Use of GUS Transformants of *Fusarium subglutinans* for Determining Etiology of Mango Malformation Disease

Stanley Freeman, Marcel Maimon, and Yaakov Pinkas

Department of Plant Pathology, ARO, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel.

Y. Pinkas is deceased.

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ABSTRACT

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Fusarium subglutinans has been associated with mango floral and vegetative malformation, although confusion exists regarding the etiology of the disease. A wild-type isolate of *F. subglutinans* causing mango malformation disease was transformed with the GUS (β -glucuronidase) reporter and hygromycin resistance genes. Five stable transformants were isolated containing varying copy numbers at different integration sites. Specific GUS activity was quantified for the transformants, whereas no

activity was recorded for the wild-type isolate. The transformants and the wild-type isolate were inoculated into healthy mango floral and vegetative buds. Typical symptoms of misshapen shoots with short internodes, stubby leaves, and bunchy, malformed inflorescences were observed 6 to 8 weeks following inoculation. The presence of GUS-stained mycelium of the pathogen viewed microscopically within infected plant organs provided unequivocal evidence that *F. subglutinans* is indeed a causal agent of mango malformation disease.

Additional keywords: *Fusarium moniliforme*, *Fusarium* spp., *Mangifera indica*.

Inflorescence and vegetative malformation of mango, *Mangifera indica* L., is one of the most important diseases of this crop and occurs in most mango-growing countries worldwide (11,22). Serious losses are caused by the disease, since malformed inflorescences do not bear fruit. Although mango malformation was first reported over a century ago in India, the epidemiology and etiology of the disease is poorly understood and confusion still exists regarding the causal agents (11,22). Physiological theories have been postulated as disease incitants such as altered C/N ratio (2) and lack of chelated iron (1,15). Viruses have also been suggested as causal disease agents (26), although results from electron microscopy, transmission, cultural, and serological studies have disproved this theory (9,10). Presence of the *Aceria* (*Eriophyes*) *mangifera* mite has been attributed to the cause of mango malformation (7, 16); however, certain studies indicate that the mite may only play a role in wounding and transfer of a fungal pathogen (22,27). Certain studies have associated the phytopathogenic fungus *Fusarium subglutinans* (Wollenweb. & Reinking) P.E. Nelson, T.A. Toussoun, & Marasas (basionym: *F. moniliforme* J. Sheld. var. *subglutinans* Wollenweb. & Reinking) with malformation (11), with further studies supporting the evidence that mango malformation is caused by the fungus *F. subglutinans* (3,14,21,31). However, additional recent publications still cast doubt on the fact that the fungus *F. subglutinans* is the responsible disease incitant (13,20). Although the pathogen reported responsible for mango malformation had been identified in the past as *F. subglutinans*, the precise taxonomic classification of the fungus remains undefined (18).

In this study, we used GUS transformants of *F. subglutinans* isolate 506/2 to assess the infection potential in mango floral and vegetative tissues and to provide unequivocal evidence that this fungus is indeed a causal agent of mango malformation disease.

MATERIALS AND METHODS

Fungal culture, growth, and transformation. The wild-type *F. subglutinans* isolate 506/2 from infected mango inflorescences at The Volcani Center plantation (originally identified by P. E. Nelson, Pennsylvania State University, and A. Z. Joffe, The Hebrew University of Jerusalem, Israel) was maintained on potato dextrose agar (PDA) at 25°C. Isolate 506/2 was transformed with a plasmid (pGPD-GUS; provided by D. Straney, University of Maryland) containing the GUS (β -glucuronidase) reporter and hygro-

Fig. 1. A partial restriction digest map of pGPD-GUS, a pBluescript-based plasmid, that is 7.8 kb in length containing the hygromycin phosphotransferase gene (*hph*) under control of the *Aspergillus niger* *PglA* promoter and *TrpC* terminator, and the β -glucuronidase (GUS) reporter gene under control of the *Cochliobolus heterotrophus* *PgpD* promoter and *NOS* terminator (provided by D. Straney, University of Maryland).

Corresponding author: S. Freeman; E-mail address: freeman@netvision.net.il

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mycin resistance genes (Fig. 1). Plasmid transformation was obtained using a polyethylene glycol-mediated protoplast protocol, after first plating on PDA amended with hygromycin (100 µg/ml) (Boehringer GmbH, Mannheim, Germany), as previously described (23). The wild-type isolate and derived transformants of *F. subglutinans* used in this study were further classified by K. O'Donnell, Peoria, IL, as a *Fusarium* sp., indicating that the pathogen is conspecific with NRRL 25266 = IMI 304063 ex *mangifera* from India (18). Identification was based on sequence and amplification of the beta tubulin gene using primers T1 × T22 and primer T21 to sequence across three introns at the 5' end of the gene (17).

Pathogenicity on mango. All stable transformants and the wild-type isolate 506/2 were tested for pathogenicity on mango trees (cv. Kent) maintained under greenhouse conditions for 30 days at diurnal temperatures of 9 to 17°C for flower induction and thereafter at 17 to 22°C. Wound inoculation was performed by

injecting 20 µl of conidial suspensions (5×10^7 conidia per ml) of each isolate into dormant apical buds, six buds per isolate on separate branches per tree. Symptoms were observed 6 to 8 weeks after inoculation, and percent infection was calculated. Inoculation experiments were also conducted on unwounded tissue by placing a 10-µl drop of conidial suspension (5×10^7 conidia per ml) on dormant buds. Inoculation experiments were conducted twice.

GUS activity. For histopathological studies, GUS activity was monitored microscopically in inoculated infected and noninoculated plant tissues that were removed from the trees 6 to 8 weeks after inoculation (6,8,19,30). Conidia of *Fusarium* sp. transformants and the wild-type fungus were isolated from infected tissue and germinated on PDA containing 50 µg of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc; Duchefa, Haarlem, the Netherlands) per ml dissolved in 10% *NN*-dimethylformamide (Sigma Chemical

Fig. 2. A, Healthy and typical floral malformation of mango artificially inoculated with B, the GUS transformants and C, the wild-type isolate of *Fusarium subglutinans*.

Fig. 3. A, Healthy and typical vegetative malformation of mango artificially inoculated with B, the GUS transformants and C, the wild-type isolate of *Fusarium subglutinans*.

Co., St. Louis). To detect GUS-stained mycelium in infected mango tissue, a reagent mix containing 50 µg of X-Gluc per ml, 0.1 M NaPO₄ (pH 7.0), 10 mM EDTA, and 0.5 mM each of Kferri- and ferrocyanide in 0.05% (vol/vol) Triton-X was vacuum-infiltrated

into plant tissues for 5 min (6,8). Plant tissues were cleared of chlorophyll by washing twice over a 48-h period in chloral hydrate (120 g per 100 ml) (Sigma Chemical Co.) and viewed using a Wild stereomicroscope (Wild, Heerbrugg, Switzerland) (28).

Fig. 4. Histochemical localization of GUS activity in *Fusarium subglutinans* from mango **A**, in leaf primordia; **C**, in sepal; **E**, within pedicel; and **G**, within nectar glands. **B**, **D**, **F**, and **H**, GUS-stained mycelia were not evident in the respective noninoculated controls. Bars = **A** and **B**, 133 µm; **C** and **D**, 100 µm; **E**, 75 µm; **F**, 50 µm; and **G** and **H**, 20 µm. Arrows denote GUS-stained mycelia within sectioned tissue.

GUS activity of the transformants and the wild-type isolate was also quantified in extracts of mycelium using the 4-methyl-umbelliferyl β -D-glucuronide (MUG) (Duchefa) fluorometric assay (6,8,19). Mycelium was grown for 7 days in still culture on potato

dextrose broth and lyophilized till dry. Thereafter, 0.16 g of mycelium of each culture was ground in liquid nitrogen and suspended in 10 ml of GUS extraction buffer consisting of 50 mM NaHPO_4 (pH 7.0), 10 mM β -mercaptoethanol, 10 mM Na_2EDTA , 0.1% so-

Fig. 5. Histochemical localization of GUS activity in *Fusarium subglutinans* from mango in **A and C**, trichomes; **E**, anther sacs; and **G**, nectar glands at fruit set. **B, D, F, and H**, GUS-stained mycelia were not evident in the respective noninoculated controls. Bars = **A**, 100 μm ; **B**, 75 μm ; **C**, 75 μm ; **D**, 50 μm ; **E and F**, 166 μm ; and **G and H**, 1.5 mm.

dium dodecyl sulfate, and 0.1% Triton X-100 (6,8). The suspension was mixed for 2 min in an Ultra-turrax T 25 (IKA-Labortechnik, Staufen, Germany) on ice, centrifuged for 20 min at $8,500 \times g$, and filtered through a 0.2- μ l filter. A MUG solution was added at a rate of 0.2 ml per 0.15 ml of filtrate and incubated in the dark at 37°C. The reactions were stopped at 0-, 30-, and 60-min intervals by adding 0.05 ml of 0.2 M Na_2CO_3 . GUS activity was measured at an optical density of 405 nm using a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, CA) and expressed as nanomoles per minute per milligrams of protein. This experiment was conducted twice.

DNA extraction and molecular analysis of integration events.

DNA extraction procedures were performed on the wild-type isolate 506/2 and the stable transformants, as previously described (4, 5,12,24,25). A digoxigenin-high prime DNA luminescent labeling and detection kit was used for nonradioactive nucleic acid labeling and detection (Boehringer GmbH). The organization of integrated pGPD-GUS in transformants was determined by digesting genomic DNA with *Kpn*I, which has a unique site on the plasmid (Fig. 1), and by performing Southern blot hybridization with luminescent-labeled pGPD-GUS.

RESULTS

GUS transformants. Twenty-two hygromycin-resistant transformants were originally obtained, whereas the wild-type *F. subglutinans* isolate 506/2 did not grow on this selection medium. Some transformed isolates did not express the GUS reporter gene after transferring to PDA plates supplemented with 50 μ g of X-Gluc per ml. Ultimately, five stable transformants (GPD-1, GPD-7, GPD-8, GPD-18, and GPD-19) expressing both the GUS reporter and hygromycin resistance genes, were selected after subculturing at least five times over a 2-month period on amended and unamended PDA.

Pathogenicity tests. All five stable transformants caused typical malformation symptoms in vegetative and floral tissues, similar to the wild-type isolate 506/2, 6 to 8 weeks after inoculation in developing mango tissues (Figs. 2 and 3). Percent infection was 67, 83, 100, 100, 100, and 100 for isolates GPD-7, GPD-1, GPD-8, GPD-18, GPD-19, and wild-type 506/2, respectively. Typical disease symptoms were also observed in unwounded inoculated tissue.

GUS staining and activity. Typical dark blue staining was observed in mycelium and conidia of transformed isolates and not in that of the wild-type control, isolate 506/2 (data not shown). GUS-stained mycelia were detected in leaf primordia (Fig. 4A), in sepals (Fig. 4C), within a pedicel (flower stalk) (Fig. 4E), and within nectar glands (Fig. 4G). GUS-stained mycelia were not evident in non-inoculated controls of leaf primordia (Fig. 4B), in sepals (Fig. 4D), within the pedicel (Fig. 4F), and within nectar glands (Fig. 4H). Likewise, GUS-stained mycelia were observed within trichomes (Fig. 5A and C), associated with anther sacs (Fig. 5E), and detected in nectar glands at early stages of fruit set (Fig. 5G), but not

in the respective noninoculated controls, trichomes (Fig. 5B and D), anther sacs (Fig. 5F), and nectar glands at fruit set (Fig. 5H). In addition, no fungi could be detected in noninoculated control tissue using aniline blue staining, whereas only typical *F. subglutinans* mycelia and conidia could be detected in mango tissue infected with transformed and wild-type isolates using this stain (data not shown).

Specific GUS activity of the transformants and the wild-type isolate was quantified in extracts of mycelium and found to range from 227.26 to 430.41 nmol/min/mg of protein for the transformants, whereas no activity was recorded for the wild-type isolate 506/2 (Table 1).

Molecular analysis of transformants. pGPD-GUS hybridized to undigested genomic DNA from each of the five transformants, but not to DNA of the wild-type isolate 506/2 (data not shown). The patterns of integration were complex and differed for all transformants when hybridized to genomic DNA digested with *Kpn*I, which has a unique site on the plasmid DNA, whereas no signal was observed for the wild-type isolate 506/2 (Fig. 6). A single integration event was observed in isolate GPD-18 denoted by two hybridizations signals, and multiple copy inserts of pGPD-GUS were observed in the transformed isolates GPD-1, GPD-7, GPD-8, and GPD-19 (Fig. 6). Although GPD-1 and GPD-19 contained multiple inserts, GUS activity was not proportional to plasmid number, since GPD-8 and GPD-18, containing single and double inserts, expressed GUS activity at a similar rate (Table 1; Fig. 6).

DISCUSSION

The cause of mango malformation disease, recognized for over a century, has been attributed to numerous agents including viral, acarological, physiological, and fungal factors (11,22). *F. subglutinans* has been the fungal pathogen most commonly associated with the disease since 1966 (29); however, a recent article does not mention this organism as a possible source of infection (13). In light of past and present uncertainty regarding the causal disease agent, this study was conducted to (i) unequivocally determine the etiology of mango malformation disease using GUS transformants of *F. subglutinans* that are known to incite disease and (ii) track the fungus in infected tissue for confirmation.

Fig. 6. Southern blot analysis of *Kpn*I-linearized pGPD-GUS and *Kpn*I-digested genomic DNA from the wild-type isolate 506/2 of *Fusarium subglutinans* and transformants (GPD-1, GPD-7, GPD-8, GPD-18, and GPD-19) probed with chemiluminescent-labeled pGPD-GUS. DNA size markers are indicated on the left.

TABLE 1. Specific activity of the GUS transformants and the wild-type isolate of *Fusarium subglutinans* as quantified by the 4-methyl-umbelliferyl β -D-glucuronide assay

Isolate	Specific activity ^a
Wild-type 506/2	0.0
Transformant GPD-1	227.26 $\pm$ 12.95 ^b
Transformant GPD-7	228.57 $\pm$ 5.83
Transformant GPD-8	456.26 $\pm$ 6.09
Transformant GPD-18	430.41 $\pm$ 2.76
Transformant GPD-19	44.28 $\pm$ 4.30

^a GUS activity (nmol/min/mg of protein) of the transformants and the wild-type isolate was quantified in extracts of mycelium, following 7 days of growth on potato dextrose broth.

^b Data are the means $\pm$ standard error. The experiment was conducted twice.

The wild-type isolate 506/2 of *F. subglutinans*, originating from infected malformed mango floral tissue, was readily transformed with the GUS reporter gene, producing transformants with stable single and multiple integration events (Fig. 6). All transformants maintained their GUS activity in vitro and in planta, and no differences in levels of staining were observed among the transformants, regardless of plasmid copy number, integration sites, or specific GUS activity. Transformants harboring multiple plasmid copies did not necessarily express elevated GUS activity compared with those containing single or double inserts. This indicates that transformants with multiple insertions may contain nonfunctional, truncated copies of the plasmid that are not expressed, besides a single functional insert. The GUS-transformed isolates expressing GUS activity remained pathogenic, causing typical vegetative and floral malformation symptoms in mango (Figs. 2 and 3). Disease symptoms were also achieved by placing a conidial drop on dormant buds without wounding, which further demonstrates the virulence of this pathogen. No other fungi were isolated or observed in non-inoculated healthy tissue. This rules out the possibility that other unidentified fungal agents were present within plant tissue. The ensuing histochemical study detecting GUS-stained mycelium in infected tissues provided further evidence that *F. subglutinans* is a causal agent of mango malformation disease. GUS-stained pollen grains were also observed (data not shown), which suggests that the fungus could be spread by insects, wind, and other means during pollination. Although mites have been associated with mango malformation, acaricides were routinely applied to plants in our experiments to ensure that mites had no effect on the current results.

In summary, the wild-type isolate 506/2 of *F. subglutinans*, transformed with the GUS reporter gene, produced transformants with stable, single, and multiple integration events. GUS-transformed isolates expressing GUS activity remained pathogenic, causing typical vegetative and floral malformation symptoms in mango. Histochemical studies detected GUS-stained mycelium in infected tissues, providing unequivocal evidence that this strain of *F. subglutinans* and its transformants are causal agents of mango malformation. Future work will concentrate on the epidemiology of disease, since long-distance and within-tree spread of the fungus is poorly understood.

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