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Real-time PCR assay to quantify *Fusarium graminearum* wild-type and recombinant mutant DNA in plant material

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Abstract

Fusarium graminearum (teleomorph, *Gibberella zeae*) is the predominant causal agent of Fusarium head blight (FHB) of wheat resulting in yearly losses through reduction in grain yield and quality and accumulation of fungal generated toxins in grain. Numerous fungal genes potentially involved in virulence have been identified and studies with deletion mutants to ascertain their role are in progress. Although wheat field trials with wild-type and mutant strains are critical to understand the role these genes may play in the disease process, the interpretation of field trial data is complicated by FHB generated by indigenous species of *F. graminearum*. This report describes the development of a SYBR green-based real time PCR assay that quantifies the total *F. graminearum* genomic DNA in a plant sample as well as the total *F. graminearum* genomic DNA contributed from a strain containing a common fungal selectable marker used to create deletion mutants. We found our method more sensitive, reproducible and accurate than other similar recently described assays and comparable to the more expensive probe-based assays. This assay will allow investigators to correlate the amount of disease observed in wheat field trials to the *F. graminearum* mutant strains being examined.
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Keywords: *Fusarium graminearum*; Fusarium head blight; Plant pathogen; Quantitative assay; Real-time PCR; SYBR green; Wheat scab

1. Introduction

Fusarium head blight (FHB), or scab, is a disease of cereal grains caused primarily by the fungal pathogen *Fusarium graminearum* (Bai and Shaner, 1994). Epidemics are intermittent in growing areas that have a humid to semi-humid climate and can significantly impact the wheat and barley industry (Bai and Shaner, 1994; Snijders, 1990). Economic losses result from a reduction in yield, poor seedling germination and contamination of seed with fungal toxins such as deoxynivalenol (DON), nivalenol (NIV) and zearalenone. Contaminated seed may be refused by livestock, cause a variety of animal diseases and necessitate expensive screening programs during grain preparation to exclude the toxins (Bai and Shaner, 1994; Snijders, 1990; Tuite et al., 1990).

Numerous virulence factors have been identified and characterized to varying degrees for *F. graminearum* (Dyer et al., 2005; Hou et al., 2002; Jenczmionka et al., 2003; Jenczmionka and Schafer, 2004; Lu et al., 2003; Seong et al., 2005; Urban et al., 2003; Voigt et al.,

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2005). The best studied virulence factor is the mycotoxin DON (Desjardins et al., 2000; Proctor et al., 1995; Rocha et al., 2005). DON toxicity to plants and animals is mediated through the inhibition of protein synthesis by interfering with ribosomal peptidyl transferases (Miller and Ewen, 1997). Wheat disease assays with DON mutants in the greenhouse and in the field have shown the important role DON plays in the disease process. Unfortunately, interpretation of the field trial data was complicated by a significant incidence of disease in control plots, generated presumably by indigenous strains of *Fusarium* including *F. graminearum* (Desjardins et al., 1996; Bai et al., 2001). Disease assessments, either FHB or toxin levels, do not provide any knowledge, *a prior*, of the fungi responsible.

Rapid advances in fungal genomic resources and technologies have led, at an unprecedented rate, to the identification of potentially new candidate virulence genes. Targeted gene disruption of the candidate genes is an important first step to assess function. The *Escherichia coli* hygromycin-B phosphotransferase gene (*HYG*), which confers resistance to the fungicide hygromycin, is a selectable marker frequently used with fungi and has been used to create a DON deficient strain of *F. graminearum* (Fincham, 1989; Proctor et al., 1995).

We describe the development of an easy and rapid, SYBR green-based real-time PCR assay for the quantification of total *F. graminearum* DNA and quantification of total *F. graminearum* DNA contributed by *HYG* tagged strains contained in DNA extracted from plant material. We found that our protocol is at least an order of magnitude more sensitive than previous SYBR green-based real-time quantitative PCR assays and is comparable in sensitivity to more expensive and complicated Taqman assays.

2. Methods

2.1. Fungal strains and culture conditions

F. graminearum strain FGSC 8630 (GZ3639) was isolated from scabby wheat in Kansas and is the progenitor strain for the two mutant strains, ΔFgOrfA-C15 (dAC) and ΔMat14 (dMat), examined in this work (Bowden and Leslie, 1992; Brown et al., 2004; Desjardins et al., 2004). All three strains produce DON in culture and *in planta*. dAC lacks a 4.5 kb portion of genomic DNA that includes the three open reading frames (ORFs) downstream of *TRI8*. dMat lacks a 9.6 kb portion of genomic DNA that includes the 4-gene MAT locus required for sexual reproduction. Hygromycin-resistant transformants were maintained on V-8 juice agar slants containing 300 μg ml^{-1} hygromycin B (Sigma, St. Louis, MO) and GZ3639 was maintained on V-8 juice agar slants.

2.2. Growth of wheat

Wheat (*Triticum aestivum* L.) cultivar Wheaton was grown in 18.7 cm plastic pots containing large coffee filters and filled with pasteurized soil (Scotts Redi-Earth Plug and Seedling Mix, Scotts Company, Marysville, OH). The pots were placed into the growth chamber (EconoAir, Model GC-16, Ecological Chambers, Inc., Winnipeg, Mb, Canada) and incubated at 15 °C with a 12 h light/dark cycle. Seedlings were fertilized with 200 ml pot^{-1} of an aqueous solution containing 1.25 g L^{-1} Peter's 20–20–20 (Spectrum Brands, St. Louis, MO) and 0.08 g L^{-1} Iron (II) Sulfate (Sigma, St. Louis, MO). After 4 to 5 weeks, pots were transferred to the greenhouse where temperatures were maintained at 23 °C during the day and 17 °C during the night. After a week, seedlings were thinned to four plants per pot. Plants were watered daily and fertilized weekly.

2.3. Production of fungal infected wheat seed

Fungal inoculum was prepared as described (Bai and Shaner, 1996). Briefly, macroconidia were generated by growth in 50 ml of mungbean media with shaking (200 rpm) at 25 °C for 4 to 5 days. Mungbean media was prepared by boiling 40 g of mungbeans in 1 L of deionized water for 10 min., filtering the resulting liquid through Miracloth (Calbiochem, EMD Biosciences, Inc., San Diego, CA) and autoclaving the filtrate. Macroconidia were harvested by low speed centrifugation and rinsed once with water. Spore concentrations were determined by hemacytometer and adjusted to 100,000 spores ml^{-1}. The third or fourth floret up from the base of the wheat head was inoculated by injection with either sterile water or with 1000–2000 macroconidia (10–20 μl from the 100,000 spores ml^{-1} stocks). After inoculation, wheat heads were enclosed in plastic bags (3 to 4 heads per bag) for three days to increase the humidity and encourage disease. Disease assessments were noted at 3 day intervals out to 21 days. Heads were allowed to mature prior to harvest and were individually threshed. Seeds from each treatment protocol (10 heads) were pooled, chopped into small pieces in a model M-2 Stein Laboratory Mill (Steinlite Corporation, Atchison, KS) and ground to a fine powder with a mortar and pestle.