TRANSFORMATION OF MINT ISOLATES OF *VERTICILLIUM DAHLIAE* TO FLUORESCE GREEN

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Abstract

To facilitate microscopic visualization of the location and movement of *Verticillium dahliae* in mint, a green fluorescing protein (gfp) gene from a jellyfish was inserted into a mint isolate of *V. dahliae*. Several fluorescing transformants were obtained. Each transformant grew equivalently to the non-transformed parent isolate, and all incited wilt symptoms in inoculated peppermint Two transfoimants that exhibit normal growth and are pathogenic to mint are being stored and maintained for use in future research efforts. At present, all transformants are confined by federal regulatory guidelines to controlled conditions in labs, growth chambers, and greenhouses.

Introduction

Pinpointing the location and visualizing the movement of microorganisms in plants typically have been tedious, time consuming and expensive, partly because microorganisms are so small and non-descript. A wilt fungus such as *Verticillium dahliae* may grow in only trace amounts through living plants before decay processes develop. Furthermore, once decay processes develop, other organisms may overgrow and obscure the pathogen.

It would be useful to have a tool to facilitate manipulation and visualization of infection processes and distribution of *Verticillium dahliae.* The gfp from the jellyfish, *Aequorea victoria,* is a very attractive system for the demonstration of heterologous expression of a cloned gene because the gene product (protein) is capable of generating a green fluorescence when illuminated with blue light. The use of this fluorescent gene product as an *in situ* tag to visualize the infection biology of filamentous fungi is a very attractive system because it is stable, very sensitive, and can be monitored by noninvasive methods in living tissues. Such a tool was recently developed in the Oregon State University Department of Botany and Plant Pathology. A transformation vector (pCT74) has been shown to work in a number of filamentous fungi including selected *V. dahliae* isolates pathogenic on potato (Sawyer, et al. 1998; Lorang et al., unpublished manuscript).

Materials and Methods

Isolates of *V. dahliae* were transformed using hygromycin resistance (HYG) as a selectable marker *(V. dahliae* is sensitive to the antibiotic hygromycin) and the gene for the green fluorescent protein (gfp) driven by the ToxA promoter from *Pyrenophora tritici-repentis* (Ciuffetti et al., 1997). The objective of this project was to transform *V dahliae* isolates pathogenic on mint Protoplasts of selected isolates of *V. dahliae* pathogenic on mint were transformed with pCT74. Expression of the gfp gene in the *V. dahliae* transformants that were hygromycin resistant was determined by the ability of the

transformed mycelia to fluoresce when illuminated with a blue light source. Stability of the integrated gfp gene was determined on a non selective medium. Growth curve assays were performed on the transformants by growing on 20, 25, 30, 35, 40, and 451_1.1 hygromycin/ml and mycelial growth was measured on days 11-18. Select isolates were tested for pathogenicity on mint. Roots of Black Mitcham peppermint were dipped in $1X10^6$ spores/ml water of the selected isolates and planted in greenhouse soil. Plants were screened for disease symptoms after 2 months.

Results and Discussion

Transformations of *V. dahliae* isolate MT-96-1-4 from Black Mitcham peppermint and pathogenic on mint yielded a high number of $HYG^+/GFP^\pm$ transformants. Five hundred fifty-six $HYG^\pm$ transformants were checked for successful insertion of the gfp gene by visualizing expression of gfp by irradiating with blue light (470nm) using a Leica microscope with an Endow GFP filter cube (exciter HQ470/40, emitter HQ525/50 with beamsplitter Q495LP).

Thirty-six successful gfp transformants were selected and screened further for growth on hygromycin-amended agar. The growth rate of the transformants was equal to the wild type (untransformed *V. dahliae).* An example of two growth curves (transformant 1441 and transformant 1451) is shown in Figure 1.

Five transformants (1331, 1323, 1411, 1441, and 1451) were tested further for pathogenic capability. All transformants tested induced typical Verticillium wilt symptoms. Plants infected with transformant 1451 showed 80-100 percent stunting and necrotic symptoms and the plants were dead. Plants infected with isolate 1441 were all stunted and showed an average of 55 percent typical Verticillium symptoms. Transformants 1451 and 1441 will be stored and used for further studies because these transformants were the most stable both on hygromycin supplemented media (selection media) and on nonselective media. These transformants maintain typical colony growth patterns, good microsclerotia production, and were equal to wild type (untransformed *V. dahliae)* with respect to pathogenic capability.

Using a gfp-isolate of *V dahliae*, mint was infected normally. Whole small roots and crude hand cut sections of plant parts were placed directly under a fluorescence microscope and the thin, fluorescing strands of fungal hyphae, or even single spores, were observed amidst non-fluorescing plant tissues and other non-fluorescing microorganisms. Previous special staining techniques were much more cumbersome and much thinner sections were required, which necessitated special handling, time delays, and greater expense. Microsclerotia of untransformed *V. dahliae* already were somewhat distinctive without the aid of fluorescence, but microsclerotia from the gfp isolates were more easily discerned, even when stem material was decayed and with many other organisms present.

The novel genes used in this transformation are considered safe, and many such gfp transformations have been made for research purposes on other living things, although

few on fungi. Nevertheless, federal regulations require that such genetically modified pathdgens be used only in the laboratory, growth chambers, or used the greenhouse. We destroy all material at the end of each experiment. Ultimately, isolates could be released for the use of other scientists under similarly controlled conditions. Administratively, federal guidelines consider L. Ciuffetti as OSU's representative responsible for the disposition of the transformants.

Literature Cited

Ciuffetti, L. M., R. P. Tuori, and J. M. Gaventa. 1997. A single gene encodes a selective toxin causal to development of tan spot of wheat. The Plant Cell 9:136-144.

Sawyer, T., L. M. Ciuffetti, R. P. Tuori, and K. Johnson. 1998. Green fluorescent protein expressed by *Verticillium dahliae.* Phytopathology 88:S78.

Acknowledgement

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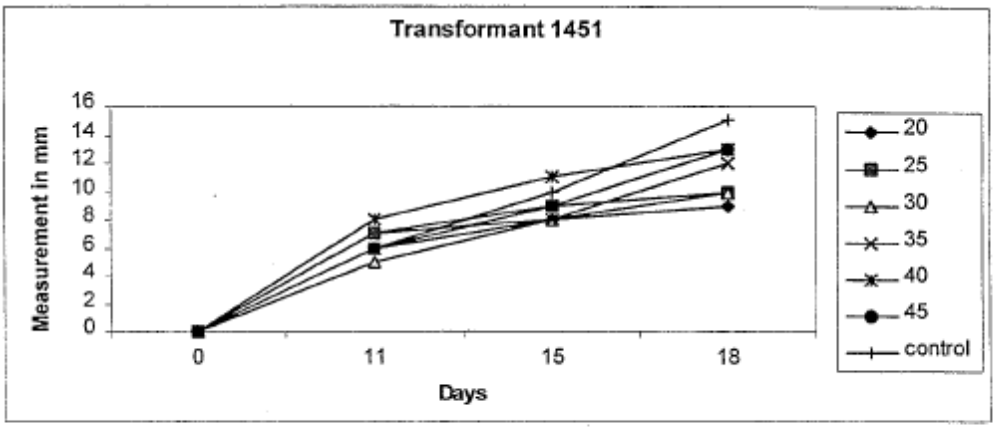

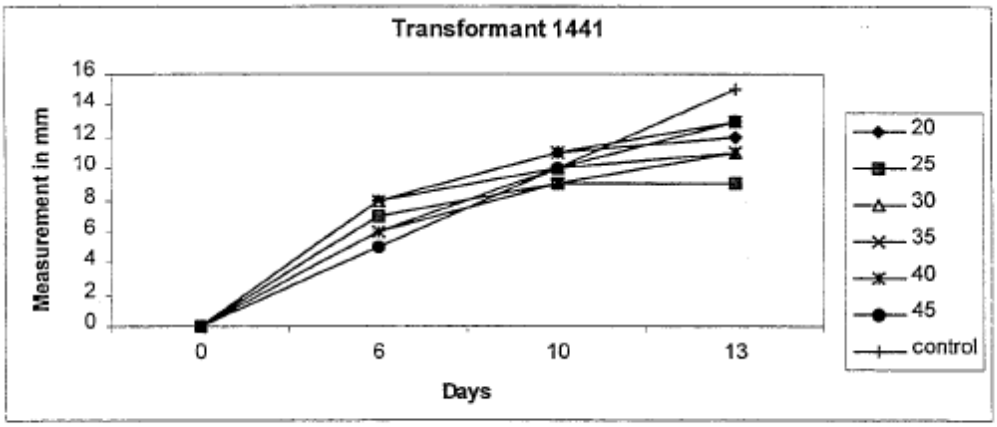

Figure 1. Growth curves of selected transformants of *Verticillium dahliae* transformed with a green fluorescent protein from jellyfish grown on media containing different concentrations of hygromycin (ug/ml agar).