

# Exploring Infection of Wheat and Carbohydrate Metabolism in *Mycosphaerella graminicola* Transformants with Differentially Regulated Green Fluorescent Protein Expression

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**A *Mycosphaerella graminicola* strain transformed with the green fluorescent protein (GFP) downstream of either a carbon source-repressed promoter or a constitutive promoter was used to investigate in situ carbohydrate uptake during penetration of the fungus in wheat leaves. The promoter region of the *acu-3* gene from *Neurospora crassa* encoding isocitrate lyase was used as a carbon source-repressed promoter. The promoter region of the *Aspergillus nidulans* *gpdA* gene encoding glyceraldehyde-3-phosphate dehydrogenase was used as a constitutive promoter. Fluorometric measurement of GFP gene expression in liquid cultures of *acu-3*-regulated transformants indicated that the *N. crassa* *acu-3* promoter functions in *M. graminicola* as it does in *N. crassa*, i.e., acetate induced and carbon source repressed. Glucose, fructose, and saccharose triggered the repression, whereas mannitol, xylose, and cell wall polysaccharides did not. Monitoring the GFP level during fungal infection of wheat leaves revealed that *acu-3* promoter repression occurred after penetration until sporulation, when newly differentiated pycnidiospores fluoresced. The use of GFP transformants also allowed clear visualization of *M. graminicola* pathogenesis. No appressoria were formed, but penetration at cell junctions was observed. These results give new insight into the biotrophic status of *M. graminicola*.**

**Additional keywords:** plant-pathogenic fungus, septoria blotch, *Septoria tritici*, UB7.

Nutrient transfer between a biotrophic pathogen and its host is fundamental to the outcome of infection. Although specialized feeding structures such as haustoria can be differentiated during leaf colonization, there is a possibility that nutrient

transfer initiates much earlier during the penetration process and plays a part in the recognition compatibility of the host-pathogen interaction.

*Mycosphaerella graminicola* (anamorph *Septoria tritici*), the causal agent of Septoria blotch of wheat, is a hemibiotroph (Parbery 1996). Penetration through stomata occurs within 12 to 48 h after inoculation (Kema et al. 1996). After penetration, the fungus behaves as a symptomless endophyte. Hyphae colonize intercellular spaces without causing cell death for a period of 3 to 5 weeks before necrosis and sporulation occur (Royle et al. 1986). Hyphae are strictly ectotrophic, and no haustoria are formed. Kema et al. (1996) reported differences between a compatible and resistant interaction 48 h or more after inoculation, with mesophyll cell ultrastructure being affected and colonization taking place only in the susceptible cultivar. This suggests establishment of an interface with the host at the beginning of infection through which various substances, including nutrients, can pass.

Until now, few data were available on the development of such an interface. In ectotrophic fungi, nutrient transfer is thought to involve specialized intercellular hyphae (Hardham 1992), described by Heath and Skalamera (1997) as “conspicuously wider than those growing nonbiotrophically in the same plant.” Evidence for fungal structure-dependent nutrient uptake has been obtained from pulse-chase experiments carried out with <sup>14</sup>CO<sub>2</sub> (Clark and Spencer-Phillips 1993; Manners and Gay 1982). Results obtained by this method, however, give only a fragmented picture of the time course of nutrient transfer during infection, which cannot be related confidently to fungal structures localized inside the host.

We used an *M. graminicola* strain transformed with either a carbon source-repressed promoter–green fluorescent protein (GFP) fusion or a constitutive promoter–GFP fusion construct in order to investigate in situ soluble carbohydrate uptake during infection. Expression of the S65T GFP variant (Sheen et al. 1995) under the control of various promoters has been reported in several fungal plant pathogens (Maor et al. 1998; Spellig et al. 1996). Recently, GFP has been used successfully to monitor gene expression during the *Ustilago maydis* infection cycle (Basse et al. 2000). The promoter region of the *acu-3* gene from *Neurospora crassa* encoding isocitrate lyase (EC

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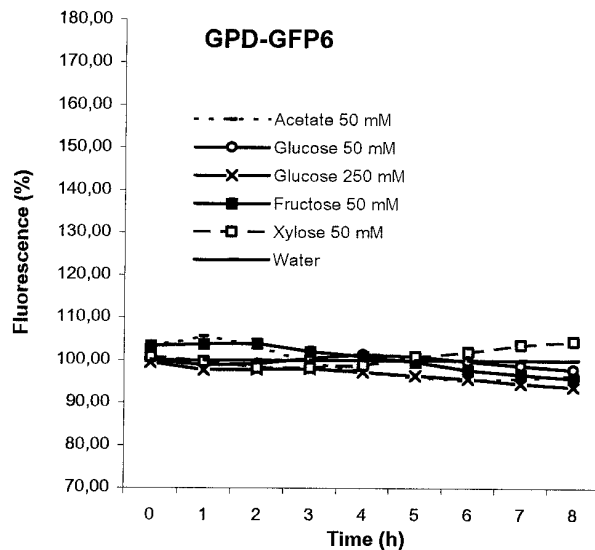
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4.1.3.1) was used as an inducible promoter. Isocitrate lyase is a key enzyme of the glyoxylate cycle that cleaves isocitrate to glyoxylate and succinate, and its synthesis has been shown to be carbon source repressed and acetate induced in *N. crassa* (Bibbins et al. 1998). The promoter region of the *Aspergillus nidulans gpdA* gene encoding glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) was used as a constitutive promoter (Kershaw et al. 1998; Punt et al. 1990). Both *acu-3* and *gpdA* promoter regulation were shown to be conserved in *M. graminicola* and readily allowed the detection of wheat-soluble carbohydrate uptake by the fungus after penetration.

## RESULTS

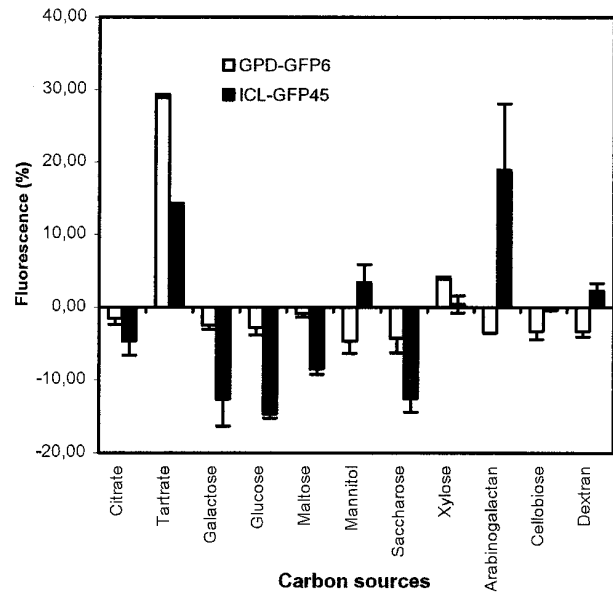
### Fluorometric monitoring of GFP synthesis in spore suspensions.

In *acu-3*-regulated *M. graminicola* transformants, GFP synthesis was strongly induced by acetate and repressed by glucose (Fig. 1). Increasing glucose concentration from 50 to 250 mM resulted in stronger repression. Among other carbohydrates tested, fructose (Fig. 1), galactose, maltose, and saccharose (Fig. 2) triggered the repression, though to a lesser extent than glucose. Xylose, fungal metabolite mannitol (Fig. 2), and the structural polysaccharides cellobiose, dextran, and arabinogalactan all failed to repress *acu-3*-regulated GFP synthesis. Acetate was the strongest inducer of the *acu-3* promoter activity that we tested. Citrate had no effect, and tartrate induced GFP synthesis in both *acu-3*- and *gpdA*-regulated transformants (Fig. 2). In *gpdA*-regulated transformants, GFP expression was not significantly repressed as a function of the carbon source. All *acu-3*- and *gpdA*-regulated transformants tested (at least two per construct and carbon source) behaved consistently with the above findings, despite showing different morphologies and levels of fluorescence. The background fluorescence of untransformed strain ST16 was negligible in our assays (less than 5% of GPD-GFP6 transformant fluorescence in water or carbohydrate media under standard settings used for fluorometric measurements).

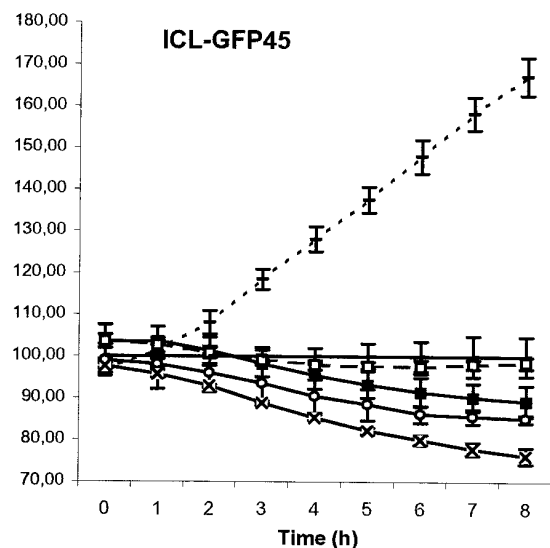


### Western blot and polymerase chain reaction (PCR) analysis of infected wheat leaves.

In carbon-starved media (Fig. 3), *gpdA*-regulated GPD-GFP6 transformant and *acu-3*-regulated ICL-GFP45 transformant expressed similar levels of GFP as detected by an anti-GFP antibody in Western blots. In infected leaves (Fig. 4),



**Fig. 2.** Fluorometric measurement of green fluorescent protein (GFP) gene expression in response to a 10-h incubation with different carbon sources in starved liquid cultures of *gpdA*-regulated GPD-GFP6 and *acu-3*-regulated ICL-GFP45 transformants. Fluorescence is expressed as a percentage of the transformant fluorescence in water. Carbon sources were added at a final concentration of 50 mM at time 0. We ensured that the fluorescence measured after 10 h was the result of a steady increase or decrease of fluorescence over the incubation period. The average and standard error of two measurements were taken from separate assays.

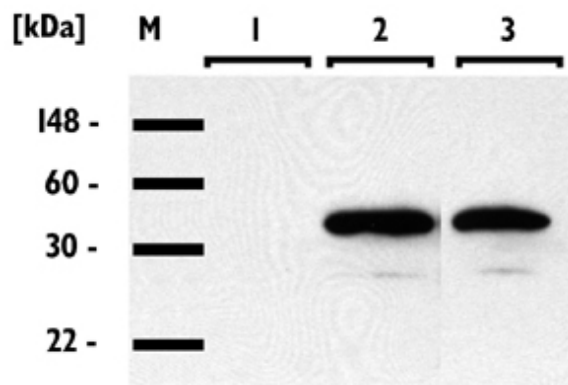


**Fig. 1.** Time course fluorometric measurement of green fluorescent protein (GFP) gene expression in response to carbon source in starved liquid cultures of *gpdA*-regulated GPD-GFP6 and *acu-3*-regulated ICL-GFP45 transformants. Fluorescence is expressed as percentage of transformant fluorescence in water. Carbon sources were added at indicated final concentrations at time 0. The average and standard error of two measurements were taken from separate assays.

GFP (approximately 30 kDa) (Fig. 4C) was detected by Western blotting in *gpdA*- or *acu-3*-regulated transformants 1 day postinoculation (dpi) (prior to penetration). At 9 dpi (after penetration), GFP was detected in leaves infected by *gpdA*- but not *acu-3*-regulated transformants. Unlike GFP, the constitutively expressed fungal glycoprotein (approximately 60 kDa) (Fig. 4B), recognized by the antibody UB7 (Mitchell et al. 1997), was detected at 1 and 9 dpi in leaves infected by both types of *M. graminicola* transformants and in the wild-type strain ST16. Quantification of *M. graminicola* DNA in infected leaves by PCR (Fig. 4D) confirmed the progress of infection between 1 and 9 dpi for every transformant and ST16. Similar results were obtained with *gpdA*-regulated GPD-GFPB3 transformant and *acu-3*-regulated ICL-GFP4 transformant in Western blot and PCR analysis, despite different intrinsic fluorescence levels. The presence of fungal penetrating structures in leaves was confirmed by fluorescence microscopy. Anti-GFP and UB7 antibodies gave no signal with healthy plant DNA (data not shown).

### Pathogenic development of GFP transformants in wheat leaves.

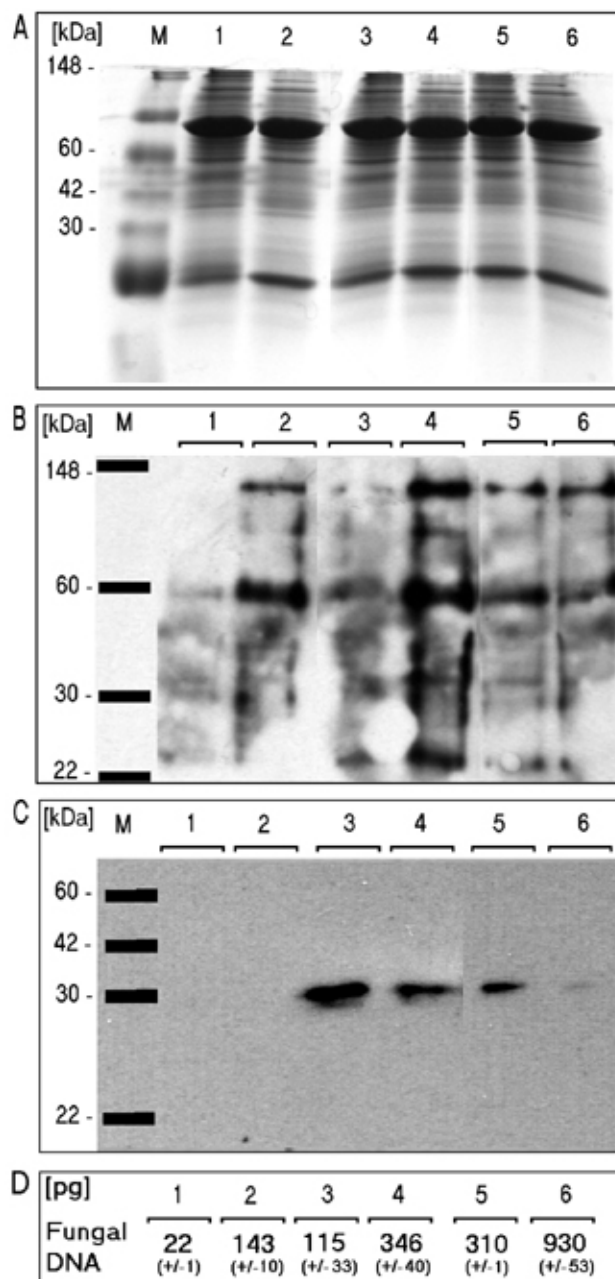
*gpdA*- and *acu-3*-regulated GFP transformants infected wheat and produced mature pycnidia 3 weeks after inoculation in the same way as the wild type. Although inoculated spores adhered everywhere on the leaf, they were found more frequently in stomatal depressions (Fig. 5A and B) and wrapped around trichomes (Fig. 5C). Germ tubes often failed to penetrate the leaf. Penetration occurred within 24 h after inoculation. Penetrating structures were observed over stomata (Fig. 5B) at the anticlinal junction of epidermal cells (Fig. 5D) and at hair base cell junctions. No appressoria were formed. Confocal microscopy confirmed actual penetration of the fungus through stomata (Fig. 5E) and between host epidermal cells (Fig. 6). Once within the leaf, mycelial strands extended longitudinally following lower junctions of epidermal cells (Fig. 6) and wrapped around mesophyll cells (Fig. 5E and H). Despite extensive hyphal growth in the apoplast, host cell wall integrity was preserved up to 4 days before sporulation.



**Fig. 3.** Western blot analysis of green fluorescent protein (GFP) expression in starved liquid cultures of *gpdA*-regulated and *acu-3*-regulated transformants. Lane 1: untransformed ST16 strain. Lane 2: *gpdA*-regulated GPD-GFP6 transformant. Lane 3: *acu-3*-regulated ICL-GFP45 transformant. Lane M: molecular mass markers.

### In planta fluorescence monitoring in *gpdA*- or *acu-3*-regulated GFP transformants infection structures.

In the various stages prior to penetration, GFP fluorescence could be readily detected in *gpdA*- and *acu-3*-regulated trans-



**Fig. 4.** Western blot analysis of green fluorescent protein (GFP) expression and polymerase chain reaction (PCR) quantification of fungal DNA in wheat leaves infected by *gpdA*- and *acu-3*-regulated transformants. Lanes 1 and 2: leaves infected by untransformed ST16 strain. Lanes 3 and 4: *gpdA*-regulated GPD-GFP6 transformant. Lanes 5 and 6: *acu-3*-regulated ICL-GFP45 transformant. Lanes 1, 3, and 5: leaves sampled at 1 day postinoculation (dpi). Lanes 2, 4, and 6: leaves sampled at 9 dpi. **A**, Coomassie-stained gel of total protein extracts. **B**, Western blot probed with fungal glycoprotein-specific monoclonal antibody UB7. **C**, Western blot probed with anti-GFP monoclonal antibody. **D**, PCR quantification of *Mycosphaerella graminicola* DNA (pg/100 ng of wheat DNA). Average and standard error of two measurements from separate PCR assays. Lane M: molecular mass markers.

formants. Maximum fluorescence intensity was reached in penetrating structures, usually appearing as bright dots on the plant surface in all transformants. After penetration, GFP fluorescence was readily observed in intercellularly growing hyphae of *gpdA*-regulated transformants (Figs. 5E and H and 6). By contrast, *acu-3*-regulated transformants stopped fluorescing immediately after penetration, whether in the substomatal chamber before reaching the mesophyll cells (Fig. 7) or after formation of a bright penetrating structure as in the case of penetration at a cell junction. No fluorescent *acu-3*-regulated transformant hyphae could be detected in the mesophyll in leaf sections by confocal microscopy. Only when pycnidia were formed could the penetrated *acu-3*-regulated transformants be detected again under UV because newly differentiated pycnidiospores were fluorescing (Fig. 5F and G).

## DISCUSSION

Fluorometric measurement of GFP gene expression in response to carbon source in liquid cultures of *acu-3*-regulated transformants indicated that the *N. crassa acu-3* promoter functions in *M. graminicola* as it does in *N. crassa*, i.e., glucose repressed and acetate induced (Bibbins et al. 1998). Repression by fructose, galactose, and saccharose is consistent with *A. nidulans* isocitrate lyase gene *acuD* promoter regulation (Bowyer et al. 1994; McCullough and Roberts 1980). While McCullough and Roberts (1980) noticed partial derepression on glutamate or proline, the complete lack of repression on C6 carbohydrates such as xylose or mannitol was not reported by other authors. None of the cell wall polysaccharides tested repressed *acu-3* promoter activity in liquid cultures. Hence, in planta fluorescence changes in *acu-3*-regulated transformants most likely reflect uptake of predominantly metabolic sugars from the plant by the fungus throughout the infection process. The *gpdA*-regulated transformants provided a good control in detection techniques because they were not subject to carbon source repression in liquid cultures and, consequently, the GFP gene was expressed at all stages of the infection process.

Western blot analysis was consistent with microscopic assessment of GFP fluorescence during infection. GFP was detected prior to penetration (1 dpi) but not during internal growth (9 dpi) in *acu-3*-regulated transformants, whereas it was detected at both infection stages in *gpdA*-regulated transformants. Consistent with the work by Mitchell et al. (1997), UB7 bound specifically to infected plant material and detected the fungus at 9 dpi in leaves infected with *acu-3*-regulated transformants. Monitoring fungal DNA levels also confirmed that, at 9 dpi, infection was in progress in all leaf samples, including those inoculated with *acu-3*-regulated transformants. Differences in DNA quantities measured between strains may be a result of a slight variation in spore size or adhesion properties because every sample was inoculated with the same spore concentration. The contribution of initial inoculum to detection at 9 dpi is likely to be reduced, considering the extensive degradation and removal of spores on the leaf surface observed at this stage in our assays.

During development on the leaf surface, GFP was strongly expressed in *acu-3*- and *gpdA*-regulated transformants. This is consistent with the fact that soluble carbohydrates are usually present on the leaf surface in very small quantities (nanograms per square centimeter) (Derridj 1996). GFP fluorescence in-

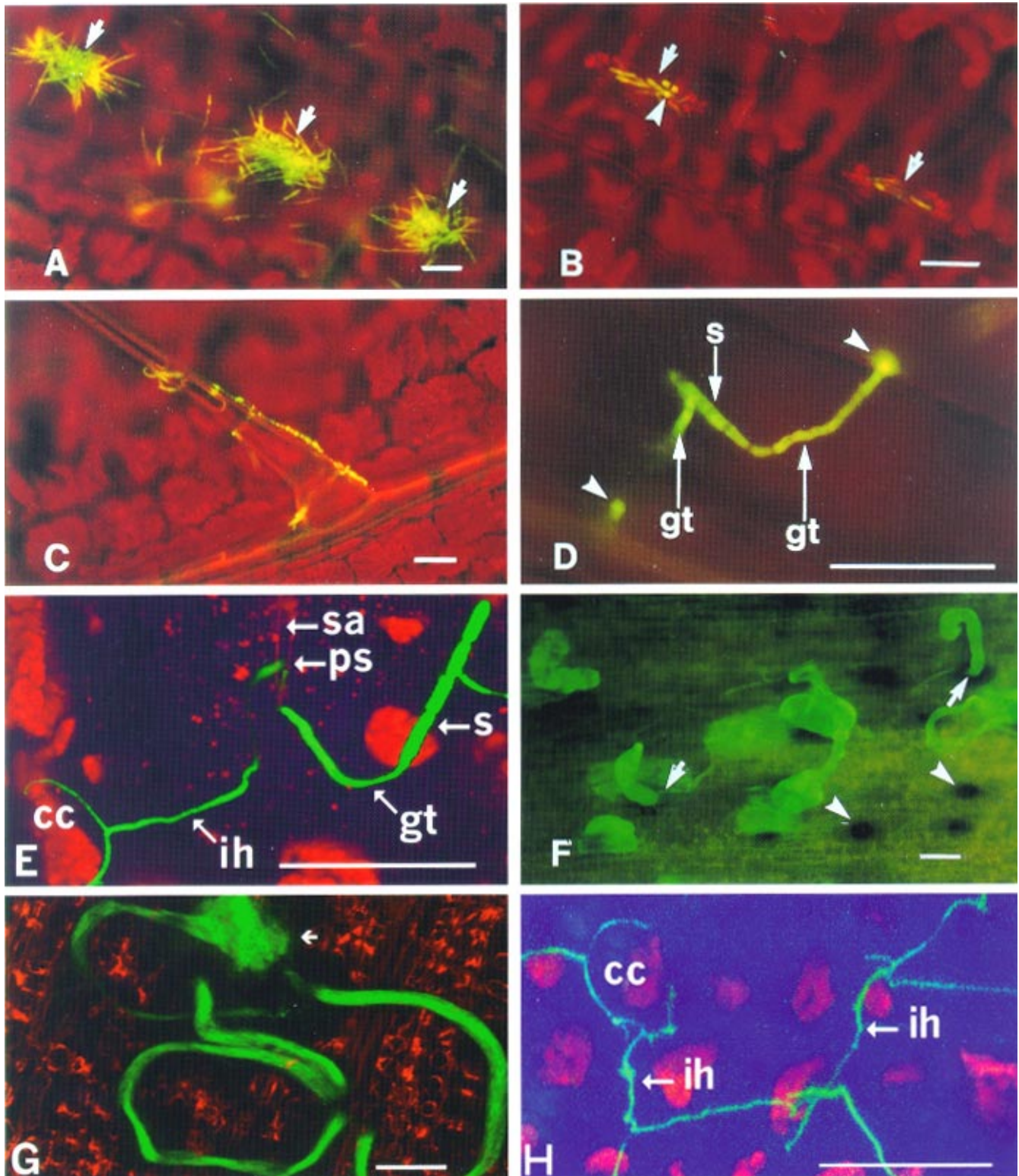
creased in epicuticular penetration structures, possibly as a result of cytoplasm condensation, enhanced protein synthesis, or the presence of vertical hyphae in the leaf plane. Repression of GFP synthesis in *acu-3*-regulated (but not *gpdA*-regulated) transformants was readily observed after penetration, indicating early carbohydrate uptake by penetrating hyphae. Penetration was always direct, without formation of an appressorium. Kema et al. (1996) used conventional light microscopy and scanning and transmission electron microscopy and did not observe penetration of the epidermis. In this study, confocal microscopy provided conclusive evidence that successful penetration between epidermal cells occurs, at least in some cultivar-isolate combinations. Hence, establishment of the host-pathogen interface does not seem to require the differentiation of specialized intercellular hyphae, at least at the beginning of the infection process. Soluble sugars are present in the apoplasm of graminaceous species at millimolar concentrations and are compatible with *acu-3* promoter repression (Tetlow and Farrar 1993). Interestingly, newly differentiated pycnidiospores were fluorescent in *acu-3*-regulated transformants, a result that gives new insight into the biotrophic status of *M. graminicola*. Parbery (1996) considered *M. graminicola* as a hemibiotroph, not a holobiotroph, because it seemed unlikely that a parasite would occupy a necrotic site rich in nutrients without utilizing them. The fluorescence of sporulating structures, however, suggests carbohydrate starvation at this stage in *M. graminicola*. Cohen and Eyal (1993) also reported that, in some cultivars, pycnidia on seedlings appear either prior to or without the formation of necrosis.

Although photosynthate uptake by epiphytic plant pathogens is well documented, there is little evidence available for endophytic fungi because an intimate host-pathogen association renders the examination of solute transport technically difficult. Clark and Spencer-Phillips (1993) reported on the assimilation of <sup>14</sup>C-labeled pea apoplastic carbohydrates by intercellular hyphae of *Peronospora viciae*. Tetlow and Farrar (1993) found decreased apoplastic carbohydrate concentrations in barley leaves infected with the brown rust fungus *Puccinia hordei* and suggested that the uptake by intercellular hyphae was a possible cause of this reduction. There is indirect evidence for soluble carbohydrate uptake by the rice blast fungus *Magnaporthe grisea* in the report by Talbot et al. (1997). They found that the population of mRNAs expressed by the fungus during infection had a greater similarity with the mRNA population expressed during in vitro growth under nitrogen deprivation than under carbon deprivation. Coleman et al. (1997) suggested that several genes from fungal pathogens that exhibit in planta-induced expression are also starvation induced. However, their data suggest that starvation occurs quite late in the infection process (14 dpi) in *Cladosporium fulvum*. None of these methods related soluble carbohydrate uptake with differentiation of specific fungal structures, with the exception of haustoria in *P. viciae*.

The use of GFP-expressing transformants has allowed in situ detection of soluble carbohydrate uptake at various infection stages in *M. graminicola*. Monitoring uptake can be improved by engineering short-lived GFPs (Andersen et al. 1998) that make fluorescence measurement quantitative in order to more closely relate GFP promoter repression to decreased fluorescence. Quantitative measurement may allow

characterization of cultivar resistance on the basis of apoplastic carbohydrate availability for fungal uptake. This study indicates that there is a conservation of the *acu-3* and *gpdA* promoter function between *M. graminicola*, whose molecular biology is poorly characterized, and model organisms such as

*A. nidulans* and *N. crassa*. The novel characteristics of GFP provide new opportunities to use the well-characterized promoters of *A. nidulans* and *N. crassa* in *M. graminicola* to monitor the pathogenic development and physiological status of fungal cells during infection.



## MATERIALS AND METHODS

### Strains, culture conditions, and constructs.

Wild-type *M. graminicola* strain ST16 and growth conditions are described in Payne et al. (1998). ST16 was cotransformed with equimolar quantities of pAN7-1 (Punt et al. 1987) that bears the hygromycin B resistance gene and either pICL-GFP or pGPD-GFP. pICL-GFP contains a 2.5-kb *PstI*-*NcoI* fragment of the *acu-3* promoter (Bibbins et al. 1998) upstream of *NcoI*-*Clal*-digested SGFP-S65T-nos (Sheen et al. 1995) cloned into *PstI*-*Clal*-digested pBluescript-KS (Stratagene, La Jolla, CA, U.S.A.). pGPD-GFP (received from M.-H. Lebrun, Aventis Crop Sciences, Lyon, France) contains the 717-bp fragment of SGFP-S65T cloned downstream of the *gpdA* promoter from *A. nidulans* in *NcoI*-*BamHI*-digested pAN52-1 (Punt et al. 1987).

### Fluorometry.

GFP expression in *M. graminicola* cells was monitored in microtiter plates with a LS 50B spectrofluorometer (Perkin Elmer, Beaconsfield, U.K.) set on 485-nm excitation and 520-nm emission wavelengths with a 10-nm slit width in both cases. An emission filter was set at 515 nm. Assays were carried out in duplicate, at 18°C into darkness. Spores from liquid cultures were harvested by centrifugation, washed extensively, and resuspended in water. Spores were starved for 12 h on a rotary shaker, and suspensions were then adjusted to an optical density at 600 nm of 0.6 (approximately  $10^7$  spores per milliliter) in water and 100  $\mu$ l was added to each microtiter plate well. Carbon sources were added to the spore suspensions at a final concentration of 50 mM. Water controls (without spores) were performed for each carbon source, and weak carbon source fluorescence was subtracted from fluorescence values measured in the corresponding cultures. Fluorescence of the untransformed strain ST16 was monitored as an additional control.

### Inoculation of plants.

Single-spore-derived transformants were grown for 5 days at 18°C on potato dextrose agar medium (Oxoid, Basingstoke,

U.K.) that contained 100  $\mu$ g of hygromycin B (Calbiochem, La Jolla, CA, U.S.A.) per milliliter. Spores were harvested in 0.01% (vol/vol) Tween 20 and suspensions adjusted to  $10^7$  spores per milliliter. Primary leaves of 10-day-old seedlings of the susceptible wheat cultivar Riband (PBI-Cambridge, Cambridge, U.K.) were grown in a controlled-environment cabinet set at 18°C in a 16-h day with 50 W per square meter of light and inoculated with a single 10- $\mu$ l droplet of spore suspension. Inoculated plants were left to stand for 30 min at room temperature and then maintained in a controlled-environment cabinet, as described above, in 100% humidity. Four replicate leaves per transformant were sampled daily for microscopic observation. Five and two leaves per transformant also were sampled for protein and DNA extraction, respectively, in a separate experiment.

### Western blot and PCR analysis.

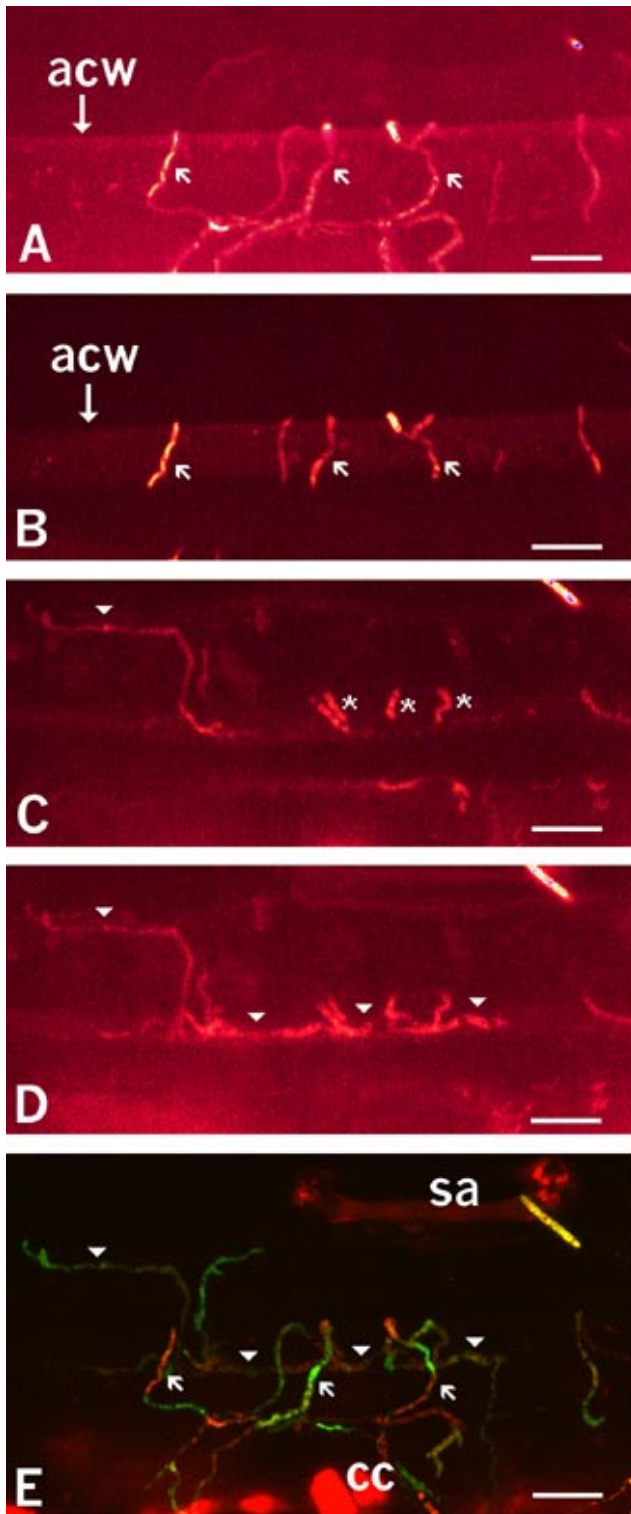
Total protein extracts were obtained through grinding in liquid nitrogen approximately  $10^7$  spores from in vitro cultures starved in water for 12 h or five infected leaf segments, and resuspending in 20 mM Tris-HCL (pH 7.4), 200 mM NaCl, 2 mM dithiothreitol, and Complete protease inhibitors (Boehringer, Mannheim, Germany). Infected leaf segments were thoroughly washed before grinding to remove excess inoculum. Protein was quantified with Bio-Rad protein assay reagent (Hemel Hempstead, U.K.), separated by 10% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli 1970), and transferred to Hybond P membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) by semidry blotting (Bio-Rad). GFP polypeptides were detected with an anti-GFP monoclonal antibody (Clontech, Basingstoke, U.K.). The monoclonal antibody UB7 (Mitchell et al. 1997) was used as a control. UB7 binds specifically to constitutively expressed fungal glycoproteins restricted to a subset of the fungi and does not bind to plant glycoproteins. Membranes were incubated with antimouse-conjugated horse radish peroxidase and, finally, with luminol (Amersham Pharmacia Biotech) before autoradiography. PCR quantification of *M. graminicola* DNA in infected leaves was carried out as described in Fraaije et al. (1999). A fragment of an *M. graminicola* beta-tubulin gene was amplified from infected-leaf total DNA with specific primers, and the PCR-amplified product was quantified by fluorometry with PicoGreen dye (Molecular Probes, Leiden, The Netherlands). *M. graminicola* DNA in infected leaves was then quantified with the use of a calibration curve generated by PCR amplification of healthy wheat leaf DNA mixed with different amounts of genomic *M. graminicola* DNA. Two infected leaf segments were washed and processed for each sample. Replicate fluorometric measurements were performed.

### Microscopy.

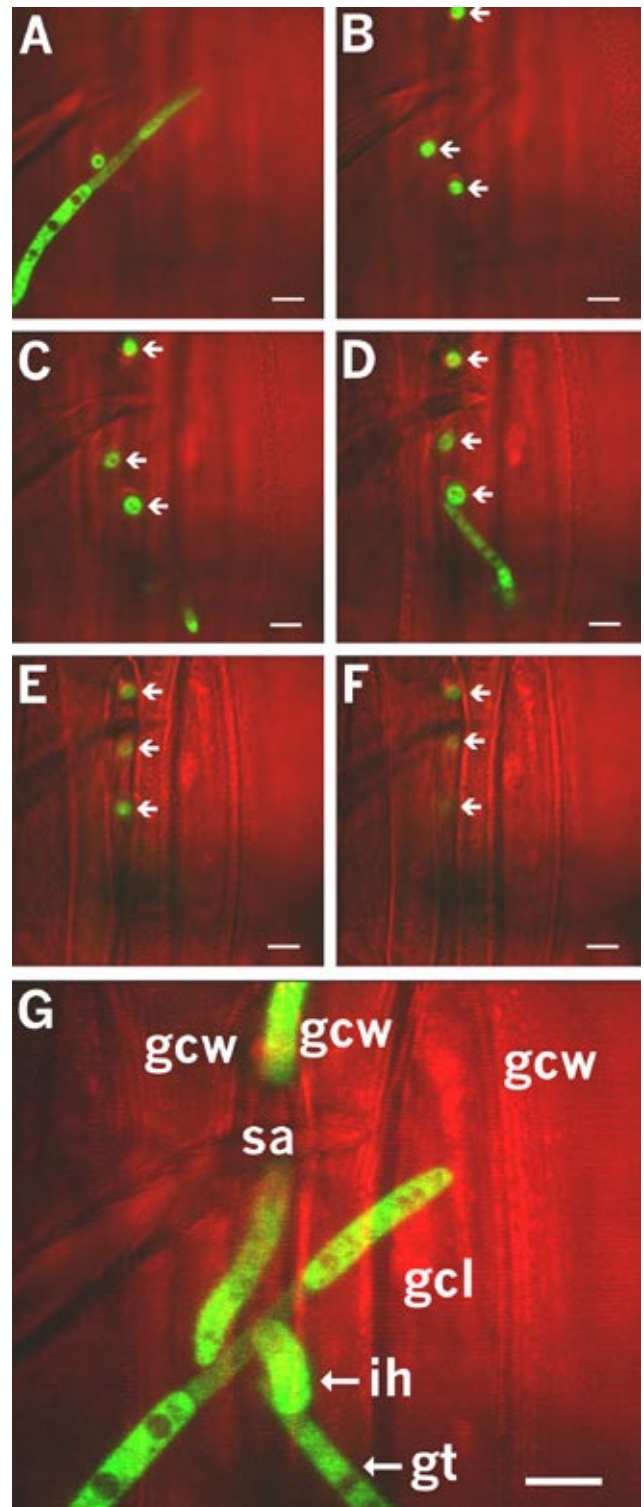
The area of the leaf around the inoculation site was excised and gently washed in 0.01% (vol/vol) Tween 20 to remove excess inoculum. Leaf fragments were mounted in glycerol and inspected with a Zeiss Axiophot epifluorescence microscope (Welwyn Garden City, U.K.). GFP was visualized with a mercury lamp and a fluorescein isothiocyanate filter set with a BP450 to BP490 excitation filter (Carl Zeiss Ltd.), 510-nm dichroic mirror, and a LP590 emission filter set (Carl Zeiss Ltd.). Confocal microscopy was performed with a Leica

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**Fig. 5.** Epifluorescence microscope (EFM) and confocal laser scanning microscope (CLSM) images of *Mycosphaerella graminicola* infection structures in wheat leaves. **A and B**, Adhesion of spores on stomatal apertures. **A**, *acu-3*-regulated ICL-GFP4 transformant, 9 days postinoculation (dpi); **B**, *gpdA*-regulated GPD-GFP6 transformant, 3 dpi; EFM images. Arrows point to stomata; arrowhead in **B** points to a bright structure corresponding to a penetration site. **C**, Germ tubes wrapped around a trichome (ICL-GFP4 transformant, EFM image, 8 dpi). **D**, Germinated spore (S) with two germ tubes (GT) (ICL-GFP4 transformant, EFM image, 7 dpi). Each germ tube ends with a bright penetration structure (arrowheads) above epidermal cell junctions. One of the germ tubes is out of focus. **E**, Spore (S) with a germ tube (GT) and an infection hypha (IH) penetrating through a stomatal aperture (SA). The infection hypha grows in the mesophyll below the penetration site (PS) and wraps around a mesophyll cell with red-fluorescing chlorophyll (CC) (GPD-GFP6 transformant, projected CLSM image, 6 dpi). **F and G**, ICL-GFP4 transformant pycnidia (arrows) oozing green fluorescent cirrhi (**F**, EFM image; **G**, projected CLSM image; 24 dpi). Arrowheads in **F** point to immature pycnidia without cirrhi. Yellow fluorescence associated with the necrosed leaf surface represents autofluorescence. **H**, Infection hyphae (IH) growing in the mesophyll and wrapping around a cell with red-fluorescing chlorophyll (CC) (*gpdA*-regulated GPD-GFPB3 transformant, projected CLSM image, 11 dpi). Scale bars = 50  $\mu$ m, except for **F and G**, which = 500  $\mu$ m.



**Fig. 6.** Direct penetration of *gpdA*-regulated GPD-GFP6 transformant hyphae at epidermal cell junction (11 days postinoculation). Confocal laser scanning microscope (CLSM) created 3  $\mu\text{m}$  optical sections collected in the plane of the leaf through 40  $\mu\text{m}$  of infected leaf tissue. **A to D**, Merged projections of three to five optical sections from the leaf surface **A**, to the mesophyll **D**. Arrows point to hyphae located on the leaf surface at the upper junction of two epidermal cells; arrowheads point to a mycelial strand forming at the lower junction, just below the epidermis. Asterisks in **C** indicate hyphae penetrating along the anticlinal cell wall (ACW). **E**, Merged projection of the 16 CLSM optical sections. CC = cell with red-fluorescing chlorophyll in the mesophyll; SA = stomatal aperture. Scale bars = 50  $\mu\text{m}$ .



**Fig. 7.** *acu-3* promoter repression inhibits green fluorescent protein synthesis in *acu-3*-regulated ICL-GFP4 transformant during penetration through stomatal aperture (11 days postinoculation). Confocal laser scanning microscope (CLSM) created 3  $\mu\text{m}$  optical sections collected in the plane of the leaf through 60  $\mu\text{m}$  of infected leaf tissue. **A to F**, Gallery of six optical sections every 9  $\mu\text{m}$  from the leaf surface **A**, to the substomatal chamber **F**. Note the decrease of fluorescence in the three penetrating hyphae (arrows). **G**, Merged projection of the 20 CLSM optical sections. GCW = guard cell wall; GCL = guard cell lumen; SA = stomatal aperture; GT = spore germ tube; and IH = infection hyphae. Scale bar = 10  $\mu\text{m}$ .

TCS-NT scanning laser confocal instrument that included an argon-krypton UV laser (Leica Lasertechnik, Heidelberg, Germany).

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