PEPTIDES PRODUCED BY *FUSARIUM OXYSPORUM* WHICH KILL PLANT CELLS

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Abstract

Fusarium oxysporum is the causal agent of vascular wilts in many different plant species. The components of the interaction between pathogen and plant that lead to susceptibility or resistance to disease are receiving intensive study. It is evident that *F. oxysporum* produces proteins which can kill plant cells and may function in plant/ pathogen interactions. A 24 kDa protein which causes necrosis in leaves of many dicots was purified from *F. oxysporum* culture fitrates. Related 24 kDa proteins are produced in the culture filtrates of many *F. oxysporum* isolates including several *formae specialis*. A 22 kDa xylanase originally isolated from *Trichoderma viride* was identified in culture filtrates of *F. oxysporum*. The xylanse induces many different defense responses in tomato and tobacco with an activity that is independent of the xylanase enzyme activity and apparently dependent upon a protein/protein interaction with a plant membrane protein. A pair of proteins, 56 and 61 kDa in size, were purified from culture filtrates of *F. oxysporum* f. sp. *lycopersici* race 1 which cause death of tomato cells lacking the I_1 gene which gives resistance to race 1 of the pathogen. In this case the proteins are thought to function as toxins. A 59 kDa glycoprotein has been isolated from spores of a *F. oxysporum* f. sp. *vasinfectum* race 1 isolate which has biological activity in a cotton variety resistant to race 1. This interaction closely resembles an elicitor/receptor model where the fungal protein acts as an avirulence factor which is recognized by a receptor in an incompatible interaction. The proteins discussed here demonstrate the wide range of activities and specificities proteins produced by *F. oxysporum* can have in plants and plant/pathogen interactions.

Introduction

Fusarium oxysporum is the causal agent of vascular wilts in many different plant species (Beckman 1987). The strains (*formae specialis*) of the fungus which cause vascular wilts are most often genetically distinct and host specific causing disease on a single plant species or a few related plant species. Symptoms of the disease may include chlorosis of the leaves, leaf abscission, and wilting, which may be limited to individual branches, and browning of the vascular system in symptomatic tissues. The mechanisms involved in disease development have been studied for many years with the majority of the efforts going toward two hypotheses. A major hypothesis continues to be that wilting occurs due to occlusion of the water transport in tissues as a result of products produced during fungal invasion (Beckman 1987). A second major hypothesis is that wilting occurs due to the activity of various fungal toxins produced within the plant vascular system which destroy the plant's ability to maintain turgor pressure (Gaumann 1957, Diamond and Waggoner 1953). The mechanisms involved in resistance have been less well studied but are the subject of more intensive efforts as a result of recent developments in this area in other plant disease interactions (Dangl 1995, van Kan 1991). Resistance to fusarium wilt is often controlled by a single dominant gene which confers resistance in a race specific manner (Beckman 1987). It is the components of the interaction between pathogen and plant that lead to susceptibility or resistance to disease which are receiving intensive study.

Discussion

We undertook to identify components found in the culture filtrate of *Fusarium oxysporum* f. sp. *erythroxyli* which might be involved in the plant/ microbe interaction. Previous studies of other plant/pathogen interactions had indicated that culture filtrates could be used to select out, identify or characterize resistant genotypes of various plant species (Selvapandiyan et al. 1989, Buiatti et al. 1985, Sutherland and Pegg 1992). Active components of culture filtrates were identified based on their ability to kill coca leaf tissue and stimulate ethylene biosynthesis. Ethylene biosynthesis is a common response to many different stresses (Mattoo, A. K. and J. C. Suttle 1991) including both susceptible and resistant disease interactions. The primary active component of *F. oxysporum* f. sp. *erythroxyli* culture filtrates was identified as a 24 kDa protein (Table 1) after purification by FPLC gel chromatography (Bailey 1995). The 24 kDa protein induces ethylene biosynthesis and causes necrosis in coca leaves. The 24 kDa protein is a major component in culture filtrates of *F. oxysporum* grown in Czapek-dox media supplemented with 1% Casamino acids. Production of the protein is limited when complex carbohydrates are used as the only carbohydrate source and repressed when plant extracts are added to the media (Bailey et al. 1997). The optimum media composition for production of the 24 kDa protein is Czapek-dox plus 1% asparagine (Bailey et al. 1997).

In order to further characterize the 24 kDa protein, polyclonal antibodies were developed to the purified protein (Bailey et al. 1997). The antiserum identified the 24 kDa protein in quantities as low as 2.5 ng after Western blotting. The antiserum to the 24 kDa protein was used to identify related 24 kDa proteins in the culture filtrates of many other *F. oxysporum* isolates including several *formae specialis*. In each case regulation of 24 kDa protein production was similar to that observed for the 24 kDa protein from *formae*

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specialis erythroxyli. The related 24 kDa protein was purified from different *formae specialis* of *F. oxysporum* and an isolate of *F. acuminatum* using similar purification techniques and found to have similar biological activity to the 24 kDa protein from *F. oxysporum* f. sp. *erythroxyli* (Bailey et al. 1997). The 24 kDa protein has biological activity in a broad range of dicot plant species regardless of the *Fusarium* isolate that produces it (Bailey et al. 1997). The relative sensitivity to the 24 kDa protein is species specific. Some species respond by producing extensive necrosis and ethylene while others only produce ethylene, necrosis or water soaking. Infiltration of cotton leaves with 1 µg/mL of 24 kDa protein results in necrosis of the infiltrated area within 24 h (unpublished data). Some dicot plant species do not respond in a measurable manner. Monocot species appear to be insensitive to the 24 kDa protein. We have been unable to identify the 24 kDa protein in plant tissue infected with *F. oxysporum* (Bailey et al. 1997), but this effort has been limited to 3 plant pathogen interactions of which hundreds exist. Problems with protein extractibility, stability, and detectability temper our conclusions.

F. oxysporum produces several different proteins which are known to have activities similar to the 24 kDa protein in plants. For example, a 22 kDa xylanase (Table 2) was identified in culture filtrates of *Trichoderma viride* which causes necrosis and ethylene production in certain solanaceous plant species (Anderson et al. 1997). Antibodies to the *T. viride* xylanase were used to demonstrate that *F. oxysporum* produces a 22 kDa xylanase with similar activity (Dean et al. 1989). The 22 kDa xylanase induced responses of tobacco and tomato have been characterized as a hypersensitive response involving most of the known events associated with plant defense. The xylanase elicitor activity is not only limited to certain species within the solanaceae but also to certain genotypes within those species. Sensitivity to the 22 kDa xylanase is controlled by a single gene in both tobacco and tomato in a manner similar to the genetics of commonly identified hypersensitive response genes in many plant/pathogen interactions. The necrosis inducing activity of the 22 kDa xylanase is independent of the xylanase enzyme activity (Sharon et al. 1993) and apparently dependent upon a protein/protein interaction with a plant membrane protein. An initial consideration in characterizing the 24 kDa protein was that it was a known enzyme. Several hydrolytic enzymes can reproduce similar responses in plant tissues as a result of their enzyme activities. We have isolated and characterized the gene for the 24 kDa protein of *F. oxysporum* f. sp. *erythroxyli*. The gene includes a single intron and codes for a leader peptide which is cleaved in the process of producing the mature excreted peptide. The amino acid sequence deduced from the nucleic acid sequence was verified by amino acid sequence obtained from the purified protein. The sequence of the gene encoding the 24 kDa protein does not show significant homology to any gene within established databases and the deduced amino acid sequence appears unrelated to any known enzyme.

Biologically active proteins were identified in culture filtrates of *F. oxysporum* f. sp. *lycopersici*, race 1 (Sutherland and Pegg 1995). A pair of proteins, 56 and 61 kDa in size, were purified from culture filtrates which cause death of tomato cells lacking the I_1 gene which gives resistance to race 1 of the pathogen (Table 3). The proteins simulate the symptoms of fusarium wilt when applied to tomato and have a much higher activity in the susceptible line than in the resistant line or other plant species. To purify these peptides, biological activity was identified based on the ability to kill protoplasts (Sutherland and Pegg 1992). The proteins were not purified in sufficient quantities to obtain sequence and therefore have not been cloned. Since antibodies to the proteins have not been produced the proteins have not been localized in infected plant tissues. In this case the proteins are thought to function as toxins or virulence factors allowing the pathogen to cause disease in susceptible tomato lines. Insensitivity to the proteins was associated with resistance to the pathogen. It was suggested that other *formae specialis* of the pathogen produce similar proteins important in their abilities to cause disease in their host but this has not been proven. The 24 kDa protein has also been purified from culture filtrates of isolates of *F. oxysporum* f. sp. *lycopersici* (Bailey et al. 1997).

In addition to the obvious differences between the *F. oxysporum* f. sp. *lycopersici* 56 and 61 kDa proteins and the 24 kDa protein in size and host specificity, the proteins also appear to differ in their activities. Although both protein preparations kill cells of sensitive host plants, the 24 kDa protein has many attributes which suggest it may actually function as a resistance inducing factor. The 24 kDa protein induces K^+ and H^+ channeling, active oxygen production and oxygen consumption in tobacco cell cultures, all responses closely associated with plant defense against pathogen attack (C. Jacyn Baker, personal communication). At present we have failed to demonstrate the induction of pathogenesis-related proteins although the genes required for ethylene biosynthesis are induced. Although we cannot rule out the 24 kDa protein functioning as a toxin, the data suggest it induces plant defense responses much in the same manner as other proteinaceous elicitors.

In contrast to the tomato/*F. oxysporum* interaction described by Sutherland and Pegg (1995), a 59 kDa glycoprotein (Table 4) has been isolated from spores of a *F. oxysporum* f. sp. *vasinfectum* race 1 isolate which has biological activity in a cotton variety resistant to race 1 but not in a susceptible line (Yi-Shen et. al. 1993). In this system, a plant protein was isolated from resistant plant tissue which binds the fungal glycoprotein (Wei, Yi-Shen et al. 1992, Yi-Shen et al. 1993). This system closely fits an elicitor/receptor model where the fungal protein acts as an avirulence factor which is recognized by a receptor in an incompatible interaction

resulting in the induction of the plant's defense responses preventing disease development. This resistance response is not only species specific but is also gene specific within a species. Although the 24 kDa protein induces many characterized defense responses, it does not, with presently used techniques and plant germplasm, show the unique specificity of the cotton/ *F. oxysporum* f. sp. *vasinfectum* interaction defined by Yi-Shen et al. (1993).

We continue not only to define the function of the 24 kDa protein but also to attempt to exploit the 24 kDa protein's attributes for increasing our understanding of induced responses in plants and for the improvement of agriculture. At present our primary efforts are aimed at knocking out expression of the 24 kDa protein and over expressing the 24 kDa protein in *F. oxysporum* to define its function in pathogenesis (Apel-Birkhold et al. 1997). It is possible that the 24 kDa protein functions as an avirulence factor inducing defense responses in plants. This hypothesis is at least superficially supported by the observation that plant extracts inhibit production of the 24 kDa protein. Unfortunately, we still can not rule out the 24 kDa protein functioning as a virulence factor or toxin. Our inability to detect the protein in diseased tissue does not preclude the protein functioning at levels below detection or ephemeral in nature. In addition we are evaluating the 24 kDa protein as a direct spray to plants. This work is modeled after recent work with another proteinacious elicitor, Harpin (Bauer et al. 1995, Wei, Laby et al. 1992). Harpin can be applied as a foliar spray (Dong et al. 1997, Theisen et al. 1997) or even a seed soak (Qiu et al. 1997) and induce resistance in the treated plants. The 24 kDa protein, because of its broad host range, offers a unique opportunity for studying and comparing the signal transduction pathways in divergent plant species.

Summary

It is evident that *F. oxysporum* produces a number of proteins which can kill plant cells. Although enzymatic activity may be involved it certainly is not required (Sharon et al. 1993). The sensitivity of a plant species to a protein may be dependent upon the presence of a single gene (Bailey et al. 1993, Sutherland and Pegg 1995, Yi-Shen et al. 1993). In some interactions proteins function as toxins promoting disease (Sutherland and Pegg 1995) whereas in other interactions proteins function as elicitors precluding disease development (Yi-Shen et al. 1993). The relative activity of the protein in a specific interaction is not dependent in all cases upon the pathogenicity of the fungal isolate or the susceptibility of the plant to disease (Dean et al. 1989, Bailey et al. 1997).

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Table 1. Characteristics of the 24 kDa protein isolated from culture filtrates of *Fusarium oxysporum* f. sp. *erythroxyli* (Bailey et al. 1997).

Source of the protein	Filtrates from 3 to 12 day old cultures of *Fusarium oxysporum*, *F. acuminatum* and *F. avenaceum*. Production is independent of *formae specialis*.
Protein Sequence	Sequence is known but is unrelated to sequences in available databases.
DNA Sequence	Genomic sequence has been determined but is unrelated to sequences in available databases.
Activity	Induces necrosis, ethylene biosynthesis, K^+ and H^+ channeling, active oxygen production, and oxygen consumption.
Specificity	The protein is active in many different dicot species. The induced responses are species dependent. The protein is inactive in monocots.
Genetic Relationships	None established.
Effect on pathogenicity	None established.

Table 2. Characteristics of the 22 kDa xylanase isolated from culture filtrates of various fungi including *Fusarium oxysporum* (Anderson et al. 1997).

Source of the protein	Filtrates from xylan-induced cultures of *Trichoderma viride*, *Fusarium oxysporum*, and many other fungi.
Protein Sequence	Sequence is known and represents a family of 22 kDa xylanases with related sequences.
DNA Sequence	Sequence is known and represents a family of 22 kDa xylanases with related sequences.
Activity	Induces necrosis, ethylene biosynthesis, Pr-protein biosynthesis, K^+ and H^+ channeling, accumulation of salicylic acid, and active oxygen production.
Specificity	The protein is active in tomato and tobacco and certain other members of the *Solanaceae*.
Genetic Relationships	Sensitivity in tomato and tobacco is dependent on a single dominant gene. A plant protein has been identified which binds the xylanase in what is proposed to be an elicitor/receptor interaction.
Effect on pathogenicity	Proposed to induce systemic acquired resistance.

Table 3. Characteristics of the 56 and 61 kDa peptides isolated from culture filtrates of *Fusarium oxysporum* f. sp. *lycopersica* (Sutherland and Pegg 1995).

Source of the protein	Filtrates from cultures of *Fusarium oxysporum* f sp. *lycopersica* race 1.
Protein Sequence	Sequence is unknown.
DNA Sequence	Sequence is unknown.
Activity	Induces necrosis.
Specificity	The protein is most active in tomato. Genetic Relationships Sensitivity in tomato dependent on a single recessive gene.
Effect on pathogenicity	The protein is proposed to act as a toxin. Sensitivity to the toxin results in susceptibility to disease.

Table 4. Characteristics of the 59 kDa peptide isolated from culture filtrates of *Fusarium oxysporum* f. sp. *vasinfectum* (Yi-Shen et al. 1993).

Source of the protein	Extracted from microconidia of *Fusarium oxysporum* f. sp. *vasinfectum* race 1.
Protein Sequence	Sequence is unknown.
DNA Sequence	Sequence is unknown.
Activity	Induces occlusion of vessels and phytoalexins.
Specificity	Most active in cotton cultivar 52-128 which is resistant to race 1.
Genetic Relationships	Sensitivity in cotton is dependent on a single dominant gene. A plant protein has been identified which binds the glycoprotein in what is proposed to be an elicitor/receptor interaction.
Effect on pathogenicity	Sensitivity to the protein is correlated with an incompatible interaction.