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Genetic transformation of *Fusarium oxysporum* f.sp. *gladioli* with *Agrobacterium* to study pathogenesis in *Gladiolus*

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Abstract *Fusarium* rot caused by *Fusarium oxysporum* f.sp. *gladioli* (*Fog*) is one of the most serious diseases of *Gladiolus*, both in the field and in bulbs in storage. In order to study the mechanisms of pathogenesis of this fungus, we have transformed *Fog* with *Agrobacterium tumefaciens* binary vectors containing the hygromycin B phosphotransferase (*hph*) gene and fluorescence reporter genes EGFP (green), EYFP (yellow) or ECFP (cyan) using the AGL-1 strain of *A. tumefaciens*. Hygromycin B (100 μg/ml) resistant colonies were observed only when acetosyringone was added to the co-cultivation medium. Transformed colonies are more clearly visible when co-cultivated on cellophane membrane than on Hybond -N^+ membrane. Transformed lines were stably maintained through four serial passages on medium containing hygromycin B, and they expressed green, yellow or cyano fluorescence. PCR with *hph*-specific primers and Southern blotting with an *hph*-specific probe were positive for Hyg^R lines but not for the untransformed isolate. The cyano fluorescence of the ECFP-transformed isolate was clearly distinguishable from the green autofluorescence of *Gladiolus* roots, signifying the potential of these lines for further histopathological investigations. Transformed lines will be useful for identifying pathogenicity related genes, screening transgenic resistance, and in studies of host-pathogen interactions.

Keywords Enhanced green fluorescent protein · Enhanced yellow fluorescent protein · Enhanced cyano fluorescent protein · Gladiolus corm rot · Histopathological investigations

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Introduction

Gladiolus is grown world-wide as an economically important ornamental crop. In 2008 the wholesale value of *Gladiolus* cut flowers in the US was $19,936,000 which is 4.9% of the total value of all cut flowers in the US (http://usda.mannlib.cornell.edu/usda/current/FlorCrop/FlorCrop-04-23-2009.txt). *Fusarium* rot caused by *Fusarium oxysporum* Schltdl. : Fr. f. sp. *gladioli* (Massey) W.C. Snyder & H.N. Hansen (*Fog*)

is one of the most serious diseases of *Gladiolus*, affecting plants in the field, causing corms to rot before digging, in storage, or after planting (Jones and Jenkins 1975). Symptoms of the disease include root-, crown- and stem rot, vascular wilts, foliage chlorosis, yellowing and necrosis, and discoloured and misshapen flowers (Heimann and Worf 1997; Agrios 2005). Occasionally the stunted plants fail to bloom (Khan and Mustafa 2005; Heimann and Worf 1997). In Florida the disease is estimated to cause an average loss of 200 dollars per acre (Magie 2000). Some of the best commercial varieties are most susceptible to the corm-rot phase of the disease (Magie 1980).

The ascomycetous pathogen *F. oxysporum* reproduces only asexually (Agrios 2005). *F. oxysporum* is a species complex with a broad host range, infecting over 100 plant species (Michielse and Rep 2009; Lievens et al. 2008); however, individual strains normally infect a single or limited number of plant species. Such pathogenically specialized strains are designated as *forma speciales* (f.sp.). There are more than 120 *forma speciales*, infecting distinct hosts (Agrios 2005). Pathogenic specializations of *F. oxysporum* have been noted both at the tissue and organ levels as well as at the molecular level (Schenk and Bergman 1969; Lievens et al. 2008; Michielse and Rep 2009). Strains of *Fog* and their reported vegetative compatibility sub-groups are known to attack several genera within the Iridaceae family (Roebroeck and Mes 1992).

Traditionally, the pathogen had been controlled using methyl bromide soil fumigation (which is now banned), hot water treatment, and to a limited extent, tolerant cultivars. Resistance against the pathogen has not been reported and tolerance is detected only in a limited number of cultivars (Löffler et al. 1997; Straathof et al. 1998). Very few novel chemical control methods have been tested (Elmer 2006), mostly due to insufficient knowledge of the disease process. Moreover, no efficient therapeutic treatments are available against the pathogen; hence eradication of infected bulbs is an important aspect of disease control methods. Despite many attempts to control this disease, the problem is still widespread (Roebroeck and Mes 1992; Riaz et al. 2010). Unlike in some other *forma speciales* of *F. oxysporum*, knowledge of the molecular biology, biochemistry and histopathology of *Fog* interaction with its host is lacking (Lagopodi et al. 2002; Lievens et al. 2008; Michielse and Rep 2009; Yang et al. 2010), necessitating a thorough study of its pathogenic interactions (Roebroeck and Mes 1992).

Agrobacterium tumefaciens-mediated transformation (ATMT) has proven to be a simple and efficient tool for transforming filamentous fungi. (Chen et al. 2000; Mullins et al. 2001; Degefu and Hanif 2003). In addition, it allows investigators an opportunity to identify genes with roles in morphogenesis, metabolism and pathogenicity of fungi through tagging genes by *Agrobacterium*-mediated insertional mutagenesis (AIM) (Tsuji et al. 2003). ATMT has the unique advantage that it can transform protoplasts, hyphae, spores, conidia, and other fungal morphogenic forms (Hanif et al. 2002), making it a powerful genetic transformation tool for fungi (Degefu and Hanif 2003).

To facilitate the histopathology and molecular investigations of *Fusarium-Gladiolus* interactions, we have developed an efficient *Agrobacterium*–mediated system of *Fog* transformation with the hygromycin B resistance gene (hygromycin B phosphotransferase or *hph* from *Escherchia coli*) and the three fluorescent reporter genes ECFP (cyano), EGFP (green), and EYFP (yellow). The transformed *Fog* mycelia grew on 100 μg/ml hygromycin B-supplemented medium and expressed appropriate fluorescence (e.g., cyano, green or yellow) both in the mycelia and microconidia when observed with a confocal laser scanning microscope (CLSM). A preliminary report of this research has been recently documented (Lakshman et al. 2010).

Materials and methods

Fungal isolate

The *F. oxysporum* f.sp. *gladioli* (*Fog*) used in this study was received from Prof. Robert McGovern (Univ. of Florida, Gainesville, FL). A single conidial isolate of the fungus was maintained on Potato Dextrose Agar (PDA) medium with occasional transfer to *Gladiolus* corms to maintain pathogenicity. The fungus had been stored as spore and mycelial suspension in 25% glycerol at −80°C. Previous literature states that 80 μg/ml hygromycin B can be used to select transformed colonies from the wild type of *F. oxysporum* (Mullins et al. 2001; Khang et al. 2006). However, because *Fog* is a different pathotype of *F. oxysporum*, we set up a kill curve using poisoned food technique

(Dhingra and Sinclair 1995) to determine the optimal hygromycin B level at which wild type *Fog* will not grow. For this assay, PDA was supplemented with 25, 50, 60, 70, 80, 90 and 100 μg ml^{-1} of hygromycin B and the growth of *Fog* was evaluated following incubation at 22°C for 21 days in dark. For transformation, conidia were harvested in sterile water from sporulated PDA plates and filtered through two layers of cheesecloth to remove spore aggregates and mycelia pieces. Spore count in the liquid medium was recorded using a haemocytometer (McDonnell 1962). Spores were stored in 4°C till co-inoculated with *A. tumefaciens*.

Plasmid and *A. tumefaciens* strains

The AGL-1 strain of *A. tumefaciens* containing the binary vectors pBGgHg (Chen et al. 2000), SK1046: pBHt2-EYFP, and SK1292:pBHt2-ECFP (Mosquera et al. 2009) were kindly supplied by Prof. Seogchan Kang (Penn State Univ., PA). The pBGgHg is a 9.6 kb plasmid construct with the hygromycin B phosphotransferase gene (*hph*) and the enhanced green fluorescence protein gene (*EGFP*) as selective and expression markers respectively, both under the influence of *Agaricus bisporus* glyceraldehyde-3-phosphate dehydrogenase promoter (Pgpd) (Chen et al. 2000). The SK1046: pBHt2-EYFP, and SK1292:pBHt2-ECFP plasmids contain the *hph* gene and the enhanced yellow fluorescence protein or the enhanced cyano fluorescence protein genes, respectively (Mosquera et al. 2009).

Fungal transformation

Transformation was based on a published protocol (Mullins et al. 2001) with modifications. A freshly grown single colony of *A. tumefaciens* AGL-1 strain was grown in minimal medium (Mullins et al. 2001) supplemented with kanamycin (50 μg/ml) for 2 days at 25°C on a rotary shaker at 100 rpm. At 0.600 OD (600λ), the cells were diluted in the induction medium (Mullins et al. 2001) in the presence of 200 μM acetosyringone and were allowed to grow for an additional 5–7 h at 26–27°C with shaking at about 200 rpm. Microconidia (1.0×10^{7}/ml) from a 10 days old *Fog* isolate were mixed in equal proportions with either acetosyringone-induced or non-induced *A. tumefaciens* cells (0.300 OD), incubated for 5–10 min at 22°C and plated on either cellophane (Bio-Rad, Hercules, CA) or Hybond N + (Amersham Biosciences, GE Health care, Piscataway, NJ) membrane spread over the co-cultivation medium (Khang et al. 2006) containing 200 μM acetosyringone. Plates were then incubated at 23°C for 48 to 60 h. As controls, the *Fog* - *A. tumefacien* mixes were also spread on cultivation medium without acetosyringone. The membranes were transferred to PDA selection medium containing 100 μg/ml hygromycin B, 200 μM cefotaxime and 100 μg/ml moxalactum; the latter two antibiotics were used to kill *Agrobacterium*. Hygromycin B resistant (Hyg^{R}) colonies of *Fog* were observed following 7–9 days of incubation at 23–25°C. Transformed lines were serially transferred 4 to 5 times in the selection medium to check for stability of growth and morphology. Hyg^{R} single conidial cultures of transformants were preserved in 25% glycerol at −80°C for long term storage and analysis.

Fungal DNA isolation

For DNA isolation, *Fog* was grown in 100 ml of potato dextrose broth (PDB) containing hygromycin B (100 μg/ml) in 300 ml Erlenmeyer flasks at room temperature on a rotary shaker at 150 rpm for 4–5 days. Mycelial and conidial bits were collected by centrifugation at 6000 rpm at 4°C, frozen in liquid nitrogen and lyophilized. Total DNA from non-transformed and transformed lines was extracted using a modified protocol of Cubero et al. (1999) using CTAB. Simply, lyophilized fungus material was ground in liquid nitrogen, suspended in pre-warmed (65°C) CTAB buffer (Cubero et al. 1999) and incubated at 65°C for 60 min. DNA was extracted once with chloroform:isoamyl alcohol (24:1) and RNA was removed from the extract by incubating with 10 μl RNAse A (Sigma Aldrich, 70 U/μl) at 65°C for 30 min and at 25°C for an additional 10 min. The chloroform extraction was repeated once more. DNA was precipitated with 0.7 volume of isopropanol at room temperature for 4–5 h followed by centrifuging at 4500 rpm for 10 min in a Sorvall Legend RT + centrifuge (Thermo Scientific). The DNA pellet was rinsed once with 70% ethanol, air dried and dissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

Molecular characterization of transformed lines

Presence of *hph* and EGFP genes in the transformed *Fog* lines were confirmed by genomic PCR. Both the transformed and untransformed *Fog* DNA were PCR

amplified with the *hph* and EGFP-specific primers HPH-F (5′-AAGCCTGAACTCACCGCGAC-3′) and HPH-R (5′-CTATTCCTTTGCCC TCGGAC-3′), EGFP-F (5′-CACATGAAGCAGCACGACTT-3′) and EGFP-R (5′-TGCTCAGGTAGTGGTTGTCG-3′). A specific band of ~1 kb should be observed to confirm transformation with the *hph* gene (Kemppainen et al. 2005) while a band of ~460 bp should be observed to confirm transformation with EGFP (Grimaldi et al. 2005). PCR was not performed for the ECFP and EYFP genes in the respective transformed lines.

Confocal laser-scanning microscopy

To visualize fluorescence generated by the expression of the reporter genes in the transformed *Fog* lines, a Zeiss™ 710 CLSM system (Carl Zeiss Inc., North America) was utilized. The images were observed using a Zeiss Axio Observer™ inverted microscope with 10×0.45 NA C-Apochromat and 25×0.83 NA Plan-Apochromat objectives. A 488-nm argon laser with a pin hole of 32 μm passing through a MBS 488 beam splitter filter with limits set between 495–543 nm was used for detection of EGFP. A 514-nm argon laser with a pin hole of 36 μm passing through a MBS 458/514 beam splitter filter with limits set between 520–554 nm was used for detection of EYFP. A 405-nm diode laser with a pin hole of 30 μm passing through a MBS 405 beam splitter filter with limits set between 454–514 nm was used for detection of ECFP.

To investigate the infection process of *Fog* on *Gladiolus* roots with confocal microscopy, a transformed ECFP-expressing *Fog* line was used. *Gladiolus* cv. Peter Pears plants were grown from corms cultured in vitro on Murashige and Skoog's medium supplemented with 3% sucrose (Murashige and Skoog 1962) and solidified with 0.2% Phytagel (Sigma Alrich). Corms were cultured under a 12 h photoperiod using cool white fluorescent light bulbs (40–60 μmol m^{-2} s^{-1}). A 5 mm diameter plug of agar was taken from a plate of a freshly growing ECFP-transformed *Fog* strain and placed approximate 1 cm from the roots of a *Gladiolus* plant in vitro. One week later the *Fusarium* had covered the plant's root and was visualized using a Zeiss™ 710 CLSM system (Carl Zeiss Inc., North America). The images were observed using a Zeiss Axio Observer™ inverted microscope with a 40×1.3 NA Plan-Apochromat objective. A lambda spectral analysis was performed using a 458 nm argon laser with a pin hole of 36 μm with limits set between 460–728 nm for detection of the spectra. The spectra was linearly unmixed to identify two different spectral curves, one for the ECFP (cyano) and the other for the autofluoroscence (green) generated by *Gladiolus* root cells. Zeiss Zen™ 2009 was used to capture the images and separate the spectra. Axiophot 4.6™ (Carl Zeiss Inc.) and Photoshop 7.0™ (Adobe Systems Inc., San Jose, California, USA) were utilized to design the figures.

Southern hybridization

Southern analysis was carried out by digesting transformed or untransformed *Fog* genomic DNA with *Hind* III (for transformation with pBHt2-EYFP, and pBHt2-ECFP vectors) or with *Eco* RV (for transformation with pBGgHg vector), both enzymes cutting once within the T-DNA insert outside the *hph* gene. Digested DNA was resolved by electrophoresis using a 0.7% agarose gel in 0.5X TBE buffer (Maniatis et al. 1982). DNA was capillary blotted to 0.45 micron pore size Whatman Nytran SuperCharge nylon transfer membrane (Sanford. ME) following depurination (0.25 M HCl for 10 min), two rounds of denaturation (1.0 M NaCl and 0.5 M NaOH for 15 min) and two rounds of neutralization (1.5 M NaCl and 0.5 M Tris-base pH 8.0 for 15 min). Prehybridization, hybridization, and post-hybridization washes, as well as detection, were carried out as described by Maniatis et al. (1982). Hybridization was carried out with the ~1 kb PCR product of pBGgHg specific for the *hph* gene (Kemppainen et al. 2005).

Inoculation of transformed pathogen on *Gladiolus* corms

Corms were inoculated with conidial suspensions of both the untransformed and transformed *Fog* lines following the procedure of Löffler et al. (1997) with minor modifications. Briefly, the *Gladiolus* cv. Peter Pears corms were dehusked and disinfected for 30 min in 1% NaOCl solution containing one drop of Tween 20 per litre followed by three rinses in sterile tap water and air-dried. Using a rubber scraper, spores from a 15 day-old culture grown on solid PDA medium were collected and suspended in 5 ml sterile water to create conidial suspensions. A lancet needle (Kendall Monolet Original Lancets, 21G, Mansfield, MA) was dipped in spore suspension and pricked up to 3 mm

deep at the outer edge of a corm. Four such inoculations were made on a single corm and 6 corms were inoculated with each transformed line. The inoculated corms were incubated in a brown paper lunch bag, closed by folding the top of the bag and stored in the dark at 25°C. The infection was evaluated following 30 days of incubation using a disease rating scale of 1 to 5 (1: no infection; 2–5: 0–5%, 6–15%, 16–50% and 51–100% of the surface covered with mycelium). For internal symptom development, 40 days after inoculation corms were horizontally cut using a razor blade and photographed. The experiment was repeated once.

Results

Sensitivity of *F oxysporum* f.sp. *gladioli* to hygromycin B and effect of membrane types on transformation efficiency and stability

The growth of the *Fog* isolate was partially inhibited at 70 μg ml^{-1} of hygromycin B, but completely inhibited at 80 μg ml^{-1} of the same antibiotics in the PDA (Table 1). We chose 100 μg ml^{-1} hygromycin B for selection of transformants. Hygromycin B resistant (Hyg^R) colonies of *Fog* appeared on support membranes on the selection medium following 7 to 9 days of incubation at 23–24°C. The colonies continually grew in the selection medium until they merged with each other. Individual transformed *Fog* colonies were better distinguishable on cellophane membrane than on Hybond N + membrane (Fig. 1). This resulted in detection of more transformed colonies on cellophane than on Hybond N^+ membrane. Following spreading of 50×10^4 microconidia on cellophane as the support membrane, an average of 29 and 32 hygromycin B resistant colonies / plate appeared when the *Agrobacterium* was pre-induced or not with acetosyringone, respectively. However, following spreading of 50×10^4 microconidia on Nylon membrane, we obtained only 1–5 colonies /plate when the *Agrobacterium* was pre-induced or not with acetosyringone, respectively. Even though pre-induction with acetosyringone was not a prerequisite for efficient transformation of *Fog*, Hyg^R colonies were observed only when acetosyringone was added to the co-cultivation medium. Stability of the transformed lines was tested by serially transferring them to fresh selection medium for at least 4 to 5 times. About 90% of the selected colonies maintained hygromycin B resistance (data not shown).

Table 1 Radial growth of *Fusarium oxysporum* f. sp. *gladioli* (*Fog*) in hygromycin-amended Potato Dextrose Agar (PDA) medium 21 days after inoculation

Hygromycin(μg/ml)	Avg. growth (mm)[a]	St dv
0	10.00	0.50
25	8.55	0.73
50	5.11	0.33
60	3.33	0.50
70	1.55	0.53
80	0.00	0.00
90	0.00	0.00
100	0.00	0.00

[a] Average of 9 readings from three poison food culture plates

PCR detection of transformants

Selected *Fog*-Hyg^R colonies were tested for the presence of both *hph* and EGFP genes by PCR of total genomic DNA using gene selective primer pairs. PCR screening of 5 randomly selected transformants gave products of the expected size (~1.0 kb for *hph* and ~460 bp for EGFP) as seen using the plasmid pBGgHg as positive control, indicating the presence of *hph* (Fig. 2b, lanes 13 to 17) and EGFP genes (Fig. 2a, lanes 4 to 8) in the same set of transformants. No PCR product specific for EGFP (~460 bp) was amplified from the wild type *Fog* isolate (lane 3). Except for some artifactual PCR products (lane 12), no *hph*-specific product of the expected size (1.0 kb) was amplified from the wild type *Fog* isolate. Even though the PCR for the ECFP and EYFP genes were not attempted from the pBHt2-EYFP and pBHt2-ECFP - transformed *Fog* lines, the *hph*-specific ~1 kb PCR products were observed in those lines (data not shown).

Southern hybridization

Hind III- or *Eco* RV-digested genomic DNA from transformed *Fog* lines mostly showed a single hybridization with the *hph* gene-specific probe, demonstrating that the majority of the transformed *Fog* lines got only one insertion of the *hph* gene into their genomes (Fig. 3). Infrequently, a double integration of *hph* gene was also observed (Fig. 3, panel a, #12). No hybridizing bands were detected with the *Hind* III- or *Eco* RV-digested DNA from the untransformed *Fog* isolate.

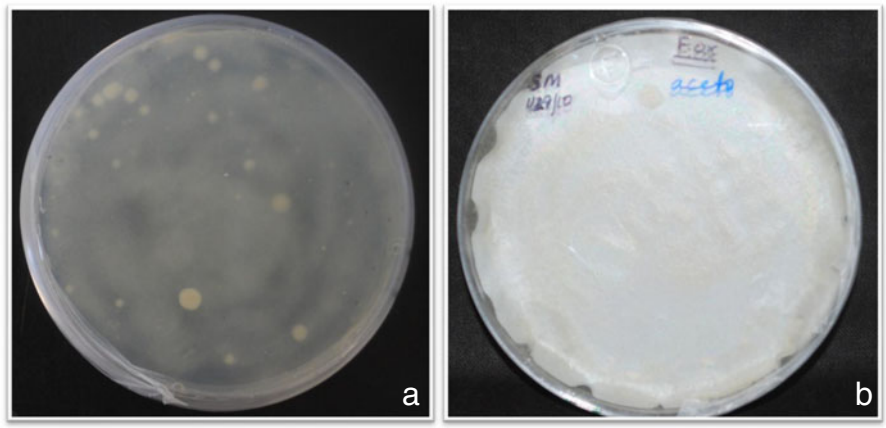

Fig. 1 Hygromycin B resistant colonies of *Fusarium oxysporum* f. sp. *gladioli* on (panel **a**) cellophane membrane and (panel **b**) Hybond-N^{+} nylon membrane

Confocal microscopic observation of transformed lines and *Gladiolus* roots

Cyano, green and yellow fluorescence was observed both in the mycelia (Fig. 4, panels a–c) and microconidia (Fig. 4a) with CLSM when the specified excitation and emission wavelengths were used for the respective *Fog* lines. No fluorescence was detected in any of the untransformed lines under above conditions and no fluorescence was detected from the transformed lines under bright field illumination (data not shown).

Histopathological observation with transformed pathogen

Since the pathogen attacks mostly underground parts, we also compared the autofluorescence of *Gladiolus* roots with the EGFP-, EYFP- and ECFP-transformed *Fog* lines. When the *Gladiolus* roots infested with the above three transformed mycelia were compared side by side, only the cyano fluorescence (ECFP) of transformed *Fog* isolate could be most clearly distinguishable from the green auto-fluorescence of the roots (Fig. 4, panel d). Following three days of inoculation with an ECFP-transformed *Fog* isolate, a network of intensely fluorescent colonized mycelia were observed on the root surface (Fig. 4, panel d). Moreover, several intracellular mycelia spanning horizontally in the root cortex (data not shown) were visible.

Infectivity of transformed pathogen on *Gladiolus* corms

No significant differences in pathogenicity of the transformed lines were observed on *Gladiolus* corms when 10 randomly selected transformed *Fog* lines were compared with the untransformed *Fog* isolate for symptom developments (Fig. 5). Cross sections of representative corms infected with either non-transformed or transformed *Fog*

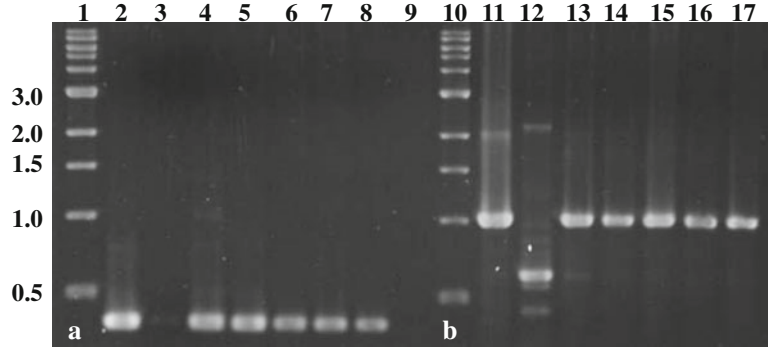

Fig. 2 PCR detection of the enhanced green fluorescent protein (EGFP) (panel **a**, lanes 4–8) and hygromycin phosphotransferase (*hph*) (panel **b**, lanes 13–17) genes in the selected transformed lines of *Fusarium oxysporum* f. sp. *gladioli* (*Fog*). The transformed *Fog* genomic DNA templates for lanes 13–17 are the same set of DNA templates used in lane 4–8. Lanes 2 and 11: PCR products of EGFP and *hph* genes, respectively, with pBGgHg plasmid as the PCR template. Lanes 3 and 12: PCR on non-transformed *Fog* DNA. Lanes 1 and 10: 1 Kb DNA marker (New England BioLabs, Ipswich, MA)

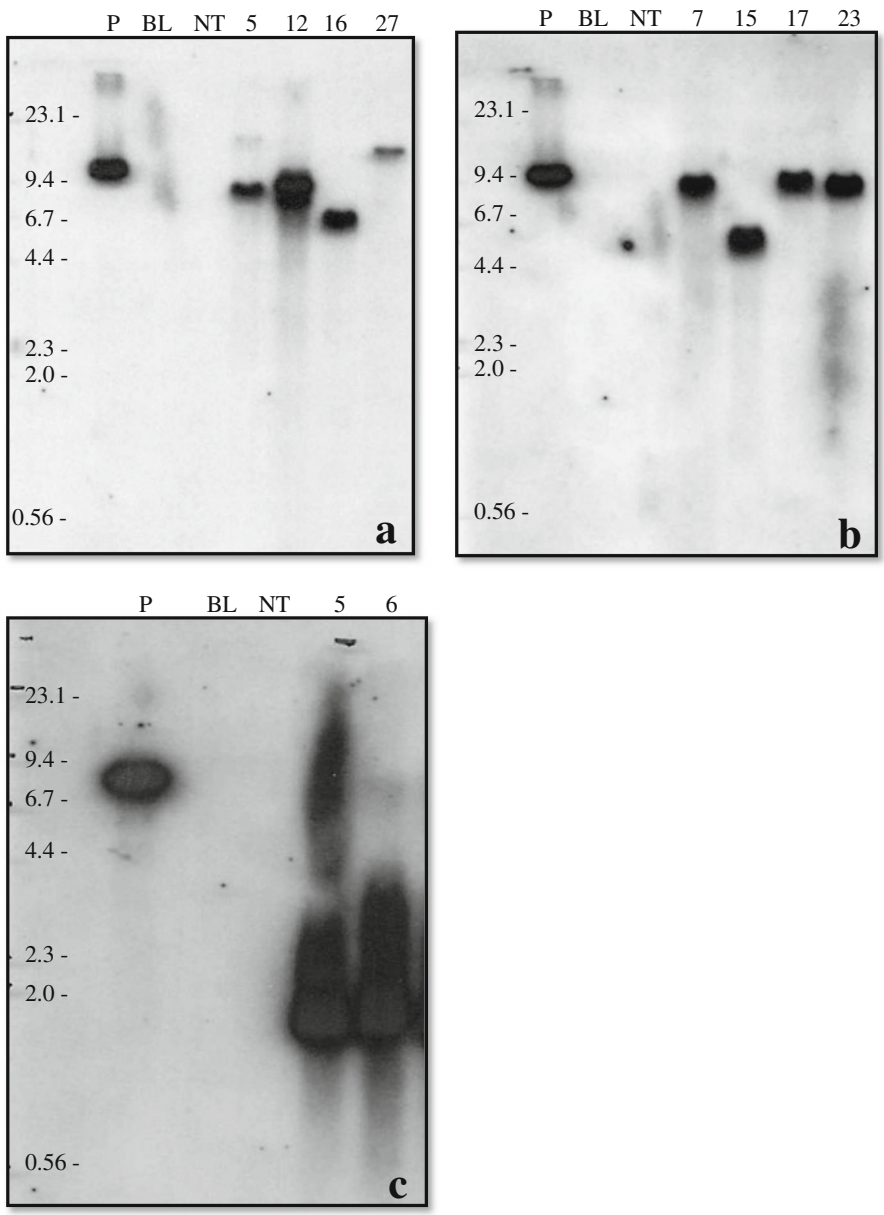

Fig. 3 Southern blot of genomic DNA isolated from *Fusarium oxysporum* f.sp. *gladioli* (*Fog*) transformed lines using the hygromycin phosphotransferase (*hph*) gene as a probe. Plasmid and genomic DNAs are digested with *Hind* III (Panels **a** and **b**) and *Eco* RV (Panel **c**). Each lane of genomic DNA contains either 1–2 μg of DNA (**a**, **b**) or 5 μg DNA (**c**). Numbers represent transformed *Fog* lines. Size markers are shown on the left in kilobases. Lane P contains approximately 50 pg of *hph* gene. BL = Blank lane. NT = non-transformed *Fog* genomic DNA digested with *Hind* III (Panels **a** and **b**) and *Eco* RV (Panel **c**)

showed no differences of symptom severity (not shown). Those observations established that the transformed lines will be useful for histopathological investigations on *Gladiolus*.

Discussion

We have developed an efficient transformation protocol for *F. oxysporum* f.sp. *gladioli* (*Fog*), one of the most important soilborne ascomycetous fungal pathogen of *Gladiolus* and successfully generated several stable transformed lines showing hygromycin B resistance and green, yellow or cyano fluorescence. To our knowledge, this is the first report on genetic transformation of this pathogen and on the histological observation with a fluorescence-tagged pathogenic isolate on *Gladiolus* roots. The transformed *Fog* lines developed in this study will be useful in molecular and histopathological investigations of *Fog*-*Gladiolus* interactions. We observed that cellophane is a better membrane substratum to get the maximum number of clearly

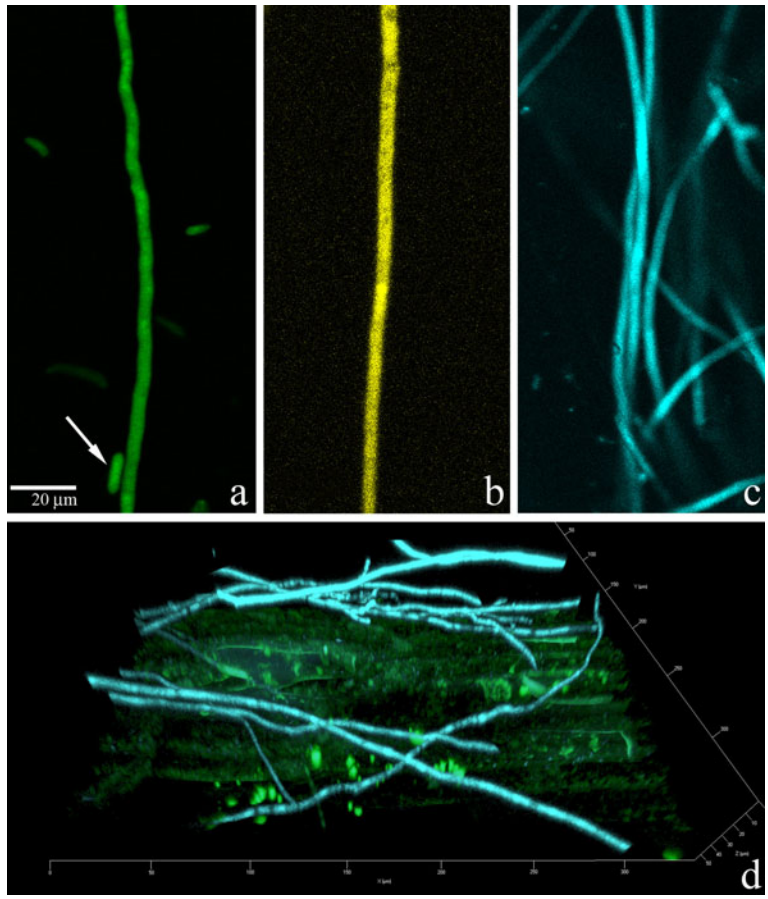

Fig. 4 Confocal laser scanning microscopy images of the EGFP- (Panel **a**), EYFP- (Panel **b**) and ECFP- (Panel **c**) transformed *Fusarium oxysporum* f. sp. *gladioli* (*Fog*) lines. EGFP-transformed *Fog* lines expressed fluorescence in microconidia (indicated with an arrow) in addition to mycelia (Panel **a**). The cyano fluorescence of the ECFP-transformed *Fog* mycelia could be clearly distinguishable from the green auto-fluorescence of *Gladiolus* root (Panel **d**)

identifiable transformants than the Hybond-N membrane. Pre-induction of *Agrobacterium* culture with acetosyringone did not improve the efficiency of transformation, even though the presence of acetosyringone in the co-cultivation medium was essential for transformation. Using cellophane membrane, we obtained an average of 30 transformed colonies / plate. This is comparable to the frequency reported for other fungi (Talhinhas et al. 2008; Zhang et al. 2008).

The presence of *hph* and EGFP genes in the transformed lines was indicated by respective positive PCR products and subsequent Southern blot analysis established that the *hph* gene has been integrated into the *Fog* genome. From the Southern analyses, we found

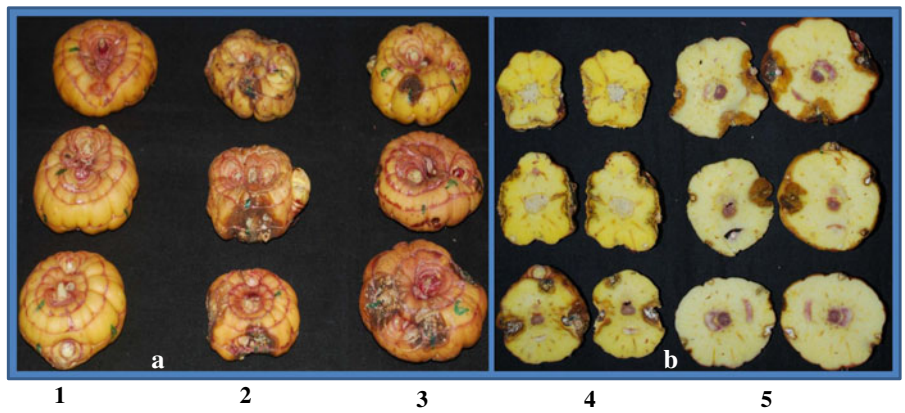

Fig. 5 Panel **a**: Comparison of pathogenicity of wild type *Fusarium oxysporum* f. sp. *gladioli* (*Fog*) spores (row 2) and spores from ECFP-transformed line 17 (row 3) on *Gladiolus* corms (cv. Peter Pears) inoculated with a lancet needle. Corms in row 1 were inoculated with sterile water only. Panel **b**: Cross section of corms infected with wild type *Fog* (row 4) and ECFP-transformed line 17 (row 5)

that most of the transformed lines have a single copy of T-DNA inserted. Confocal laser scanning microscopy (CLSM) showed that the EGFP, ECFP and EYFP genes were expressed in the respective transformants. We also observed using CLSM that the cyano fluorescence from ECFP-transformed *Fog* lines is clearly distinguishable from the green autofluorescence of *Gladiolus* roots, thus demonstrating the potential of the cyano fluorescence-transformed pathogen for conducting histopathological investigations on *Gladiolus* roots. In addition, we have shown with CLSM that following 3 days of incubation, the pathogen colonized the root surface with a network of intensely fluorescing mycelia. Also the transformed *Fog* lines retain pathogenicity of the wild type (untransformed) fungus as evidenced by their virulence on the *Gladiolus* corms.

Agrobacterium-mediated insertional mutagenesis (AIM) has been used to generate mutants with complete or partial loss of pathogenicity and other biochemical properties of several fungi, including selected *forma speciales* of *F. oxysporum* (Mullins et al. 2001; Kilaru et al. 2009; Michielse et al. 2009; Kim et al. 2011). Subsequent characterization of point of insertions led to identification of fungal genes with roles in pathogenesis and virulence (Michielse et al. 2009; Michielse and Rep 2009). Southern analysis in our investigation and others' observation on transformed *F. oxysporum* f. sp. *lycopersici* (Michielse and Rep 2009) suggest that the majority of AIM mutants carry single T-DNA insertions; thus the transformants will be useful for tagged mutagenesis. Currently, we are testing the virulence of several transformed *Fog* lines on *Gladiolus* corms to discover potentially pathogenic variants which will be useful to investigate roles of specific genes in pathogenicity (Talhinhas et al. 2008; Ushimaru et al. 2010). Moreover, we have developed transgenic *Gladiolus* lines expressing various fungal inhibitory genes (Kamo, unpublished data). Transformed *Fog* lines will be utilized to microscopically study interactions of the pathogen with those *Gladiolus* lines to understand transgene-induced resistance mechanisms. Similar studies with transgenic pathogen have been recently reported by Thirugnanasambandam et al. (2011).

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