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Cloning and Sequence Analysis of the Gene Encoding Endopolygalacturonases in the Cotton Black Spot Disease Fungus *Alternaria macrospora*

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The fungus *Alternaria macrospora* is the causal pathogen of cotton black spot disease in cotton seedlings from the cotyledon period to the seedling stages, causing black spots on leaves and their damping off. Endopolygalacturonases (endoPGs) play major roles in pathogen penetration into plants at the initial phase of invasion. In this phase, the pectic polysaccharides in the plant primary cell walls used as potential barriers against pathogens are digested. The present study aimed to clone and characterize the endoPG-encoding gene *ampg-1* in *Alternaria macrospora*. A 1241 bp open reading frame of *ampg-1* was cloned. The nucleotide and its deduced peptide sequence alignment showed that it shared quite high identities with two other *Alternaria* fungi, i.e. *Alternaria alternata* and *Alternaria citri*. The analysis of the predicted amino acid sequence of *ampg-1* revealed that the endopolygalacturonase in *Alternaria macrospora* had four *PbH1* domains, which was less than those of five other species. Phytopathogenesis was implicated in the quantity of the *PbH1* domains. Therefore, the cotton black spot disease prolonged the latency.

Keywords: *Alternaria macrospore*, Enodpolygalacturonases, Sequence analysis, Protein motif.

Plant pathogenic microorganisms invade hosts by producing a variety of enzymes with polysaccharide-degrading abilities. These enzymes break the pectin-rich plant cell walls, as well as penetrate and colonize the host cells. Among such enzymes, poly-(1,4-β-D-galacturonide) glycanohydrolases (EC 3.2.1.15) or endopolygalacturonases (endoPGs) have been demonstrated to be significant virulent factors (D'Ovidio *et al*., 2002). They are the first wall-degrading enzymes secreted by phytopathogenic microbes during an invasion process. One of the important barriers that plants use to fight pathogens is the polysaccharide-rich cell wall. However, these polysaccharides could be degraded into small oligosaccharides by pathogen-produced endoPGs at the initial phase of infection. Consequently, cell walls collapse and nutrition is provided to the pathogens (De Lorenzo and Ferrari, 2002; Desiderio *et al*., 1997). Fungal endoPGs have been reported as the pathogenic elements of some fungal pathogens such as *Alternaria citri* (Isshiki *et al*., 2001), *Botrytis cinerea* (Have *et al*., 1998), and *Claviceps purpurea* (Oeser *et al*., 2002). The bacteria *Ralstonia solanacearum* (Huang *et al*., 2000) and *Agrobacterium tumefaciens* (Rodriguez-Palenzuela *et al*., 1991) also need PGs to infect plants. PGs are also found in many types of yeast

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(Luh *et al*., 1951) and some plant parasitic nematodes (Jaubert *et al*., 2002). Even in higher plants (Brummell *et al*., 2001; Giovannoni *et al*., 1991; Sakai *et al*., 1993), PGs are considered to play important roles in fruit softening (Giovannoni *et al*., 1991) and in the texture changes of fruits during ripening. Fruit deterioration necessary for seed dispersion ultimately occurs (Brummell and Harpster, 2001). Oligogalacturonides are also released during a fungus invasion as elicitor-active signals (Vorwerk *et al*., 2004) to trigger plant defense responses. Such responses include the accumulation of phytoalexins (Nothnagel *et al*., 1983), lignifications in plants (Robertsen 1986), expression of proteinase inhibitor I and β-1,3-glucanase, as well as production of reactive oxygen species (Ridley *et al*., 2001). These responses are triggered when PG activity is controlled by polygalacturonase-inhibiting proteins (PGIPs) (Cervone *et al*., 1989; Ridley *et al*., 2001) located in the cell wall of many dicotyledonous plants (Cervone *et al*., 1997). PGIPs curb cell wall degradation and consequently hamper the infection process.

The fungus *Alternaria macrospora* is the causal agent of a serious devastating disease in China known as the cotton black spot disease (CBSD). This disease attacks from the cotyledon period to the seedling phase, causing a considerable loss of cotton production each year. Studies on the roles of endoPGs in the pathogenicities of *A. citri* and *Alternaria alternata* (Isshiki *et al*., 2001) have been reported, but none for *Alternaria macrospora*. The present study aimed to clone and characterize the gene that encodes endoPGs in *A. macrospora*. Basic knowledge on the physiology and biology of the CBSD fungus is expanded. The structure of the fungal pathogenic proteins, endoPGs, is also elucidated.

MATERIALS AND METHODS

Fungal culture, growth conditions, and gene extraction

Alternaria macrospora provided by Dr. Yiancheng Zhang (Jiangsu University,Zhenjiang, China), was rejuvenated on potato dextrose agar (PDA) media by tube culture for 7 d at 28 °C without shaking. The activated fungi were inoculated into fresh liquid PDA media (containing 200 g of potato, 20 g of sugar, and 1 g/l agar) in a 250 ml conical flask for amplification culture. The flask was placed in a shaker at 28 °C and vibrating velocity of 180 rpm for 4 d. The filamentous fungi were harvested by filtration through a four-layer clean gauze, washed with distilled water thrice, and stored at -79 °C for genomic DNA and RNA extractions. The total genomic DNA of *A. macrospora* was isolated from the freeze-dried fungal mycelia according to the protocol of Möller and Bahnweg (Möller *et al*.,1992), and stored at -20 °C. Total RNA was extracted with TRIzol reagent (Invitrogen) according to the instructions, and was stored at -70 °C for succeeding experiments.

DNA Manipulations

Species that are phylogenetically close to one another have highly similar relevant gene sequences. Consequently, 4 primers pairs were designed by the Primer5 program resulting from the contrast of DNA sequences of the endoPGs from fungi *A. alterata* and *A. citri* (access nos. AB047682 and AB047543, respectively). Only one pair designated as AM-S:CGAACCGC TGGGAACAA and AM-A:CCAATGCCCCG ATTACCT was used to amplify the endoPG fragment *ampg-1*, with an assumed length of about 1860 bp for cloning. The selection was based on the standard requirement that PCR results should conform to length predictions and be the larger fragment. The PCR protocol was run as follow: (1) initial denaturation at 95 °C for 5 min, (2) 35 cycles of denaturation at 95 °C for 45 s, as well as (3) annealing at 57 °C for 45 s, extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min.

RNA Manipulations

The primers for the amplification of *ampg-1* from the total RNA were AM-S1: ATGGTTGCCTTAACTCTTGGTA and AM-A1: TACGAACACTTGGTAACG CTG. Reverse transcription (RT) PCR was performed in two parts. The RT part was performed using a RevertAidTM First Strand cDNA Synthesis Kit (Fermentas Life Science) according to the instructions. The PCR part was performed using the total first strands of cDNA as templates following the same protocol mentioned in the preceding subsection.

DNA and RNA cloning

The PCR products were electrophoretically purified, reclaimed from agarose

gels using an EZ Spin Column PCR Product Purification Kit (Bio Basic Inc., Canada), cloned into a pMD18-T vector (TaKaRa Biotechnology Co.), and then sequenced by the Shanghai Sangon Biotechnological Company (China).

Sequence and bioinformatics analysis

The sequences were analyzed using DNAStar and used to draw a phylogenetic tree using CLUSTALX, as shown in TreeView. *ampg-1* cDNA and peptide sequence alignments were completed using CLUSTALX. Secondary structure predictions were made using web-based motif analysis tools (http://smart.embl-heidelberg.de/).

RESULTS

Cloning and sequence analysis of ampg-1

The sequence of an RT-PCR fragment from *A. macrospora* total RNA with primers AM-S1 and AM-A1 was found to be 1241 bp in size as well as 99.8% and 99.5% identical with that of the endoPGs of *A. alternata* (accession no. AB047682)

Table 1. The *PbH1* domains of PGs in some species

Species	Protein accession no.	*PbH1* domains	Appearance of obvious infection (days)	Reference
Alternaria macrospora		4	10	Bashan, 1986
Alternaria alternata	BAB32924	5	7–8	Gilchrist et al., 1976
Alternaria citri	BAB32923	5	7	Timmer et al., 2000
Colletotrichum gloeosporioides	CAC14022	5	5	Bautista-Bañoset al., 2003
Cochliobolus carbonum	AAA79885	6	2	Ransom et al., 1997.
Aspergillus niger	CAA74744	6	1–2	Cervone et al., 1987

Fig. 1. The sequence encodes for endopolygalacturonase in the cotton black spot fungus *A. macrospora* (*ampg-1*).Two introns, which locations were found in 679-740, and 947-995, respectively, have been underlined

1 MVALTLGIFF TSLAASAVAA PAPAITPAPK PEVVKRASSC TFSGSNGAAE ASKSQSSCAT
61 MVLSDVAVPS GTTLDLSSLA DGTTVIFEGT TTWGYSEWKG PLLDIQGKKI TVKGAEGSVL
121 NGDGARWWDG KGGNGGKTKP KFFSAHKLTD STITGITIKN PPVQVVSING CDGLTITDMT
181 IDASDGDKDE QGHNTDGFDI GSSNNVIIDG AKVYNQDDCV AVNSGTEITF KNGLCSGGHG
241 LSIGSVGGRD DNTVDTVTFS NSEVTKSVNG VRVKAKVGTT GKINKVTYED ITLSEISKYG
301 VLIEQNYDGG DLHGDADTGV PITALTPDNV TGGVSSSGYD VVVTCGKGSC TGWTWTGVDV
361 TGGKTYDKCS NVPSVTKCS

Fig. 2. The sequences of deduced amino acids from *ampg-1*. Four *PbH1* domains, which locations were found in 171-202, 203-224, 254-275, and 283-305, respectively, have been underlined

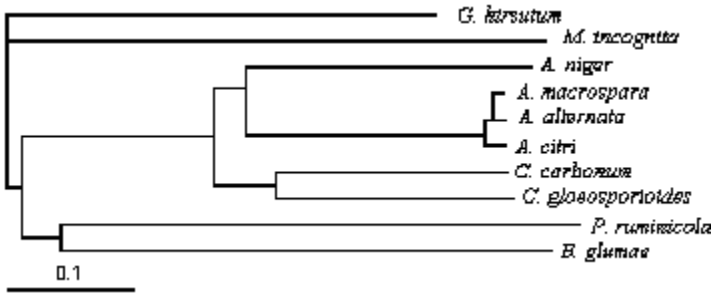

Fig. 3. Phylogenetic tree of PG amino acid sequence of *A. macrospora* and other nine species

and *A. citri* (accession no. AB047543), respectively. Compared with other pathogenic fungal species, *ampg-1* shared quite low identities, such as 34.0% with that of *Cochliobolus carbonum* (M55979), 32.0% with that of *Colletotrichum gloeosporioides* (AJ291494), and 37.0% with that of *Aspergillus niger* (Y14386). The sequence of ampg-1 and its deduced amino acids are shown in Figures 1 and 2. The deduced peptide sequence of AMPG-1 had 379 amino acids and a molecular mass of a 39.8 kDa, with a theoretical isoelectric point of 4.70. Secondary structure prediction plots showed that AMPG-1 had four *PbH1* domains, which was characteristic of endopolygalacturonases, and belongs to glycosyl hydrolase family 28 (data not shown).

Phylogenetic relationship

A phylogenetic tree based on evolutionary distances was drawn using the PG peptide sequence of *A. macrospora* and nine other species, including five fungi (*A. alternata*, accession no. BAB32924; *A. citri*, BAB32923; *C. carbonum*, AAA79885; *C. gloeosporioides* f. sp. malvae, CAC14022; and *A. niger* (CAA74744), two bacteria (*Burkholderia glumae*, BAC98359; and *Prevotella ruminicola*, BAA78557), one nematode (*Meloidogyne incognita*, AAM28240), as well as one higher plant (*Gossypium hirsutum*, ABO31369). As expected, fungi were in the same cluster and bacteria formed another cluster. In contrast, the nematode and higher plant were placed in two separate clusters (Figure 3).

DISCUSSION

In the present study, the nucleotide sequence of *ampg-1* was cloned. This gene encodes for endoPG in the cotton black spot fungus *A. macrospora*. The considerably high identities of the gene with the endoPGs of two other *Alternaria* species (*A. alternate* and *A. citri*) showed that the cloned gene was indeed an endoPG, and was designated as *ampg-1*.

Proteins containing *PbH1* domains are most often enzymes with polysaccharide substrates. The endoPGs of *A. macrospore*, which belongs to glycosyl hydrolase family 28, had four *PbH1* domains. The *PbH1* domains were involved

in phytopathogenesis. Compared with the endoPGs of five other species (Table 1), the endoPG of A. macrospore had the least number of *PbH1* domains. Therefore, the appearance of obvious infection by *A. macrospore* was longest among the six species. In fact, infection took 10 d to appear in *A. macrospore* with four *PbH1* domains, but took only 2 d in *C. carbonum* and *A. niger*, both with six *PbH1* domains (Table 1).

Phylogenetic analysis revealed that the species were clustered according to evolutionarily different classes (i.e., fungi, bacteria, nematode, or higher plant). The fungi cluster was divided into two groups, revealing that different ranks also existed within the same phylogenetic cluster during the evolutionary process. Three *Alternaria* species were in the same group, indicating that *A. macrospore* was a near relative of the other two species.

In conclusion, an endoPG-encoding gene of *Alternaria macrospora* was cloned and sequenced. The gene, named *ampg-1*, was closely related to the endoPGs of two other *Alternaria* species. However, there were less *PbH1* domains in ampg-1.

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