasites, causing different symptoms (Cooper 1983; Cooper 1984; Walton 1994). Only two pathogens that cause maceration, *Aspergillus flavus* on cotton bolls and *Botrytis cinerea* on tomato (Shieh et al. 1997; Ten Have et al. 1998), have been tested. Although these pathogens have multiple genes encoding endoPGs, in both fungi the disruption of a single endoPG gene decreased virulence. Most recently, Rogers et al. (2000) reported that disruption of a single pectate lyase gene did not reduce virulence of *Nectria haematococca* on pea, but disruption of two PL genes drastically reduced virulence. Taking a different approach to the question of the role of pectinases and other cell wall-degrading enzymes in pathogenesis, Tonukari et al. (2000) disrupted *ccSNF1*, a gene required for expression of catabolite-repressed genes under limitation of glucose, in the maize pathogen *Cochliobolus carbonum*. The expression of genes encoding endoPG and exoPG and enzymatic activities were down regulated in the *ccSNF1* mutant, and virulence of the mutant on its host, maize, was decreased significantly (Tonukari et al. 2000).

The present study was undertaken to understand the role of endoPG (poly[1,4- α -D-galacturonide] glycanohydrolase, E.C. 3.2.1.15) in phytopathogenesis by the filamentous fungus *Alternaria* spp. The species of *Alternaria* used in this study cause different symptoms on citrus. *Alternaria citri* Ellis & N. Pierce causes Alternaria black rot, a postharvest fruit disease on a broad range of citrus cultivars (Brown and McCornack 1972; Brown and Eckert 2000). *A. citri* infects either at the stylar or stem end of the fruit and causes internal black discoloration of the fruit core during storage (Brown and McCornack 1972; Brown and Eckert 2000). The other pathogen, *A. alternata* rough lemon pathotype, causes brown spot disease on a narrow range of citrus cultivars, e.g., rough lemon and rangpur lime (Kohmoto et al. 1979; Ruehle 1937; Timmer et al. 2000). This pathogen causes necrotic brown spots surrounded by a chlorotic halo on young leaves as a result of production of a host-selective toxin, ACR-toxin

In this study, we compared the pathogenicity role of endoPG in these two pathogens, which are closely related but cause different symptoms (rotting and necrotic leaf spot) on the same host. Mutation of the endoPG genes of these two fungi had different effects on their virulence, demonstrating a distinct role of endoPG in their respective pathogenicities.

A. citri endoPG was purified from 25-day-old culture filtrates by the method used to isolate endoPG from *A. alternata* rough lemon pathotype (Isshiki et al. 1997). Both endoPGs had molecular masses of 60 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), pH optimum of approximately 5.0, and K_{ms} of 0.15 mg of polygalacturonic acid per ml. Unlike the endoPG from *A. alternata* rough lemon pathotype (Isshiki et al. 1997), the endoPG of *A. citri* was not glycosylated (data not shown).

Total DNA of the recipient strain AC325 of *A. alternata* or transformant PGD3 also was digested with the same restriction enzymes, and the blot was probed with an internal fragment of *Aapgl* (Fig. 2B). The wild type showed single bands of 6.3 kb for *EcoRI*, 7.7 kb for *EcoRV*, and 6.5 kb for *SacI*, whereas strain PGD3 showed two bands of 4.4 and 6.5 kb for *EcoRI*, a single band of 14.0 kb for *EcoRV*, and a single band of 12.7 kb for *SacI* (Fig. 2B). These hybridization patterns

indicate homologous integration of a single copy of pATE2 (Fig. 2D).

Characterization of endoPG mutants.

The *Acpg1*-disrupted mutant M60 was still able to utilize pectin as a sole carbon source for growth in liquid medium, but dry weight of mycelial mats after 25-day growth was reduced by 42% compared with the wild type (average of three independent repeats; data not shown). M60 grew as well as the wild type on medium containing glucose. The *Aapg1* mutant PGD3 grew as well as the wild-type strain on medium containing pectin or glucose. Total endoPG activities in culture filtrates were reduced in both mutants. EndoPG in M60 was reduced by $83\% \pm 5$ (average of three independent repeats), and endoPG in PGD3 was reduced $30\% \pm 7$ (average of three independent repeats).

Expression of *Acpg1* and *Aapg1* was determined by RNA blotting. mRNA from the wild type showed hybridizing bands

of the expected size (approximately 1.5 kb), whereas mRNAs of the endoPG genes were undetectable in the two mutants (data not shown). This confirms that the two endoPG genes had been mutated.

To evaluate production of endoPGs in the mutants, endoPG was purified in parallel from culture filtrates by ammonium sulfate precipitation, carboxymethyl (CM)-Sephacrose, and cation-exchange high-pressure liquid chromatography (HPLC) (Isshiki et al. 1997) (Fig. 3). In the case of M60, a peak of polygalacturonase (PG) activity from cation-exchange chromatography in fractions 13 and 14 in the wild-type strain (Fig. 3A) had disappeared completely in M60 (Fig. 3B). A low level of activity found in the wild type at fraction 15 was still present in PGD3, whereas a major peak at fractions 16 and 17, corresponding to the targeted endoPG, was missing in PGD3 (Fig. 3C and D). Throughout the purification procedures, a considerable amount of exoPG activity (detectable by reducing sugar but not viscosity assay) was in the nonabsorbed fraction from the CM-Sephacrose column in wild types and mutants. No significant change was detected in the amount of exoPG activity in the mutants compared with the wild types (data not shown).

M60 showed no significant reduction in radial growth on glucose as the sole carbon source, but it was reduced severely on pectin, polygalacturonic acid, or purified citrus cell wall (Fig. 4A). PGD3, however, grew as well as the wild type on all of these media (Fig. 4B). Both endoPG mutants 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). ACR-toxin production by PGD3 also was not affected (data not shown).

Evaluation of endoPG-disrupted mutants for symptom development on citrus.

Wild-type *A. alternata* rough lemon pathotype and mutant PGD3 were spray inoculated on rough lemon leaves, which are susceptible to the wild type. No obvious differences were found between the strains in lesion number and size or on the

Fig. 2. Homologous recombination mediated gene disruption of endoPG genes. **A**, Southern blot analysis of *Alternaria citri* wild type (W) and endoPG mutant (M) probed with the deleted fragment of *Acpg1*. **B**, Southern blot analysis of *Alternaria alternata* rough lemon pathotype wild type (W) and endoPG mutant PGD3 probed with the deleted fragment of *Aapg1*. **C**, Restriction maps of *A. citri* wild type (upper panel) and predicted restriction map of mutant M60 (lower panel), assuming tandem homologous integration of pATEC1. **D**, Restriction maps of *A. alternata* rough lemon pathotype wild type (upper panel) and predicted restriction map of mutant PGD3 (lower panel), assuming integration of a single copy of pATE2. The bar with diagonal lines in **C** and **D** indicates the target DNAs for homologous recombination. Black shading in **C** and **D** indicates the gene encoding hygromycin phosphotransferase. The hybridization patterns shown in **A** and **B** are consistent with the predicted integration patterns in **C** and **D**. EI = *EcoRI*, EV = *EcoRV*, SI = *SacI*.

Fig. 3. Cation exchange high-pressure liquid chromatography analysis of endoPG activities in the wild types and mutants. **A**, ● = enzyme activity in wild-type *Alternaria citri* as measured viscometrically with phosphoglyceric acid as the substrate. The solid line is absorbance at 280 nm. **B**, ● = enzyme activity in endoPG mutant M60 of *A. citri*. **C**, ● = enzyme activity in wild-type *Alternaria alternata* rough lemon pathotype. **D**, ● = enzyme activity in endoPG mutant PGD3 of *A. alternata* rough lemon pathotype.

rate of necrosis development, up to 3 days after infection. Typical necrosis development after 3 days from spore inoculation (5×10^5 spores per ml) on rough lemon leaves of two different ages is shown in Figure 5A. Additionally, there was no difference on necrosis development with a lower inoculum level (10^3 spores per ml; data not shown).

The wild-type strain of *A. citri* and the mutant M60 spray inoculated onto rough lemon leaves caused no visible symptoms, even after 7 days (data not shown). In contrast, M60 showed a remarkable reduction in disease development on citrus peel. A mycelial block of M60 or wild-type *A. citri* was

placed on the internal surface of a sterilized piece of citrus peel. The wild type grew until the entire surface of the peel was covered by mycelia (5 days), whereas M60 exhibited much less growth (Fig. 5B). The wild type also grew on the peels of grapefruit (cv. Marsh), iyo tangor (Fig. 5B), navel orange (cv. Washington), oroblanco (cv. Sweetie), rough lemon, Satsuma mandarin, and Valencia orange, whereas M60 failed to grow on peels from any of these cultivars. Wild-type *A. alternata* and the mutant PGD3 grew equally, but poorly, on these peel segments and, unlike wild-type *A. citri*, the mycelium never covered the segments (data not shown).

A droplet of a spore suspension or a mycelial block of *A. citri* was placed under the pedicels of sterilized citrus fruits. After 3 weeks, the wild-type strain caused rotting, mainly in the central axis of the inoculated fruit, and the fungus spread into the juice sac area through the vascular bundles (Fig. 5C). Growth of M60, however, was restricted to the inoculation site, and rotting never developed in the central axis or juice sac area (Fig. 5C). Spore suspensions also were injected directly into the central axis of citrus fruits. M60 growth was limited to the injected area, whereas the wild-type strain developed severe rotting (Fig. 5D). The same results were obtained when these inoculation assays were repeated on iyo tangor, rough lemon, navel orange, Satsuma mandarin (Fig. 5C), or Valencia orange (Fig. 5D). In this assay, the wild-type *A. alternata* and the mutant PGD3 could not cause rotting on any citrus fruit tested (data not shown).

To confirm that *AcpG1* is expressed in planta, *AcpG1* transcripts were detected by RT-PCR with total RNA extracted from areas of fruit showing symptoms. A product of the appropriate size (1.2 kb) could be detected in rotting navel orange fruit but not in RNA from sites inoculated with M60 (Fig. 6). Although no visible symptoms were observed, a RT-PCR product of *AcpG1* also was amplified from the total RNA of rough lemon leaves inoculated with the wild type but not from equivalent sites inoculated with M60 (Fig. 6).

A. alternata wild type and M60 were inoculated onto potato tubers to examine their ability to macerate. Droplets of spores were inoculated on the top of cubes of sterilized potato tuber, and maceration was evaluated after 7 days. The maceration ability of M60 was reduced 85% compared with the wild-type strain (Fig. 7).

DISCUSSION

Our results show that the endoPG-disrupted mutant M60 of *A. citri* is reduced significantly in its ability to cause black rot symptoms on citrus as well as maceration of potato tissue. In nature, *A. citri* invades citrus fruit through cracks or openings on either the button or stylar end, and black rot symptoms progress from the infected site through the vascular bundles and down the center of the fruit into the juice sac area (Brown and McCormack 1972; Brown and Eckert 2000). In contrast, M60 caused light rotting only in a limited area around the inoculated site. The reduced symptoms caused by M60 are most likely caused by a reduction in maceration ability as a result of its lack of endoPG. This conclusion is supported by the fact that M60 also showed severe reduction in its ability to macerate potato tuber.

A similar reduction in maceration ability has been observed with pectinase-deficient mutants of phytopathogenic bacteria

Fig. 4. Growth of *Alternaria citri* and *Alternaria alternata* rough lemon pathotype wild types and endoPG mutants on various carbon sources. **A.** *A. citri* wild type and mutant M60 were grown on agar plates containing pectin, polygalacturonic acid, purified citrus cell walls, or glucose as the sole carbon source. ● = wild type; ○ = M60. **B.** *A. alternata* wild type and mutant PGD3 were grown on various carbon sources under the same condition. ● = wild type; ○ = PGD3. Vertical bars represent the standard deviation of three independent replicates.

such as *Erwinia*, *Pseudomonas*, and *Ralstonia* species (Barras 1994; Collmer and Keen 1986). “Soft-rotting” bacteria, however, usually have multiple pectinase genes, and a significant reduction of symptoms has been observed only when multiple genes were mutated (Barras 1994; Collmer and Keen 1986), except in the case of *P. viridiflava*, in which a single PG gene reduced soft rot development (Liao et al. 1988). In contrast, most studies with pathogenic fungi, up to this point, have mu-

Fig. 5. Evaluation of virulence of endoPG-disrupted mutants. **A**, Spore suspension (5×10^5 spores/ml) of *Alternaria alternata* rough lemon pathotype wild type (WT) or mutant PGD3 (Mutant) was spray inoculated on rough lemon leaves of different ages. Typical necrosis development and uninoculated leaves (Control) is shown. **B**, Mycelial block of *Alternaria citri* wild type (WT) or M60 (Mutant) mycelium was placed on the internal surface of sterilized peel segments of citrus fruit (iyo tangor). Typical growth after 5 days incubation is shown. **C**, Block of wild type (WT) or M60 (Mutant) mycelium was placed under the pedicel of a sterilized citrus fruit (satsuma mandarin), and the inoculated fruit was incubated for 3 weeks. Typical development of rot symptoms is shown. **D**, Spore suspension of wild type (WT) or M60 (Mutant) was injected directly into the central axis of Valencia orange fruit. Inoculated fruits were incubated for 3 weeks; typical development of rot symptom is indicated.

tated only a single pectinase gene, and most of the mutants have not shown a decrease in virulence (Bowen et al. 1995; Di Pietro and Roncero 1998; Gao et al. 1996; Scott-Craig et al. 1990). Disruption of individual pectinase genes in two soft rot fungi, *A. flavus* and *B. cinerea*, caused a modest decrease in virulence (Shieh et al. 1997; Ten Have et al. 1998). With the use of two different assays, the disruption of *P2c* encoding endoPG in *A. flavus* decreased invasion of cotton bolls by 23 to 33% (Shieh et al. 1997). A strain of *B. cinerea* that was disrupted in *Bcpg1* encoding endoPG showed no defect in primary infection but decreased lesion expansion, indicating that the endoPG encoded by *Bcpg1* is not important for penetration and the initial steps of infection but has a role in spread (Ten Have et al. 1998). The *Bcpg1*-deficient mutant also was predicted to possess additional PGs encoded by other members of an endoPG gene family (Ten Have et al. 1998).

In two cases, fungal pathogens that are disrupted in multiple pectinase genes have been created (Rogers et al. 2000; Scott-Craig et al. 1998). Scott-Craig et al. (1998) disrupted two genes, *PGN1* and *PGX1*, encoding endoPG and exoPG, respectively, and found decreased growth on pectin but no effect on virulence on maize. The disruption of two pectate lyase genes, *pelA* and *pelB*, reduced virulence of *Nectria haematococca* on pea, whereas disruption of either gene alone did not (Rogers et al. 2000). Our results indicate that single pectinase genes can contribute significantly to pathogenesis because disruption of the single-known endoPG of *A. citri* dramatically reduced its ability to cause citrus fruit soft rot.

There are two reasons why disruption of *AcpG1* in *A. citri* gives a dramatic reduction in virulence. First, the disrupted gene was probably the only endoPG gene existing in this strain. We could not detect any trace of other endoPG activity in either the wild type or mutant M60 of this pathogen. Southern blot results also showed no evidence of related genes, and the mutant lost 100% of its endoPG activity and 83% of its total PG activity. The residual 17% of total PG activity is therefore concluded to be the result of exoPG activity. Because the mutant barely could grow on plates containing pectin or polygalacturonic acid as the sole carbon source, the exoPG activity apparently is insufficient to support growth. Second, because of the tissues it colonizes and the way in which it invades plant tissue, pectin degradation probably is

Fig. 6. *AcpG1* expression during infection. An internal region of the *AcpG1* gene was amplified by reverse transcription-polymerase chain reaction (RT-PCR) with total RNA of a sample of tissue (navel orange) inoculated with the wild type (WT) or M60. A RT-PCR product also was amplified from total RNA from rough lemon leaves inoculated with the wild-type strain (WT) or mutant (M60). Additionally, RT-PCR was performed with wild-type total RNA from mycelium grown on pectin as a positive control (Control), and RT-PCR with RNA from uninoculated leaves was used as a negative control. The predicted product of 1.2 kb is indicated on the left.

very important in successful pathogenesis by *A. citri*, which invades tissues that are very rich in pectin, unlike leaves. Therefore it makes sense that pectinase is important (Brown and McCornack 1972; Brown and Eckert 2000). This feature of *A. citri* might lead to the strong inhibition of growth in citrus fruit by disruption of the endoPG gene. *A. citri* may grow in pectin rather than other cell wall components because a lack of pectin degradation caused by the endoPG disruption allowed for the reduction of fitness in the plant as well as enabling its maceration ability. Contrary to this case, it is reasonable to accept that there was no obvious change on the pathogenicity of endoPG mutant PGD3 of *A. alternata* rough lemon pathotype, a necrotrophic pathogen producing host-selective ACR-toxin (Akimitsu et al. 1989; Gardner et al. 1985; Kohmoto et al. 1979; Nakatsuka et al. 1986). The strong toxicity of ACR-toxin could induce necrotic spot, without extensive penetration and colonization.

In this study, we isolated endoPGs and identified the endoPG genes *Acpgl* and *Aapgl* from two morphologically similar pathogens, citrus brown spot and black rot, which belong to the same genera, *Alternaria*, but cause completely different symptoms. *Acpgl* and *Aapgl* are highly homologous (99.6%) and share all of the general features of fungal endoPGs. The only difference that might be significant is that *Acpgl* contains a single predicted N-glycosylation site and *Aapgl* contains two sites. Furthermore, *Aapgl* has been experimentally shown to be glycosylated, whereas *Acpgl* has not (Isshiki et al. 1997). The lack of glycosylation, which is most likely caused by the lack of one glycosylation site, is probably not important to the enzyme activities because biochemical features of these enzymes are not significantly dif-

ferent. Indirect effect of the lack of glycosylation such as stability of this enzyme in plant tissue currently is not known.

Some phytopathogenic fungi grow and develop in the middle lamella between the cuticle and outer epidermal cell wall (Cooper 1983; Cooper 1984; Isshiki et al. 2000), which is mainly composed of pectin (Carpita 1993; Darvill et al. 1980). These pathogens tend to establish a biotrophic relationship with their hosts, and their endoPGs are often large, preventing diffusion through the wall matrix or wall bound that results in localized degradation of the wall to reduce host damage (Cooper 1983; Cooper 1984). *A. citri* also develop in pectin-rich tissue parts (Brown and McCornack 1972; Brown and Eckert 2000), but the pathogen is not considered as biotrophic fungus. Moreover, the purified, secreted endoPG (approximately 60 kDa) from *A. citri* does not have any of the special characteristics of biotrophic endoPGs such as being large or wall bound.

We cannot ignore the remaining endoPG activity as a possible explanation for the retention of full pathogenicity by mutant PGD3. Throughout our purification procedures, we detected only a single endoPG activity from the *A. citri* culture filtrate, whereas *A. alternata* rough lemon pathotype possessed another minor endoPG activity, which was detected in cation-exchange HPLC. The minor activity did not increase after disruption of the major endoPG in *A. alternata*, yet it did remain in the mutant PGD3. Although the major endoPG in the culture filtrate was lost completely, the remaining activity of the minor endoPG and exoPG may have more of a role in planta, or the role of the disrupted endoPG in pathogenicity may be compensated for by the activity remaining in the mutant.

Here, we describe the importance of endoPG for symptom development and colonization by the postharvest disease pathogen *A. citri*. A lack of endoPG, however, showed little effect on the pathogenicity of the *A. alternata* rough lemon pathotype. Differences in symptom development of the wild type and endoPG mutants of each pathogen clearly showed a distinct role for endoPG in their respective pathogenicities. We hypothesize that there may be no general role of cell wall-degrading enzymes in the pathogenicity of fungal pathogens, but that endoPG is more likely to have a function in the pathogenicity of pathogens that grow in pectin-rich plant tissues.

MATERIALS AND METHODS

Fungal cultures and enzyme purification.

A. alternata rough lemon pathotype (strain AC325) (Akimitsu et al. 1989; Kohmoto et al. 1979), *A. citri* (ATCC 58171) (Cotty and Misaghi 1984), and their endoPG mutants were grown in liquid pectin medium, as described previously (Isshiki et al. 1997). EndoPGs were purified from culture filtrate, as described for endoPG of *A. alternata* rough lemon pathotype (Isshiki et al. 1997). Fungi were grown on potato dextrose agar (PDA) and agar plates containing a single carbon source of either 1% (wt/vol) pectin (P-9135; Sigma, St. Louis, MO, U.S.A.), 1% (wt/vol) polygalacturonic acid (P-1879; Sigma), 1% (wt/vol) glucose, or 1% (wt/vol) purified citrus cell walls. Citrus cell walls were purified according to English et al. (1971).

PG activity was measured by either a relative viscosity assay (Walton and Cervone 1990) or a reducing group assay

Fig. 7. Maceration of potato tuber by *Alternaria citri* wild type (WT) and endoPG mutant M60. A droplet of spore suspension was placed on the top of a surface-sterilized cube of potato tuber and incubated for 1–7 days. Macerated tissue surrounding the inoculation site was removed and its wet weight determined (Walker et al. 1994). Vertical bars represent the standard deviation of three independent replicates.

with 2-cyanoacetamide (Gross 1982). The ECL Glycoprotein Detection System (Amersham Pharmacia Biotech, Uppsala, Sweden) was used to determine protein glycosylation of the isolated PG. The molecular mass on SDS-PAGE, a pH optimum, and a K_m for the substrate polygalacturonic acid of the purified endoPG were determined, as described previously (Isshiki et al. 1997).

Nucleic acid manipulations.

Wild-type and mutant strains were grown in 50 ml of potato dextrose broth (PDB) in 200-ml Erlenmeyer flasks at 28°C for 2 to 3 days on a shaker (120 rpm). Genomic DNA was isolated from the mycelia grown in PDB, in accordance with Yoder (1988). Total RNA was isolated from mycelia grown at 24°C for 25 days in Richard's medium containing 1% (wt/vol) pectin (Isshiki et al. 1997), according to Masunaka et al. (2000).

For Southern blot analysis, an internal 481-bp region of *Acpg1* or 683-bp *Aapg1* was amplified by PCR, and the products were labeled with the DIG system (Boehringer Mannheim, Mannheim, Germany). PCR was performed in a PerkinElmer DNA Thermal Cycler (Wellesley, MA, U.S.A.) with *Taq* DNA polymerase (2.5 units; Takara, Shiga, Japan) for 30 cycles (1 min at 95°C, 2 min at 55°C, and 1 min at 72°C) with 0.5 μ M primer (each), 2.5 mM deoxynucleoside triphosphate (dNTP) mixture (each), 100 ng of genomic DNA, and reaction buffer supplied with the *Taq* DNA polymerase (Takara). The primers used were PG#3 (forward) 5'-TACCATGGTTCCTTTCCGA-3' and PG#2 (reverse) 5'-RCARTCRCTC-YTGRIT-3' (where R is G or A; and Y is T or C) for *Acpg1*; PG#28 (forward) 5'-TTCTTCAATATCAAGCAT-3' and PG#2 (reverse) 5'-RCARTCRCTCYTGRIT-3' for *Aapg1*, respectively. Restriction enzyme digestion, hybridization, and detection conditions were described previously (Isshiki et al. 1997; Masunaka et al. 2000).

For Northern blot analysis, the RNA probe was prepared from PCR products used for the DNA probe, as described above, and cloned into the pSPT18 vector by in vitro transcription for labeling with the DIG RNA Labeling Kit (Boehringer Mannheim). Total RNA (8 μ g) was loaded on per lane of agarose-formaldehyde gels and transferred to Hybond N⁺ membrane (Amersham Pharmacia Biotech) by capillary transfer (Sambrook et al. 1989). RNA on the membrane was stained with 0.02% (wt/vol) methylene blue in 0.3 M sodium acetate (pH 5.2) and destained in 20% (vol/vol) ethanol. Staining of the rRNAs with methylene blue was used to confirm equal RNA loading. Hybridization was performed at 68°C overnight with a nonisotope-labeled RNA probe (100 ng per ml). Detection was carried out, as described previously for Southern blots (Isshiki et al. 1997; Masunaka et al. 2000).

RT-PCR was performed with total RNA with the ThermoScript RT-PCR System (GIBCO-BRL, Gaithersburg, MD, U.S.A.). Total fungal RNA was isolated according to Masunaka et al. (2000). Total RNA from citrus leaf or fruit tissues, including macerated fruit tissues, was isolated with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The tissues were cut out with a sterilized razor and frozen immediately in liquid nitrogen. The tissues were then homogenized with a mortar and pestle, and 0.1 g of sample was used for total RNA purification. PCR primers PG#6 (forward) 5'-AC-AATGGTTGCCTTAAC-3' and PG#8 (reverse) 5'-ACGAA-

CACTTGTAACGC-3' were synthesized for this RT-PCR. Total RNA (6 μ g) was reverse transcribed with the use of oligo(dT) primer and the ThermoScript RT-PCR System. The reaction contained *Taq* DNA polymerase (2.5 units; Takara) and were run for 30 cycles (1 min at 95°C, 2 min at 55°C, and 1 min at 72°C) with 0.5 μ M primer (each), 2.5 mM dNTP mixture (each), 100 ng of cDNA as template, and reaction buffer supplied with the *Taq* DNA polymerase.

Isolation of endoPG gene.

To obtain the full-length cDNAs, 5' and 3' RACE were performed with the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA, U.S.A.). Total RNA (6 μ g) was reverse transcribed with oligo(dT) primer and the ThermoScript RT-PCR System. Second-strand cDNA synthesis and adapter ligation was performed with the Marathon kit. Subsequent PCR was conducted with the Clontech Adapter Primer 1 (5'-CCATCCTAATACGACTCACTATAGGGC-3') and either forward primer PG#3 (3' RACE) or reverse primer PG#2 (5' RACE). The products were subcloned into pT7Blue-2 T-vector (Novagen, Madison, WI, U.S.A.). The sequences of 5' and 3' RACE products were obtained from both strands by the dideoxy chain termination method (Sanger et al. 1977) with the use of an ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, U.S.A.) and an automated fluorescent DNA sequencer (Model 310; Applied Biosystems).

Another set of PCR primers, PG#22 (forward) 5'-CTTTTC-TTCAATATCAAG-3' and PG#21 (reverse) 5'-CCTCACCTT-TCCGAACAT-3', from the upper end of the 5' RACE product or downstream of the 3' RACE product, was used to amplify the entire region of the endoPG cDNA by RT-PCR from total RNA. DNA sequences were aligned with CLUSTAL W (Thompson et al. 1994), and homology analysis was performed with BLAST at the DNA Databank of Japan.

To identify genomic DNA sequences corresponding to the upstream sequence of *Aapg1* cDNA, we recovered genomic DNA from PGD3 flanking pATE2, the vector used for endoPG disruption in *A. alternata* rough lemon pathotype, by plasmid rescue (Yelton et al. 1984). Genomic DNA was digested with *SacI*, and the fragments were ligated and transformed into *Escherichia coli* XL1-Blue cells. A clone (pRC5) containing pATE2 and flanking genomic DNA from PGD3 genome was selected on Luria-Bertani plates containing ampicillin. We used the flanking sequences of the pATE2 insertion site obtained by plasmid rescue to design gene-specific forward primer PG#20 5'-CAGATGTGGCGTTTTGCG-3' from the genomic DNA. Primers PG#20 and PG#21 amplified the entire endoPG genes by PCR from genomic DNA of *A. alternata* rough lemon pathotype and *A. citri*. The PCR product was cloned into pT7Blue-2, and the entire region was sequenced.

Homologous recombination-mediated gene disruption.

The endoPG disruption constructs of both fungi were made by inserting a 481-bp region of *Acpg1* or 683-bp of *Aapg1* into plasmid pSH75 (Kimura et al. 1993) containing the *hph* gene as a selectable marker. The insertion region was amplified by PCR with PG#3 and PG#2 primers for *Acpg1* and PG#28 and PG#2 for *Aapg1*, as described above.

Protoplasts were prepared with 10 mg of Novozyme per ml and 30 mg of Driselase per ml, in accordance with Akamatsu

et al. (1997). Transformation was performed with approximately 10^7 protoplasts and 3 μ g of the plasmid by the polyethylene glycol method (Akamatsu et al. 1997). Following transformation, the protoplasts were plated directly onto a regeneration medium containing 0.1% (wt/vol) Bacto yeast extract, 0.1% (wt/vol) Bacto casein digest, 1 M sucrose, and 1.5% (wt/vol) Bacto agar and incubated at 24°C for 24 h. The plates were then overlaid with 5 ml of the same medium containing 100 μ g of hygromycin B per ml and incubated at 24°C for an additional 2 to 3 days. The colonies growing on the selective medium were replated to PDA containing 50 μ g of hygromycin B per ml and purified to nuclear homogeneity by single-spore isolation. Spore germination, appressorium, and infection hypha formation of mutants were examined on celophane membranes (Masunaka et al. 2000). ACR-toxin production was determined by leaf necrosis assay (Akimitsu et al. 1989; Kohmoto et al. 1979).

Pathogenicity and maceration activity of endoPG-disrupted mutants.

For the endoPG mutant of *A. alternata* rough lemon pathotype, a spore suspension containing 5×10^5 spores per ml or 10^3 spores per ml was spray inoculated onto rough lemon leaves, and the leaves were incubated in the dark at 24°C for 3 days. The leaves were monitored daily until they died.

For the endoPG mutant of *A. citri*, a piece (3 mm³) of mycelium growing on a V-8-medium agar plate was placed on the internal surface of peel segments (1 $\times$ 1 cm) from Marsh grapefruit (*Citrus paradisi* Macf.), iyo tangor (*C. iyo* Hort. Ex Tanaka), Washington navel orange (*C. sinensis* (L.) Osbeck), Sweetie oroblanco (*C. paradisi*), rough lemon (*C. jambhiri* Lush.), Satsuma mandarin (*C. unshiu* [Mac.] Marc.), or Valencia orange (*C. sinensis*). The peel segments were presterilized by soaking for 1 h in 1% (vol/vol) sodium hypochlorite and then rinsed in sterilized water several times. The peels were incubated in a sealed sterilized plastic plate at 24°C for 5 days. Mycelial growth was monitored daily.

A block (3 mm³) of mycelium growing on a V-8-medium plate or 50 μ l of spore suspension containing 10^6 spores per ml was placed under the pedicels of sterile citrus fruits. A spore suspension (50 μ l of 10^6 spores per ml) was injected directly into the central axis of sterilized citrus fruits in accordance with Singh and Khanna (1966). Citrus cultivars such as iyo tangor, navel orange, rough lemon, Satsuma orange, and Valencia orange were used for these inoculation assays. Citrus fruits were sterilized by soaking for 1 to 3 h in 1% (vol/vol) sodium hypochlorite and then rinsed in sterilized water. The inoculated fruits were incubated in a sealed sterilized plastic box at 24°C for 3 weeks, and the fruit was sliced in half for observation of symptom development.

Maceration of potato tuber was examined by the method described for bacterial pathogens, with some modifications (Walker et al. 1994). Cubes of potato tuber (2 cm³) were surface sterilized by soaking for 10 min in 1% (vol/vol) sodium hypochlorite and then rinsed in sterilized water and air dried. A droplet of 30 μ l of spore suspension containing 10^6 spores per ml was placed on the top of the potato cube and incubated in a sealed sterilized plastic box at 24°C. After incubation for 1 to 7 days, macerated potato tissue surrounding the inoculation site was removed and its wet weight determined (Walker et al. 1994).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp>).