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The role of a *fadA* ortholog in the growth and development of *Colletotrichum graminicola in vitro* and *in planta*

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ABSTRACT

A transposon-based split-marker protocol was used to produce insertional mutations in the *fadA* ortholog of the maize anthracnose pathogen *Colletotrichum graminicola*. The mutants grew more slowly in culture, produced fewer oval spores, produced fusiform rather than falcate phialospores, lost their normal clockwise spiral growth pattern in culture, and were significantly reduced in their pathogenicity to maize stalks and leaves. The differential effect of the *fadA* mutation on oval spore versus phialospore production suggests that there are differences in the signaling pathways that regulate these two types of sporulation. It has been suggested that oval spores function in anthracnose lesion extension. In maize stalks, production of oval spores appeared to be relatively unaffected in the mutant strains, but production of vegetative hyphae and elongation of primary lesions were both reduced. This suggests that vegetative hyphae play a more important role than oval spores in primary lesion development. However, production of discontinuous secondary lesions in maize stalks infected by mutant strains did not appear to be seriously affected, and thus oval spores may play a more important role in that process.

1. Introduction

Colletotrichum graminicola (Ces.) Wils. causes anthracnose stalk rot and leaf blight of maize (reviewed by Bergstrom and Nicholson, 1999). *C. graminicola* produces two types of asexual propagule. Falcate conidia (phialospores) are produced on the surfaces of lesions from conidiogenous cells (phialides) in acervuli, and play a major role in dispersal of the pathogen from plant to plant. In contrast, oval spores are produced directly from the hyphae inside infected plant tissues, and are apparently not released to the outside (Panaccione et al., 1989). The role of the oval spores in the disease cycle is unknown, but it has been suggested that they are responsible for lesion expansion by facilitating systemic movement of the pathogen through the vascular tissues in leaves and stalks (Panaccione et al., 1989; Bergstrom and Nicholson, 1999). Our recent cytological studies implied that hyphae, and not spores, were mainly responsible for lesion expansion, as they colonized and grew through the fibers that are associated with the vascular bundles and stalk rind (Venard and Vaillancourt, 2007). The goal of the work described here was to create and characterize a *C. graminicola* mutant that is altered in sporulation in order to investigate further the developmental biology of oval spores, and their role in pathogenicity.

The *Aspergillus (Emericella) nidulans fadA* gene encodes a Gα subunit that regulates sexual and asexual development and secondary metabolism (Hicks et al., 1997; Adams et al., 1998; Yu and Keller, 2005). The dominant *fadA* mutation G42R (conversion of glycine 42 to arginine) results in constitutive activation of the GTPase activity of FADA. The mutant exhibits a "fluffy" phenotype, due to proliferation of the vegetative mycelium, and a reduction in asexual conidiation (Adams et al., 1998). Dominant activation of *fadA* also decreases production of a toxic secondary metabolite called sterigmatocystin (Hicks et al., 1997). Interfering mutations in FADA have the opposite effect, resulting in overproduction of sterigmatocystin, slower growth, and increased sporulation. The *A. nidulans flbA* gene encodes a protein with a regulator of G-protein signaling (RGS) domain (Hicks et al., 1997). In the current model in *A. nidulans*, FADA is negatively regulated by FLBA, which allows sporulation to proceed, while vegetative growth is inhibited (Hicks et al., 1997; Adams et al., 1998). In the absence of regulation by FLBA, FADA becomes activated, resulting in repression of sporulation and vegetative proliferation.

By analogy with *A. nidulans*, a negative mutation in the *fadA* ortholog of *C. graminicola* would be expected to result in an increase in the production of spores, and a decrease in vegetative growth. The goals of this study were to compare the effects of a

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mutation in the *fadA* ortholog of *C. graminicola* on the production of oval versus falcate spores, and to investigate the effect of the mutation on pathogenicity to maize leaves and stalks.

2. Materials and methods

2.1. Fungal strains

Colletotrichum graminicola strain M1.001, also known as CgM2 (Forgey et al., 1978), was used for this study. Fungal wild-type and transformant strains were cultured on potato dextrose agar (PDA, Difco) at 23 °C in continuous light. Falcate spores were collected from 3-week-old cultures by adding 10 ml of sterile water and rubbing the surface gently with a sterile plastic mini pestle. The conidial suspension was filtered though sterile glass wool, and the conidia were washed three times in sterile water before diluting for use in experiments.

2.2. Production of "split-marker" deletion constructs for insertional mutagenesis

A tBLASTN search of a DuPont *C. graminicola* genome database with the *fadA* sequence from *A. nidulans* identified a similar 2.1 kb contig sequence. Primers CgFadA1 (5′-TGTCGGCGCCCACTGTT TACC-3′) and CgFadA2 (5′-GAGGATGATGGAGGTCTTGATGAACC-3′) were used to amplify the 2.1 kb fragment from M1.001 genomic DNA. The polymerase chain reaction (PCR) mix contained 3.4 μg of genomic DNA template, 50 pmol of each primer, and 1× QIATaq Master Mix (QIAgen Inc.). Thermocycling conditions were 95 °C for 15 min, followed by 30 cycles consisting of 95 °C for 45 s, 63 °C for 30 s, and 72 °C for 3 min 30 s, followed by one cycle of 72 °C for 7 min.

The PCR product was cloned into pCR®2.1-TOPO, using the TOPO® TA Cloning kit (Invitrogen), according to the manufacturers instructions. *Escherichia coli* strain DH10 was transformed by electroporation with 1 μl of the TOPO reaction mix. One of the resulting plasmids, named pSM1267, was selected for transposon tagging.

Tn5-mediated transposition was used to produce insertion constructs. The *E. coli* hygromycin phosphotransferase (*hph*) gene, under the control of the aldolase promoter of *Magnaporthe grisea* and the β-tubulin terminator of *Neurospora crassa,* was cloned into the transposon construction vector pMOD-Tn5™ (Epicentre® Biotechnologies) to produce pMOD/Tn551. The EZ::TN™ transposase kit (Epicentre) was used to generate disruptions in pMOD/Tn551 according to the manufacturer's instructions, except that half of the recommended amount of transposase was used in the reaction. Each reaction consisted of 0.2 μg of the target plasmid (pSM1267); 0.2 μg of the donor plasmid (pMOD/Tn551); 1 μl of 10× buffer; and 0.5 μl of transposase. The reaction was desalted by drop dialysis, and 1 μl was used to transform *E. coli* by electroporation. Plasmids containing the transposed *hph* gene (tn/*hph*) within the 2.1 kb sequence were used as templates for PCR with the CgFadA1 and CgFadA2 primers. The PCR cycle consisted of 95 °C for 15 min, followed by 25 cycles of 95 °C for 45 s, 62 °C for 30 s, and 72 °C for 3 min 30 s. The position and orientation of the tn/*hph* fragment in the 2.1 kb sequence was determined in each case by sequencing the PCR product outward from two primers located at either end of tn/*hph*, Tn551-5′end (5′-TTCCTTGCCCTTAGGTTCCAC-3′) and Tn551-3′end (5′-TAGATTCCAAGTGTCTACTGC-3′).

2.3. Fungal transformations

All fungal transformations were performed using a PEG-mediated protocol (Thon et al., 2000). The pCT74 plasmid (Lorang et al., 2001), linearized with EcoR1, or the pSM615 plasmid (Bourrett et al., 2002) linearized with BamHI, were used to produce green fluorescent protein (GFP)-expressing transformants. The pAN8-1 plasmid (Mattern et al., 1988), linearized with EcoR1, was used to produce phleomycin-resistant transformants.

For production of split-marker transformants, pairs of overlapping truncated PCR products were produced for each *tn/hph* disruption construct. These corresponded to opposite ends of the 2.1 kb sequence, and overlapped by about 500 bp within the inserted *hph* gene sequence in each case. The *hph* primers HygR-1 (5′-CCACGGCCTCCA GAAGAAGATG-3′) and HygR-2 (5′-GAT ATGTCCTGCGGGTAAATAGCTGC-3′) were used in combination with CgFadA1 or with CgFadA2 to amplify both ends of each of the disrupted sequences. The PCR conditions consisted of 94 °C for 3 min; followed by 30 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 3 min. For each disrupted construct, 500 ng of each member of the appropriate pair of overlapping PCR products was used to transform *C. graminicola* protoplasts. All transformants were single-spored at 100× magnification by using a spore-cutter device (Microscore, Georgetown, KY), and stored on silica at −80 °C (Perkins, 1962).

To measure the stability of the split-marker transformants in culture, the strains, including a M1.001 hygromycin-resistant pCT74 transformant as a control, were subcultured eight times on either PDA or PDA-Hyg 50 μg/ml. Subcultures were made by removing a plug from the edge of a 2-week-old PDA culture and transferring it to the center of a fresh plate. The cultures were finally transferred to plain PDA for spore production. Falcate spores were harvested after 3 weeks, washed, and then germinated overnight on PDA. For each subcultured isolate, single germinated spores were transferred into each of two 24-well plates containing PDA, and two containing PDA-Hyg 50 μg/ml. The number of survivors in each case was counted after a week of incubation at room temperature.

2.4. Sequencing

DNA sequencing was done in the Advanced Genetics Technology Center (AGTC), at the University of Kentucky using an ABI machine with the ABI Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems), according to the manufacturers instructions.

2.5. Southern and Northern blotting

Preparation of fungal genomic DNA and Southern blotting were as described (Thon et al., 2000). To prepare RNA, fungal strains were grown in Fries complete medium (Fries, 1947) shaking cultures for 4 days at 28 °C, then the mycelium was harvested by vacuum filtration and flash-frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Each RNA sample was suspended in DEPC treated water, and the concentration was determined by using a spectrophotometer. Northern blot analysis and synthesis of random primed ^{32}P radioactively labeled probes were performed as described by Shah et al. (1999). RT-PCR reactions were performed as follows: a mix of 6 μg of total RNA, water (to a volume of 10 μl), and 1 μl of oligodT primer was incubated for 15 min at 65 °C, and immediately chilled on ice. Four microliters of 5× RT buffer, 2 μl of 0.1 M DTT, 1 μl of 10 mM dNTPS, 1 μl RNAsein (Promega), and 1 μl of Superscript II (Invitrogen) were successively added. The reaction mix was incubated for 1 h at 42 °C. The DNA/RNA complexes were denatured at 65 °C for 15 min, and then 20 μl of DEPC treated water was added. One microliter of cDNA was used as a template for the PCR reaction. PCR cycling conditions consisted of 93 °C for 3 min, then 35 cycles of 93 °C for 30 s, 63 °C for 30 s, and 72 °C for 3 min, and finally one cycle of 72 °C for 1 min. The primers CgFadA18 (5′-CGCACAGCCGCAGGTTCTCTTG-3′) and CgF-