Plant Cell Tiss Organ Cult (2015) 121:459–467
DOI 10.1007/s11240-015-0716-4

ORIGINAL PAPER

Expression of a synthetic antimicrobial peptide, D4E1, in Gladiolus plants for resistance to *Fusarium oxysporum* f. sp. *gladioli*

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Received: 22 September 2014 / Accepted: 11 January 2015 / Published online: 21 January 2015
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Abstract The main pathogen of Gladiolus plants is *Fusarium oxysporum*, a soilborne fungus that infects roots and corms resulting in death of the plant. Purified D4E1, a synthetic antimicrobial peptide, was previously reported (De Lucca et al. 1998) to inhibit *F. oxysporum* spores from forming mycelial colonies in vitro at a concentration of 3 μM making it a candidate gene for genetic engineering of Gladiolus for resistance to *F. oxysporum*. Gladiolus cv. Peter Pears plants were transformed by particle bombardment with plasmid DNA containing a 90 bp D4E1 gene that was under the control of the duplicated CaMV 35S promoter. Five of the 14 independently transformed plant lines were evaluated for resistance to *F. oxysporum.* Transgenic plants were tested in vitro for resistance to *F. oxysporum*, and several lines appeared to be more resistant than the control plants that lacked D4E1. Cell extracts of transgenic Gladiolus lines 6(1) and 7(1) inhibited germinated spores of *F. oxysporum* f. sp. *gladioli* from forming mycelial colonies by 34 and 38 %, respectively, in vitro. *F. oxysporum* f. sp. *gladioli* was transformed with the ECFP (cyan) gene allowing us to follow the growth of *F. oxysporum* during infection of D4E1-transformed and non-transformed roots. Fluorescence observations using confocal laser scanning microscopy showed that 3–10 days after infection, *F. oxysporum* covered the surface of the root and formed pseudo-appressoria, but hyphae were never observed to penetrate cells of the root. Ten days after infection with *F. oxysporum*, non-transformed roots had completely disintegrated whereas transgenic roots of line 7(1) were just beginning to lose their cellular integrity. Cell extracts from the five transgenic lines showed either an inhibition of *F. oxysporum* mycelial colony formation or less fungal hyphae were observed to infect their roots as compared to non-transformed Gladiolus plants.

Keywords Flower bulb crops · Antifungal · Ornamentals · Transformation · Confocal laser scanning microscopy

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
Fog	*Fusarium oxysporum* f. sp. *gladioli*
MIC	Minimum inhibitory concentration
MS	Murashige and Skoog's medium
PDA or PDB	Potato dextrose agar or broth medium

Electronic supplementary material The online version of this article (doi:10.1007/s11240-015-0716-4) contains supplementary material, which is available to authorized users.

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Introduction

Gladiolus is a popular flower world-wide that is enjoyed as a cutflower and in gardens. The wholesale value of

Gladiolus was $15,290,000 of the $342 million for all cut flowers produced in the US (USDA Floriculture Crops 2012 Summary). A major pathogen of flower bulb crops, including Gladiolus, is *Fusarium oxysporum (Fog)*. *Fog* is characterized as infecting through a wound in the root and then travelling through the vascular system of the plant resulting in the wilting symptoms that often precede death of the plant (Ori et al. 1997; Li et al. 2013). Gladiolus plants infected with *Fog* have yellowing of the leaves, damping off, and basal stem and corm rot (Straathof et al. 1998; Nosir et al. 2011). Methyl bromide was previously used to control Fusarium but is no longer an option due to regulation, and currently effective alternatives are unavailable to commercial growers. There are no commercially important cultivars of Gladiolus that have been found resistant to Fusarium.

Natural antimicrobial peptides (AMPs) are present in organisms ranging from bacteria to humans. Currently there are more than 2,000 AMPs identified (Fox 2013). They are recognized for their ability to kill bacteria although many AMPs are specifically antifungal (van der Weerden et al. 2013). AMPs range from 15 to 50 amino acids long, and they act by forming ion channels in the pathogen's membrane that results in leakage of the cellular content. The cell's normal metabolism is disrupted, and consequently cell death occurs (Pelegrini et al. 2011). D4E1 is a linear peptide that changes to a beta sheet conformation when in solution (De Lucca et al. 1998). The interaction of D4E1 with a membrane results from the charges of its amino acids, and consequently ion channels in the lipid membrane are formed. D4E1 interacts with ergosterol and cholesterol present in the fungus. D4E1 is active against both non-germinated and germinated spores as non-germinated conidia of *Fusarium moniliforme* contain cholesterol that is replaced by ergosterol during germination of the spore. The activity of D4E1 against germinated and non-germinated spores differs for each fungus species. Rajasekaran et al. (2001) observed an increased number of vacuoles and an abnormal cytoplasm present in the germ tubes of germinating spores of *Aspergillus flavus* when the spores were cultured in the presence of 25 μM D4E1. More recently AMPs have also been found to modulate host-defense systems (Fox 2013; Goyal et al. 2013).

The antimicrobial activity of synthetic D4E1 has been tested in vitro by determining its inhibition of fungal and bacterial growth (Rajasekaran et al. 2001), and D4E1 was found to be more effective against bacteria than fungi based upon the minimum inhibitory concentrations (MIC). The two bacteria tested, *Pseudomonas syringae* pv. *tabaci* and *Xanthomonas campestris* pv. *malvaearum* race 18 showed MICs for D4E1 of 2.25 and 1.25 μM, respectively. Of the fungi tested, *F. moniliforme* and *F. oxysporum* were highly sensitive to D4E1 with MICs of 3–12.5 μM (De Lucca et al. 1998). The other fungi tested were *Phytophthora cinnamomi*, *Phytophthora parasitica*, and *Verticillium dahliae* with MIC values of 4.67, 4.67, and 5.25, respectively (Rajasekaran et al. 2001).

Tobacco plants transformed with D4E1 showed resistance to *Colletotrichum destructivum*, the fungal pathogen that causes anthracnose (Cary et al. 2000). Cell extracts from the transgenic tobacco leaves inhibited growth of germinating conidia significantly, 65–99 %, for *Aspergillus flavus* and *V. dahliae*. Transgenic poplar trees expressing D4E1 did not exhibit resistance to *Hypoxylon mammatum*, a fungal pathogen, but one transgenic line showed reduced symptoms following infection with *Agrobacterium tumefaciens* and *Xanthomonas populi* (Mentag et al. 2003). Protein extracts from leaves of transgenic cotton plants expressing D4E1 inhibited the growth of *Fusarium verticilloides* and *V. dahliae* (Rajasekaran et al. 2005). These transgenic cotton plants showed a reduction in the disease symptoms caused by *Thielaviopsis basicola*, the fungal pathogen that causes black root rot in cotton, and seeds were found to inhibit colonization of *Aspergillus flavus* in their cotyledons and seed coats.

There are previous reports studying the effectiveness of various antimicrobial peptides on *Fusarium oxysporum* when expressed in transgenic plants (Epple et al. 1997; Chakrabarti et al. 2003; Lee et al. 2003; Chan et al. 2005; Oard and Enright 2006; Lee 2008; Kostov et al. 2009; Abdallah et al. 2010). This is the first study determining the effectiveness of the synthetic peptide, D4E1, against *Fusarium oxysporum* when expressed in plants.

Materials and methods

Plant materials

Small corms of Gladiolus cv. Peter Pears were sterilized in 20 % Clorox with 15 drops of Tween 20/L followed by three rinses, 5 min each rinse, in sterile water and then germinated on MS medium composed of MS salts (Murashige and Skoog 1962) supplemented with 3 % sucrose, 1.0 mg/L glycine, 1.0 mg/L thiamine, 0.5 mg/L pyridoxine, 0.5 mg/L nicotinic acid, 0.2 % Phytagel (Sigma-Aldrich, St. Louis, MO, USA), and pH 5.8. Gladiolus plants were grown in vitro in Magenta jars at 25 °C under a 12 h light photoperiod using cool white fluorescent lights (40–60 μmol $m^{-2}s^{-1}$). Embryogenic callus was induced from the base of the plants by culturing plants on MS medium containing 0.5 mg/L 2,4-D for 3–6 months. Embryogenic callus was separated from the plants and maintained in the dark on MS medium with 0.5 mg/L 2,4-D. All callus and plant cultures were transferred monthly to

fresh medium. Suspension cells were initiated from the embryogenic callus by placing callus in liquid MS medium supplemented with 0.5 mg/L 2,4-D. Each 125 ml flask contained 30 ml of liquid medium, and cells were subcultured every 3 weeks to fresh medium. Suspension cells were grown in the dark on a gyratory shaker at 120 rpm. Plants, callus, and suspension cells were grown at 25 °C.

Plasmids and PCR analysis

The 90 bp D4E1 gene was under control of the duplicated CaMV 35S promoter in pBS-d35S-D4E1-nos (Fig. 1). Selection of putative transformants was achieved by co-bombarding with p35SAc (received from AgrEvo, Somerville, NJ, USA) which contains the PAT gene that codes for phosphinothricin acetyltransferase under control of the CaMV 35S promoter. Plasmid DNAs for bombarding the suspension cells were isolated from DH5α using alkaline lysis followed by purification on a cesium chloride gradient (Maniatis et al. 1982).

Primers used for amplification of the 90 bp D4E1 gene were forward: 5′-CCATGGGATTTAAGTTGAGAGCT AAGATTA-3′ and reverse: 5′-TTTGAACGATCGGGGA AATTCGAGCTC-3′. PCR samples were amplified using a PTC-200 Microcycler (MJ Research, Waltham, MA) programmed for 94 °C for 2 min, 30 cycles (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min), 72 °C for 10 min. PCR products were run on a 1.5 % agarose gel and then viewed by staining with ethidium bromide.

Transformation

Plasmid DNAs (pBS-d35S-D4E1-nos and p35SAc) were combined in equal amounts and used to coat 0.8 μm gold particles according to the method of Sanford et al. (1993). On the day of bombardment, liquid medium that the suspension cells were growing in was removed and replaced with the same MS medium supplemented with 0.125 M mannitol. Four hours later the suspension cells were spread out on a Whatman No. 4 filter paper that was placed on solid MS medium containing 0.125 M mannitol and bombarded using the PDS-1000/Helium gene gun (Bio-Rad, Hercules, CA, USA) that was set with a 1 cm gap and 1 cm flying membrane distance. The target distance was 12 cm. Following bombardment the cells were transferred to MS medium containing 0.5 mg/L 2,4-D and 0.2 % Phytagel.

One week after bombardment the suspension cells were transferred to selection medium that was solid MS medium supplemented with 0. 5 mg/L 2,4-D and 0.1 mg/L bialaphos (Meiji Seika Kaisha, Tokyo, Japan). After one month, cells were transferred to MS medium containing 1.0 mg/L bialaphos. Cells were grown in the dark at 25 °C and transferred monthly to fresh selection medium. Plantlets that regenerated from the callus were placed on MS medium containing 0.1 mg/L bialaphos, and larger plants that developed were transferred to Magenta jars containing MS medium with 1.0 mg/L phosphinothricin (glufosinate received from AgrEvo). Plants were maintained under a 12 h light photoperiod.

Southern hybridization

Genomic DNA was isolated from suspension cells of Gladiolus plants using the method of Dellaporta et al. (1983). Each DNA sample was digested with *Hind* III prior to electrophoresis on a 1 % agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). Transfer to Nytran nylon membrane (Schleicher-Schuell, Keene, NH, USA) was accomplished by capillary movement (Maniatis et al. 1982).

A probe to the D4E1 gene was prepared by digesting pBS-d35S-D4E1-nos with *Hind* III and *Eco* RI and then separating the fragments on a 0.9 % agarose gel. The 1 kb insert was gel-purified using a QIAquick gel extraction kit (Qiagen, Inc. Valencia, CA, USA) and then random prime labeled with α-[^{32}P] dCTP using the DECAprime II kit (Ambion Inc, Austin, TX, USA). The DNA blot was prehybridized in a solution of 7 % sodium dodecyl sulfate (SDS), 1 % bovine serum albumin, 1 mM EDTA, and 250 mM $NaPO_4$, pH 7.2 for 1 h at 42 °C prior to hybridization with the α-[^{32}P]-labeled probe at 58 °C for 16 h. Following hybridization in buffer consisting of 6× SSC, 0.01 M EDTA, 5× Denhardt's solution, 0.5 % SDS, and 100 μg/ml denatured salmon sperm DNA (Maniatis et al. 1982), the blot was washed in a series of washes-2× citric acid trisodium salt (SSC), 0.2 % SDS, then 1× SSC, 0.2 %

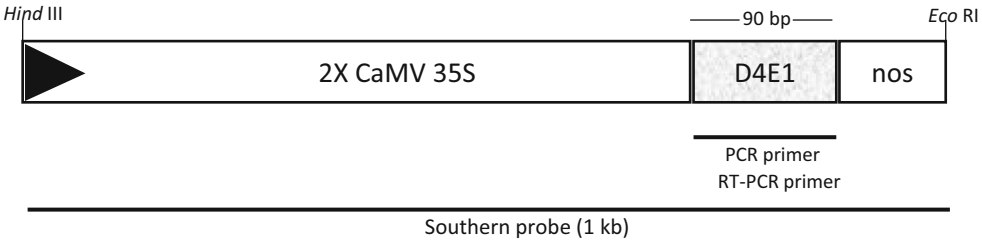

Fig. 1 Diagram of pBS-d35S-D4E1-nos used to transform Gladiolus. Regions covered by primers used to amplify genomic DNA and RNA, and the 1 kb region that was used as the probe in Southern hybridization are shown

SDS, and lastly 0.1× SSC, 0.2 % SDS, 15 min each wash at 55 °C. The DNA blot and intensifying screen were exposed to X-ray film for 1 day at −70 °C.

Semi quantitative RT-PCR

RNA was isolated from roots and shoots of plants growing in vitro on MS medium using a Qiagen RNeasy kit according to the manufacturer's instructions. Reverse transcription of 174 ng RNA was performed using an Invitrogen ThermoScript RT-PCR kit (Life Technologies, www.lifetechnologies.com) to produce cDNA (20 µl reaction with 1 µl RNaseH). A PCR reaction contained 1 µl cDNA, 100 nM of each primer, buffer, and 1.5 mM $MgCl_2$ in a 25 µl volume. Primers (forward: 5′-CCATGGGATTTAAGTTGAGAGCTAAGATTA -3′ and reverse 5′- TTTGAACGATCGGGGAAATTCGAGCTC-3′) amplified the 90 bp D4E1 gene (Fig. 1). The PCR conditions were 94 °C for 2 min, 31 cycles (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min), and 72 °C for 10 min. A second amplification was performed using 1 µl of the first PCR reaction run under the same conditions as for the first reaction.

The *GUBQ1* gene was amplified using the primers (forward: 5′-ATGCAAATTTTCGTCAAGACCC-3′ and reverse: 5′-ACCACCACGGAGGCG-3′), and the PCR product was run on an agarose gel as an RNA loading control. Amplification was performed as described above for D4E1.

Inhibition of *F. oxsporum* f. sp. *gladioli* (*Fog*) mycelial colony formation by either D4E1 or plant extracts

The minimum inhibitory concentration of D4E1 needed to prevent *Fog* spores from forming mycelial colonies was determined by inoculating each Petri plate containing PDA with 300 µl of PDB containing 135 µl of *Fog* spores and the desired D4E1 concentration (0, 6.25, 12.5, 25.0, 37.5, or 50.0 µM). Three plates were done for each D4E1 peptide concentration tested. Spores had been germinated by incubating at 30 °C for 8 h. Plates were incubated in the dark at 25 °C for 2 days before the number of spores that had germinated were counted by viewing the number of mycelial colonies that had originated from each spore (Suppl. Figure 1).

The inhibition of *Fog* spores by cell extracts of Gladiolus expressing the D4E1 gene was determined. Leaf samples of Gladiolus did not yield much liquid cell extract when ground in liquid nitrogen so a comparison was made with or without the addition of 10 mM phosphate buffer, pH 5.5 that was added after grinding the plant tissue. Leaves (2 g fresh weight) were collected from each sample, and 1 g portions of the leaves were ground in liquid nitrogen using an autoclaved mortar and pestle. Ground leaves (1 g) with its cell extract (no buffer added) were placed in a microcentrifuge tube on ice, and the other 1 g of ground leaves was placed in another microcentrifuge tube to which 1 ml of 10 mM phosphate buffer, pH 5.5 was added. Samples were centrifuged at 10,000*g* at 4 °C for 5 min. The supernatants from the sample without phosphate buffer (225 µl) and with phosphate buffer (450 µl) were each placed in a microcentrifuge tube along with 25 µl of a *Fog* spore suspension (1×10^5/ml). *Fog* spores had been germinated by incubating them at 30 °C for 8 h in PDB. Tubes were gently mixed by hand and then placed at 30 °C for 1 h. An aliquot, either 50 or 100 µl was taken from the sample without phosphate buffer or with phosphate buffer, respectively, and spread on PDA medium. Three plates were done for each sample, and this was replicated twice. Plates were incubated at 25 °C, and the number of spores that formed mycelial colonies were counted 2 days later. Significance of the *Fog* spore inhibition was analyzed by performing a one way ANOVA followed by a Tukey's test comparing the number of mycelial colonies that developed when incubated in the presence of cell extracts from Gladiolus leaves.

Transgenic plant challenge in vitro

Gladiolus plants with newly formed roots were placed in a Petri plate containing water agar [0.7 % wt/vol Phytoblend (Caisson Labs, www.caissonlabs.com)]. Plates were incubated in the light at 25 °C for 3 days prior to inoculation with a 5 mm plug of actively growing *Fog* taken from a Petri plate containing PDA and placed at a 1 cm distance from a root of each Gladiolus plant. Three-four days following inoculation with *Fog*, the plants were stained with lactophenol cotton blue (Larone 1995) for visualization of the *Fog*. The degree of infection was viewed under a dissecting microscope and given a value: 1-few hyphae, 2-moderate amount of hyphae, 3-much hyphae. Six roots were evaluated for each replicate, and 3 replicates done to collect data from 18 roots total/plant line. The value for each transgenic line was compared to that of non-transformed Gladiolus 'Peter Pears' using a student's *t* test. Lines with different letters are significant at $P < 0.001$. Photos were taken of roots using a Zeiss Observer.Z1 inverted microscope.

Confocal laser scanning microscopy

Previously *Fog* had been transformed with ECFP (Lakshman et al. 2012), and the *Fog* that fluoresced cyan was used

to visualize infection of Gladiolus roots. Gladiolus plants with newly formed roots were taken from Magenta jars and placed in Petri plates containing water agar. The ECFP-transformed *Fog* was inoculated onto PDA medium in a Petri plate, and a 5 mm plug containing actively growing *Fog* was removed from the Petri plate and placed 1 cm away from the Gladiolus root. Roots were observed 1–10 days following inoculation using a Zeiss™ 710 confocal laser-scanning microscope (Carl Zeiss Inc., Thornwood, NY). 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 pinhole of 36 µm with limits set between 460 and 728 nm for detection of the spectra. The spectra were linearly unmixed to identify two different spectral curves, one for the cyan (ECFP) fluorescence and the other for the green autofluorescence from Gladiolus roots. Zeiss Zen™ 2012 (Carl Zeiss microscopy) was used to capture images and separate the spectra. Photoshop 7.0™ (Adobe Systems, Inc., San Jose, California, USA) and Zen 2012 were used to design the figures.

Results

Integration and expression of D4E1 in Gladiolus plants

Southern hybridization with a 1.0 kb probe consisting of the 2× CaMV 35S-D4E1-nos region confirmed multicopy (2–12) integration of the promoter-transgene-terminator region in all five lines of transgenic Gladiolus plants (Fig. 2a). PCR amplification confirmed the presence of the 90 bp D4E1 gene in each of the five transgenic plant lines (Fig. 2b).

Expression of D4E1 was highest in both roots and leaves of line 14(1) based on D4E1 RNA levels (Fig. 3). Leaves and roots of the other four lines showed similar levels of D4E1 transgene expression.

Inhibition of *Fog* mycelial colony formation

The minimum inhibitory concentration of the D4E1 peptide using pre-germinated spores of *Fog* was 12.5 µM D4E1 (data not shown).

Cell extracts from transgenic lines 6(1) and 7(1) were found to significantly inhibit the development of mycelial colonies from *Fog* spores regardless of whether or not buffer was added to the cell extract (Fig. 4). Cell extract from line 2(3) inhibited the formation of mycelial colonies when buffer was added to the cell extract but not when buffer was omitted. Cell extracts from lines 2(1) and 14(1) did not significantly inhibit the formation of mycelial colonies.

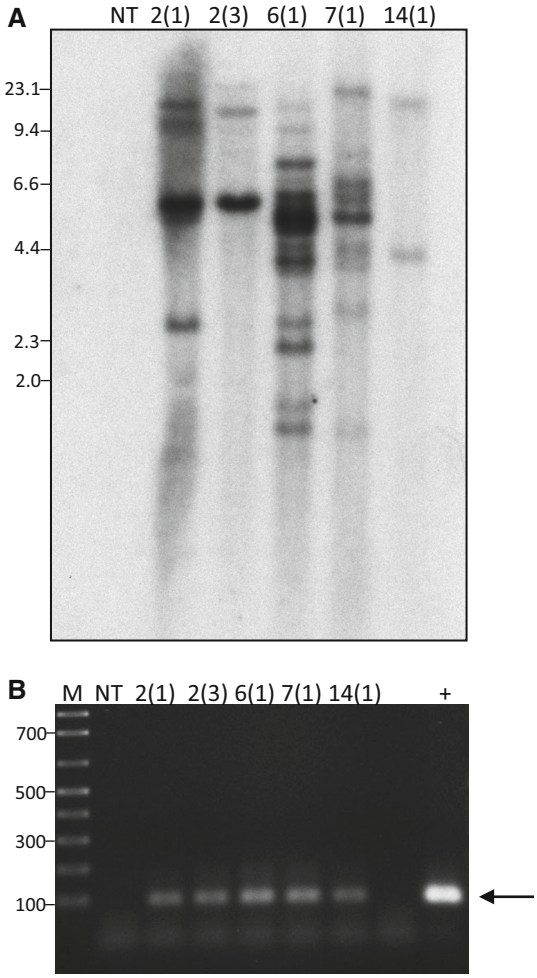

Fig. 2 **a** Southern hybridization confirms integration of the D4E1 gene in transgenic Gladiolus plants. Each *lane* contains 30 µg of genomic DNA from either a *transgenic line* indicated above the *lane* or a non-transformed plant (NT). DNA blots were hybridized with a 1.0 kb probe consisting of the 2× CaMV 35S-D4E1-nos region labeled with α-[^{32}P] dCTP. The size markers (M) from *Hind* III-digested λ are indicated at the *left* in kb. **b** PCR amplification of the 90 bp D4E1 gene (*arrow*) in genomic DNA from transformed Gladiolus lines. A 100 bp ladder was used as the size marker

Growth of *Fog* hyphae on shoots and around roots of transgenic Gladiolus plants

Fog was first observed at the shoot–root junction of Gladiolus plants growing in vitro, and then the shoot eventually succumbed to the mass of hyphae that covered its leaves. Most of the roots appeared to be free from hyphae for about a month. Staining plants with lactophenol, however, revealed that the roots were becoming covered with hyphae 3–5 days following inoculation with a plug of *Fog* placed 1 cm from the roots. Both transgenic lines 2(1) and 7(1) were judged to have significantly less fungal hyphae around their roots than non-transformed Gladiolus (Table 1; Fig. 5a, b, c).

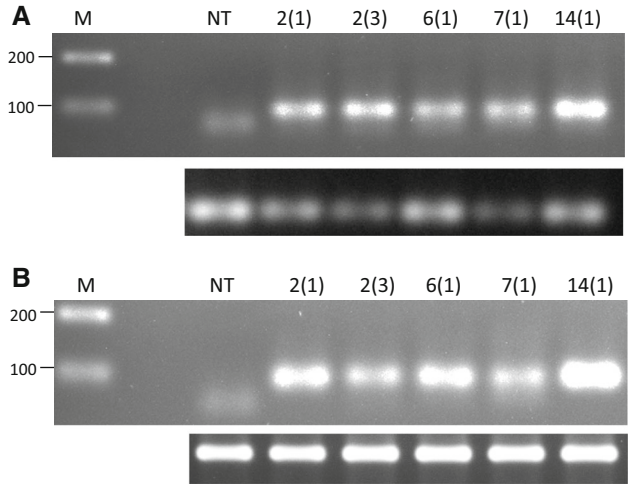

Fig. 3 Expression of the D4E1 gene was confirmed by semi-quantitative PCR in roots (**a**) and leaves (**b**). Transgenic lines are shown above each *lane* and compared to a non-transformed (NT) control plant. The RNA loading control is shown using primers that amplify the *GUBQ1* gene (*lower panel* in **a** and **b**)

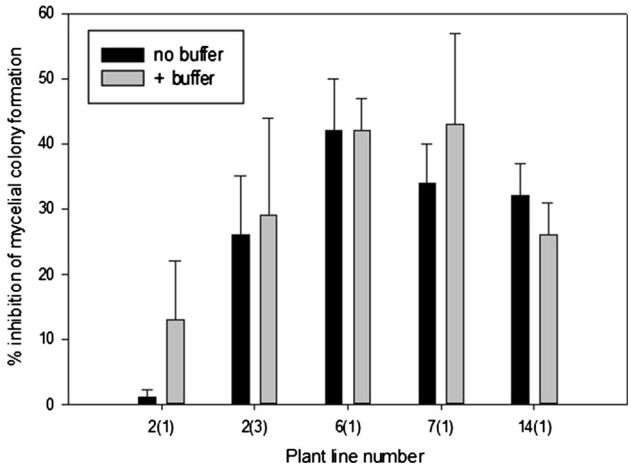

Fig. 4 Inhibition of mycelial colony formation from pre-germinated spores of *F. oxysporum* f. sp. *Gladioli* that were incubated 2 days with cell extracts from transgenic Gladiolus plants diluted with or without 10 mM phosphate buffer, pH 5.5. Standard *error bars* shown. The inhibition of mycelial colony formation from germinated spores was found to be significantly different for cell extracts from several *transgenic plant lines* as compared to a non-transformed control plant as indicated by a *star* above the *bar*. Significance was determined by a one way ANOVA followed by a Tukey's test ($P < 0.001$)

Initially the *Fog* hyphae formed pseudo-appressoria or enlarged hyphae that possibly facilitated their attachment to the non-transformed 'Peter Pears' root prior to forming the mycelial mat over the root as reported for banana, Gladiolus, and *Arabidopsis* (Mendgen et al. 1996; Michielse and Rep 2009; Li et al. 2011; Lakshman et al. 2012) (Fig. 5d). A mycelial mat forms prior to penetration of hyphae. Seven days after inoculating roots of both non-transformed 'Peter Pears' and transgenic line 7(1), the cells

Table 1 Amount of hyphae observed around roots of transgenic Gladiolus cv. Peter Pears plants 3–4 days after inoculation with *F. oxysporum* f.sp. *gladioli*

Line	Infection value ± SD
F-2(1)	1.39 ± 0.18[a],**
NT PP	2.37 ± 0.13
F-2(3)	2.17 ± 0.18
NT PP	1.78 ± 0.21
F-6(1)	2.21 ± 0.15
NT PP	2.21 ± 0.14
F-7(1)	1.07 ± 0.17**
NT PP	2.15 ± 0.13
F-14(1)	1.80 ± 0.16
NT PP	2.15 ± 0.13

[a] Categorized plants by viewing roots under a dissecting microscope: 1-few hyphae, 2-moderate amount of hyphae, 3-much hyphae. Plants were grown on a water agar plate for 3 days prior to inoculating with a plug of *Fog* placed at a 1 cm distance from a root of each plant. Plants were stained with lactophenol for improved visualization and viewed under a dissecting microscope 3–4 days after inoculation. Six roots were evaluated for each replicate, and three replicates were done to collect data from 18 roots total/plant line. The value for each transgenic line was compared to that of non-transformed 'Peter Pears' (NT PP) using a Student's *t* test

** Lines that are significant at $P < 0.001$

of the roots were intact (Fig. 5f and h, respectively). Ten days after inoculation, cells of non-transformed 'Peter Pears' had disintegrated (Fig. 5g) as compared to cells of line 7(1) that were just beginning to lose their structural integrity as indicated by their irregular shape (Fig. 5i). Shoots of non-transformed 'Peter Pears' plants were completely infected 10 days after inoculation while line 7(1) leaves were intact and green until 14–16 days after inoculation when they became covered with *Fog* (Fig. 5e).

In conclusion, cell extracts from transgenic lines 7(1) and 6(1) of Gladiolus cv. Peter Pears inhibited the development of mycelial colonies from *Fog* spores. There were less hyphae on the roots of transgenic line 7(1) 3–4 days after inoculation, but by 10 days after inoculation, the cells of the 7(1) root were beginning to lose their integrity. The five transgenic lines evaluated showed either an inhibition of mycelial colony formation or a decreased level of hyphal infection as compared to the non-transformed plants demonstrating that the antimicrobial peptide D4E1 appeared to inhibit *Fog* growth for a period of time before the *Fog* was able to overcome the protective effect from D4E1.

Discussion

Previously De Lucca et al. (1998) reported that the minimal inhibitory concentration of the purified D4E1 peptide

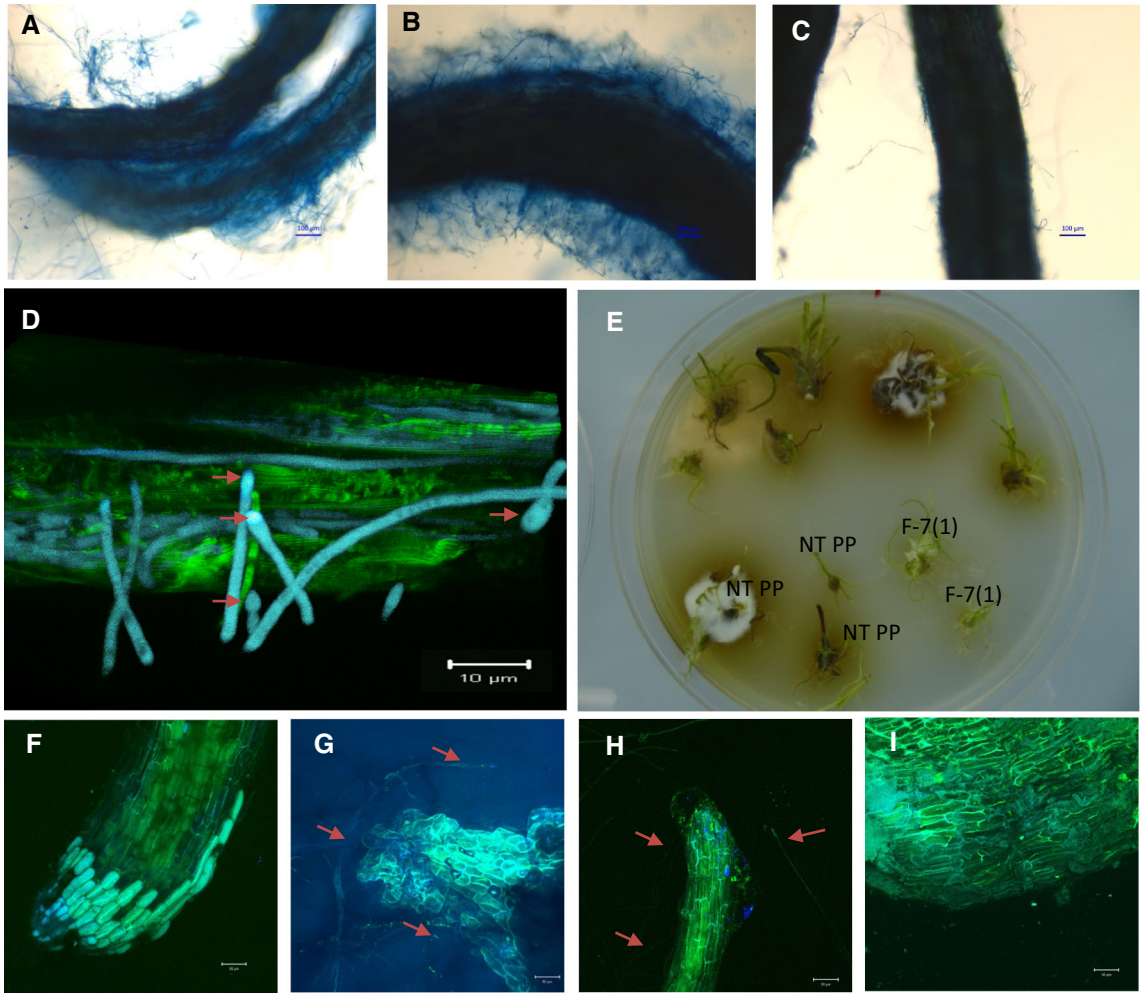

Fig. 5 Roots of **a** non-transformed 'Peter Pears' (NT PP), **b** a susceptible transgenic line, or **c** transgenic D4E1 line F-7(1) viewed 3–4 days following inoculation with *Fog*. **d** Initially it appeared that the *Fog* hyphae attached to the non-transformed Gladiolus root by pseudo-appressoria or enlarged hyphae (*arrows*) prior to forming the mycelial mat over the root. The *Fog* was transformed with the ECFP gene that fluoresced cyan while the NT PP root autofluoresced green. Seven days after inoculation with *Fog*, the NT PP and transgenic line F-7(1) roots were intact (**f** and **h**, respectively), but 10 days after inoculation, the NT PP root had disintegrated (**g**), and cells of the transgenic line F-7(1) root were just beginning to lose their structural integrity (**i**). *Fog* hyphae are indicated by *arrows*. The *magnification bars* for **a**, **b**, and **c** represent 100 µm and 50 µm for **f**, **g**, **h**, and **i**. **e** Ten days after inoculation with *Fog*, plantlets of NT PP were well-infected while the transgenic 7(1) plantlets were still green and intact

needed to kill both germinated and non-germinated spores of *F. oxysporum* and *F. moniliforme* (= *F. verticillioides*) was 3 µM indicating that D4E1 acts more effectively against these two species of Fusarium as compared to germinated spores of *Aspergillus flavus*, *A. niger*, and *A. fumigatus* that required a minimal inhibitory concentration of 12.5–25 µM. D4E1 was able to inhibit mycelial colony formation from germinated but not ungerminated spores of *Aspergillus flavus* which was why germinated spores of *Fog* were used in this study.

Cyzmmek et al. (2007) used time lapse documentation in conjunction with confocal and multi-photon microscopy to view *Arabidopsis* infection by *F. oxysporum*. They observed changes within the cell that occurred prior to physical penetration of the affected cell which suggests that *F. oxysporum* secreted phytotoxic compounds that caused changes in the autofluorescence of the vacuole and endoplasmic reticulum prior to penetration of the cell by hyphae. Fusaric acid, a phytotoxic compound produced by *Fog*, appears to result in death of Gladiolus (Remotti and Loffler 1997; Nosir et al. 2011). In this study *Fog* was never seen to penetrate the root using the Confocal Laser Scanning Microscope to view sections of the roots before their cells disintegrated supporting the observation by Remotti and Loffler (1997) and Cyzmmek et al. (2007) that *Fog* secretes phytotoxic chemicals that kill the plant cells before the *Fog* hyphae actually penetrate the cells.

It is unclear as to why line 14(1) showed the highest level of D4E1 RNA expression in both its roots and leaves as compared to the other four lines (Fig. 3), but the cell extract from line 14(1) did not show a significant effect on inhibiting mycelial colony formation from germinated *Fog* spores (Fig. 4), and the amount of hyphae around its roots was not inhibited as compared to non-transformed Gladiolus plants (Table 1). It has been reported that highly expressed mRNAs do not necessarily have high levels of protein expression (Gygi et al. 1999; Tsai-Morris et al. 2004), and although the D4E1 mRNA level in line 14(1) was high, its D4E1 protein concentration may have been low. This indicates the complexity of genetic engineering for Fusarium resistance in Gladiolus. Although high RNA and protein levels of the transgene are assumed to be desirable for achieving resistance to Fusarium, only a low level of resistance to *Fog* was observed in the line 14(1) Gladiolus plants as compared to line 7(1) that had a lower level of D4E1 RNA expression than line 14(1) but showed more resistance to *Fog* than line 14(1). Possibly another antifungal gene is needed in combination with D4E1 to develop Gladiolus with a high level of resistance to this devastating pathogen.

Acknowledgments Mention of a trademark, proprietary product or vendor does not imply its approval to the exclusion of other products or vendors that may also be suitable. Chris Pooley (USDA, Beltsville, MD) is thanked for assistance with preparing the confocal microscopy figures. Siobhan O'Connor provided excellent technical assistance with the Southern hybridizations and RT-PCR. The *Fog* was received from Professor Robert McGovern (Univ. of Florida, Gainesville, FL). Assistance with the spore germination experiments were provided by Ruichi Pandey and Bula Choudhury.

Conflict of interest None of the authors have any conflict of interest with this research.

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