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Use of Green Fluorescent Protein To Detect Expression of an Endopolygalacturonase Gene of *Colletotrichum lindemuthianum* during Bean Infection

BERNARD DUMAS,* SYLVIE CENTIS, NATHALIE SARRAZIN, AND MARIE-THÉRÈSE ESQUERRÉ-TUGAYÉ

UMR 5546 CNRS-UPS, "Signaux et Messages Cellulaires chez les Végétaux," Pôle de Biotechnologie Végétale, Auzeville 31326, Castanet-Tolosan, France

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The 5′ noncoding region of *clpg2*, an endopolygalacturonase gene of the bean pathogen *Colletotrichum lindemuthianum*, was fused to the coding sequence of a gene encoding a green fluorescent protein (GFP), and the construct was introduced into the fungal genome. Detection of GFP accumulation by fluorescence microscopy examination revealed that *clpg2* was expressed at the early stages of germination of the conidia and during appressorium formation both in vitro and on the host plant.

Endopolygalacturonases (endoPGs; poly-α-1,4-galacturonide glycanohydrolase, EC 3.2.1.15) have long been proposed to play an important role in fungal pathogenicity by degrading the homogalacturonan regions of pectin, a major component of plant cell walls (10). In addition, research on endoPGs has been widened by the finding that these proteins are able to elicit plant defense responses through the release of active oligosaccharides and pectic fragments (1, 7). EndoPGs have been characterized and the corresponding genes have been cloned from a number of pathogenic fungi (2–4, 9, 11, 14–16). During fungal saprophytic growth, expression of endoPG genes is subjected to substrate induction as well as to catabolite repression by glucose. In *Colletotrichum lindemuthianum* it is also induced by the two neutral sugars arabinose and rhamnose, which are components of plant cell walls (12). Recently, the use of reverse transcription (RT)-PCR allowed the detection of endoPG transcripts during pathogenesis (4, 9, 16) and showed that endoPG genes are also induced during infection of the host.

In previous work, we cloned and characterized two endoPG genes, *clpg1* and *clpg2*, from *C. lindemuthianum*, a hemibiotroph fungal pathogen that causes anthracnose on bean seedlings (3, 4). Expression studies using specific probes for each gene showed that *clpg1* encodes the major produced enzyme, both in axenic culture of the fungus and at the onset of the necrotrophic stage of host colonization (4). *clpg2* was shown to be induced transiently and rapidly in vitro in the presence of pectin but not during the development of the necrotrophic stage of infection, indicating that *clpg1* and *clpg2* are differently regulated (4). As a first step to identify the signals and transduction pathways leading to the induction of endoPG genes, we have investigated the possibility of using a gene encoding a modified version of the *Aequoria victoria* green fluorescent protein (SGFP-TYG; 5) as a new vital reporter gene in *C. lindemuthianum*.

In order to express *gfp* under the control of the 5′ putative regulatory sequences of *clpg2*, a fragment comprising 668 bp located upstream of the initiation codon and the first six codons of the coding sequence were amplified by PCR and cloned in frame with the sequence encoding the SGFP-TYG. The plasmid pPG2GFP was introduced into *C. lindemuthianum* via protoplast transformation along with the plasmid pAN7-1 (13), which contains a cassette conferring hygromycin resistance. For a rapid screening of GFP-expressing strains, the mycelium of each colony was grown on minimal medium containing pectin as the sole carbon source (4) and subsequently examined under fluorescence microscopy. Of the 13 colonies growing in the presence of hygromycin, 8 were fluorescent, thus showing a cotransformation rate of about 60%. No fluorescence background was detected in the wild-type strain or in a strain transformed with a promoterless vector. The presence of GFP was stable even when the transformants were subcultured on a nonselective medium, i.e., without hygromycin. The phenotypes of GFP-expressing strains were unchanged compared to the wild-type strain. The rate of growth, conidiation, and pathogenicity were also unmodified by the accumulation of GFP, suggesting that this protein does not interfere with the physiology of *C. lindemuthianum*. It was found that transcription of the reporter GFP gene truly reflects transcription of the wild-type *clpg2* gene. Indeed, *gfp* transcript and fluorescence accumulation paralleled *clpg2* gene expression when the mycelium was grown on pectin as the sole carbon source, whereas expression of both genes was almost undetectable when the mycelium was grown on glucose (data not shown).

Expression of GFP under the control of the *clpg2* promoter was monitored by fluorescence microscopy at different stages of development of the fungus. Since all transformants showed a similar level of fluorescence, in Fig. 1 we present the results obtained for only one of them, namely the strain H2. To study the expression of GFP during saprophytic growth, conidia from GFP-expressing strains were used to inoculate enzyme-linked immunosorbent assay plates containing 50 μl of pectin medium per well. Fluorescence was undetectable at the beginning of the experiment. However, after incubation of the plates for 12 h at 25°C, the germinating conidia but not the germ tube appeared fluorescent (Fig. 1A1 and A2), whereas at 24 h of incubation the fluorescence was easily detectable along the germ tube (Fig. 1B1 and B2). Fluorescence was very weak in conidia germinating in water on a glass slide (Fig. 1C1 and C2), except when they differentiated an appressorium in which a high level of fluorescence was detected (Fig. 1D1 and D2). In

* Corresponding author. Mailing address: UMR 5546 CNRS-UPS, "Signaux et Messages Cellulaires chez les Végétaux," Pôle de Biotechnologie Végétale, 24 Chemin de Borde-Rouge, BP17, Auzeville 31326, Castanet-Tolosan, France. Phone: (33) (0) 5-62-19-35-03. Fax: (33) (0) 5-62-19-35-25. E-mail: dumas@cict.fr.

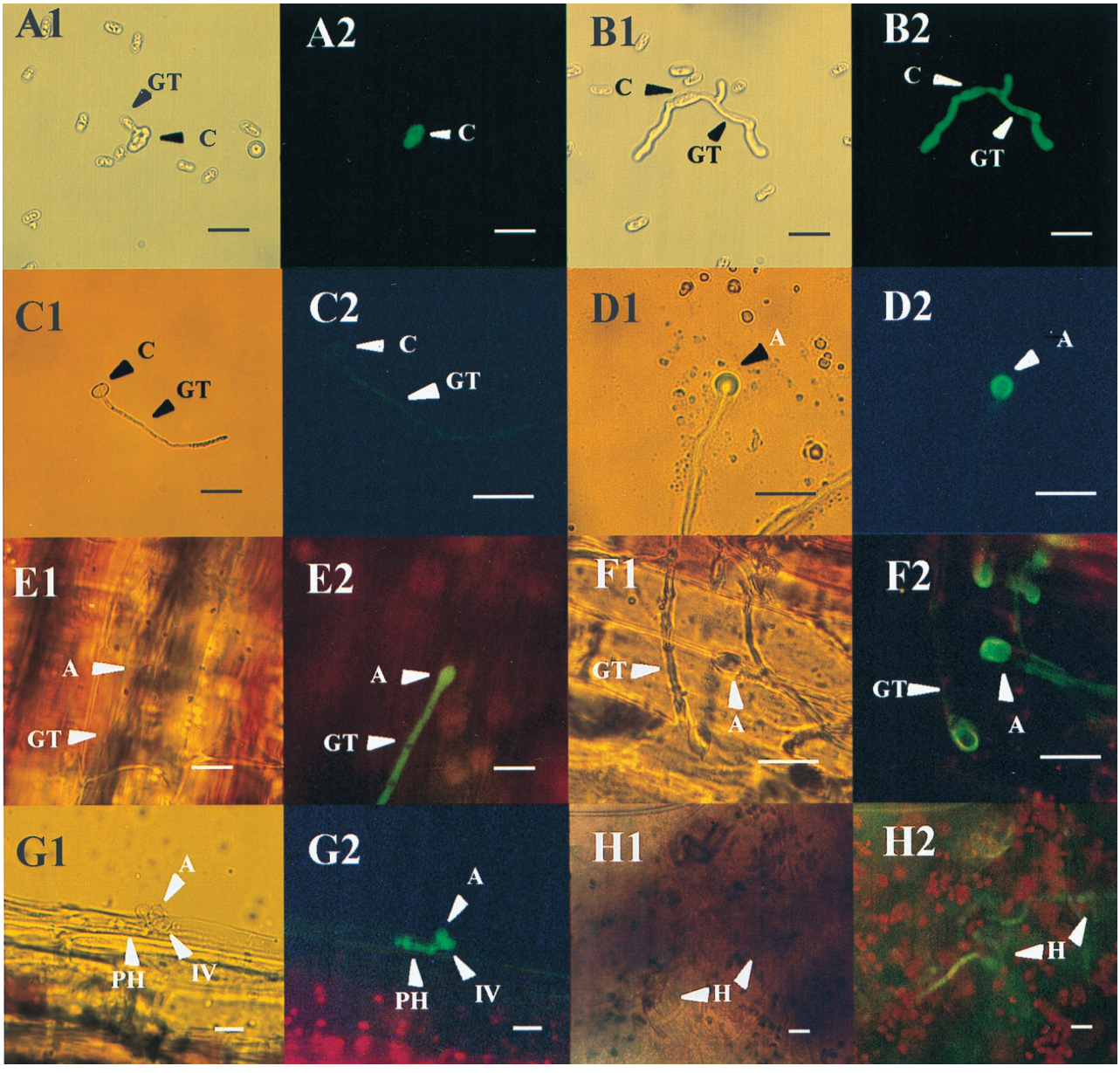

FIG. 1. Developmental expression of *gfp* under the control of the *clpg2* promoter. Conidia of *C. lindemuthianum* H2 were allowed to germinate in vitro either on pectin medium cleared by filtration (A and B) or on a glass slide in water (C and D). They were also used to inoculate bean hypocotyls (E through H). Samples shown in panels A through D were assayed for green fluorescence after 12 h (panels A and C) and 24 h (panels B and D). Infected bean hypocotyls were examined 24 h (panels E and F), 48 h (panel G), and 15 days (panel H) after inoculation. Samples were successively analyzed by light microscopy (subpanels 1) or fluorescent light (subpanels 2). A, appressorium; C, conidium; GT, germ tube; IV, infection vesicle; PH, primary hyphae. Bar = 20 μm.

order to look for the expression of *gfp* during infection of the host, bean hypocotyls of the susceptible Early Wax cultivar were inoculated with H2 conidia. The epidermis was peeled off 24 h after inoculation and analyzed by fluorescence microscopy. As shown in Fig. 1, fluorescent germ tubes and swelling appressorium were detected at the surface of the infected plant tissue (Fig. 1E1 and E2). At later stages, fluorescence of the germ tube decreased whereas fluorescence in the appressorium increased, likely reflecting migration of the cytoplasm into this swelling structure (Fig. 1F1 and F2). The penetrating hyphae were also fluorescent (Fig. 1G1 and G2), whereas fluorescence was not detected at the onset of necrosis (not shown). However, after prolonged incubation, i.e., 15 days postinoculation, fluorescent hyphae could be detected in heavily macerated tissue (Fig. 1H1 and H2), possibly reflecting reinoculation of the tissue with the resident fungus.

To confirm that the detection of GFP by fluorescence microscopy was correlated with the accumulation of *clpg2* transcripts at the very first stages of infection, the sensitive method RT-PCR was used. Oligoprimers that span the intron-containing region of *clpg2* were designed. The primers, which were tested on cloned *clpg2* cDNA and on the genomic sequence of *clpg2* (Fig. 2), enabled us to distinguish between RNA products (size without intron, 343 bp; lane 2) and DNA (406 bp; lane 3). Using RNA from the epidermis of infected bean hypocotyls collected 24 h after inoculation, a fragment of the right size,

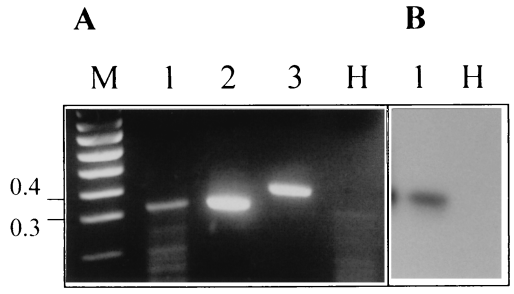

FIG. 2. Analysis by RT-PCR of *clpg2* expression in planta. Total RNA extracted from bean hypocotyl epidermis 24 h after inoculation of the susceptible cultivar Early Wax with *C. lindemuthianum* race β (lanes 1) or from the corresponding healthy plant (lanes H) was used for RT-PCR analysis. To differentiate between genomic and mRNA-derived fragments, PCRs were done by using cloned cDNA (lane 2) and cloned genomic DNA (lane 3) corresponding to *clpg2*. The PCR products were analyzed by gel electrophoresis followed by ethidium bromide staining (panel A) and by Southern blotting by using a *clpg2* probe (panel B). DNA size markers (100-bp ladder) were loaded on lane M. The sizes of two bands of the ladder, expressed in kilobase pairs, are indicated on the left.

which was undetected in healthy tissues (Fig. 2A, lane H), was amplified (Fig. 2A, lane 1). Southern blot analysis performed by using a *clpg2* probe confirmed that the amplified cDNA fragment corresponded to the transcript of *clpg2* (Fig. 2B, lane 1) which accumulated early during pathogenesis.

The correlation between the early induction of *gfp* expression and the accumulation of *clpg2* transcripts detected by RT-PCR allowed us to conclude that transcriptional activation of *clpg2* occurs rapidly when the fungus enters its parasitic stage. These observations are consistent with recent reports describing early secretion of pectinases by *Cochliobolus sativus*, *Uromyces viciae-fabae*, and *Claviceps purpurea* during pathogenesis (6, 8, 16), suggesting that these enzymes participate in host penetration by degrading the subcuticular pectin layer. It remains to be elucidated whether induction of *clpg2* in planta is mediated by pectin. Identification and deletion of pectin-responsive elements in the *clpg2* promoter should help to clarify this point.

Very few investigations have relied on the use of reporter sequences to study the regulation of pathogenicity genes. Since strains of *C. lindemuthianum* expressing the *gfp* construct showed a normal phenotype, the accumulation of GFP does not seem to alter the physiology of the fungus during its life cycle and makes *gfp* a suitable vital marker gene for *C. lindemuthianum*. To our knowledge, this is the first report on the use of *gfp* for studying the transcriptional regulation at a single cell level of a fungal gene encoding an hydrolytic enzyme induced during interaction with the host. This should help in identifying the complex signalling pathways leading to the induction of cell wall degrading enzymes during pathogenesis.

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